DIFFERENTIAL MODULATION OF FIBROBLAST PROPERTIES IN SCLERODERMA

by

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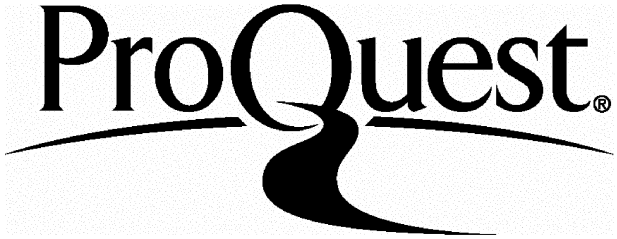

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ABSTRACT

Connective tissue fibrosis is a hallmark of many human diseases. One of these is scleroderma (SSc), where the skin and various internal organs undergo a progressive scarring or fibrosis leading to the loss of normal tissue structure and function. The underlying cause(s) of SSc are not known but autoimmune, vascular abnormalities, fibrogenic cytokines, genetic susceptibility and environmental factors have all been implicated. An early pathogenic event in SSc is infiltration of inflammatory cells into affected tissues, following endothelial cell damage. Subsequently, immune cell derived cytokines and growth factors are likely to be early modulators of fibroblast phenotype. The work described in this thesis focuses on fibroblast matrix synthesis and adhesion molecule expression, the extrinsic modulation of these properties, and the altered patterns of modulation observed in SSc. Modulation of fibroblast phenotype by growth factors e.g. endothelin-1 (ET-1), connective tissue growth factor (CTGF), extracellular matrix (3-dimensional collagen gel culture) and direct leucocyte-fibroblast interactions (Intercellular adhesion molecule-1, ICAM-1) expression were investigated. As part of this project, long-term temperature sensitive SSc fibroblast cell lines were generated.

Since SSc is characterised by excessive collagen deposition the fibroblasts have been cultured and studied. Both dermal and lung fibroblasts from SSc patient lesions exhibit elevated collagen type (I) production, and fail to down-regulate collagen type I mRNA in gel culture despite normal gel contraction. An increased activation of the collagen gene is observed in SSc cell strains compared with controls, suggesting that the elevated collagen (I) mRNA levels are due, at least partly, to transcriptional activation. The production of CTGF, which is selectively induced by transforming growth factor beta (TGFβ), was markedly greater for SSc fibroblasts than normal strains and was correlated with the increased collagen synthesis. In contrast, modulation of fibroblast synthesis of collagen and other matrix molecules by ET-1 shown reduced responses for SSc fibroblasts. Impaired SSc fibroblast response to ET-1 was associated with reduced expression of ET_A receptors. SSc fibroblasts express elevated levels of

surface and steady-state mRNA of ICAM-1 and shed higher levels of sICAM-1 *in vitro*. ET-1 also operated as a pro-inflammatory mediator, up-regulating fibroblast ICAM-1 expression. Finally, long-term temperature sensitive SSc fibroblast cell lines retain high collagen biosynthesis and ICAM-1 expression following retroviral transduction with the SV40 tsT antigen.

In conclusion, the data presented in this thesis provide further information regarding the abnormalities in the phenotype of fibroblasts from SSc and support the hypothesis that an alteration in the production of cytokines and in the response they provoke are related to the scarring and fibrosis seen in SSc.

PUBLICATIONS

Work included in this thesis appears in the following publications:

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

Shi-Wen X, Vancheeswaran R, Bou-Gharios G, O'Hare M, Olsen I, Abraham DJ, Black CM: Scleroderma-derived human fibroblasts retain abnormal functional and phenotypic characteristics following retroviral transduction with the SV40 tsT antigen. *Exp Cell Res* 1995; 220: 407-414

Shi-Wen X, Denton CP, McWhirter A, Bou-Gharious G, Abraham DJ, duBois RM and Black CM: Scleroderma lung fibroblasts exhibit elevated and dysregulated collagen type I biosynthesis. *Arthritis Rheum* 1997; 40: 1237-1244

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Shi-Wen X, Denton CP, Dashwood MR, Abraham DJ and Black CM: Endothelin-1 mediated regulation of intercellular adhesion molecule-1 expression in normal and scleroderma fibroblasts. [1997; in preparation]

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LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
AFI	Average fluorescent intensity
BSA	Bovine serum albumin
COL1A2	Pro-α2(I) collagen gene
CTGF	Connective tissue growth factor
DcSSc	Diffuse cutaneous systemic sclerosis
DLR	Dual-Luciferase™ Reporter
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
ET	Endothelin
FACS	Flow cytometry
FASSc	Fibrosing alveolitis in systemic sclerosis
FCS	Foetal calf serum
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon gamma
LcSSc	Limited cutaneous systemic sclerosis
LFA	Lymphocyte function-associated antigen
mAb	Monoclonal antibody
MHC	Major Histocompatibility Complex
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
RP	Raynaud's phenomenon
RT-PCR	Reverse transcriptase-Polymerase chain reaction
SSc	Scleroderma; Systemic sclerosis
SV40	Simian Virus 40
TdR	Thymidine
TGFβ	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
tsT	Temperature-sensitive SV40 large T antigen

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Overview

Fibrosis is the pathologic hallmark of many human diseases, including pulmonary fibrosis, hepatic cirrhosis, chronic glomerulonephritis, post-surgical adhesions and keloids. A prototypic example of an acquired fibrotic disorder is scleroderma (SSc), in which the skin, lungs, heart, gastrointestinal tract, and kidneys are major targets for progressive and often relentless fibrosis. The aetiopathogenesis of scleroderma is uncertain, but immune activation and microvascular dysfunction is increasingly recognised as important aspects of early disease. It is also clear however that dysfunctional activity of the fibroblast is central to disease progression. Indeed, it is the persistent activation of the genes encoding ECM proteins in scleroderma fibroblasts which distinguishes controlled repair, such as normal wound healing, from uncontrolled connective tissue accumulation, which results in pathological fibrosis.

Excessive collagen deposition in affected tissues is a central event in the pathogenesis of scleroderma, and is responsible for most of the clinical manifestations of this incurable disease. Therefore, an understanding of the intrinsic mechanisms responsible for the normal regulation of fibroblast function, and the modulation of this process by extracellular signals is likely to provide clues to the understanding of the molecular alterations in connective tissue cells that occur in scleroderma. This, in turn, may lead to the rationale design of novel therapeutic interventions. This introductory chapter reviews topics of relevance regarding fibroblast function in scleroderma and highlights the role that specific cellular mediators may play in the development of fibrosis. A selective review, focused on the current state of knowledge about scleroderma pathogenesis is also included.

1.2 Systemic sclerosis: General

Systemic sclerosis (Scleroderma, SSc) is one of the most deadly of the autoimmune rheumatic diseases. Although uncommon it has a significant mortality, largely through the resultant connective tissue fibrosis. The pathological hallmarks of scleroderma are vascular and microvascular

abnormalities characterised by capillary obliteration, endothelial injury, and intimal proliferation. This occurs is association with medial thinning and the appearance of a distinctive adventitial/interstitial cuff of collagen. Perivascular and tissue infiltration of mononuclear inflammatory cells is also apparent and an increased deposition of normal matrix components in the skin and internal organs. This deposition would appear to be in response to a disruption of the normal steady state of connective tissue turnover and regulated repair (Black, 1995).

In 1753 Curzio provided the first detailed description of a scleroderma-like disease: his patient was a young woman with excessive tension and hardness of the skin. In 1847 Gintrac introduced the term scleroderma (skleros, hard; derma, skin), emphasizing that the skin was the most obvious organ involved (Barnett, 1996). Vascular dysfunction (Raynaud's phenomenon; RP) was first noted by Raynaud in 1862 and the association of scleroderma with RP was described in 1896 by Hutchison. The extensive involvement of other organs was not appreciated until after the careful work by Von Notthaft in 1898 and Matsui in 1924. These observations led to the hypothesis that both lung parenchymal interstitial change and vascular abnormalities were directly caused by scleroderma. The presence of other visceral lesion was described in a detailed review by Goetz in 1945 when he first used the term "systemic sclerosis".

Recent data from the United Kingdom suggests that the incidence of scleroderma around 1 in 100,000 (Silman, 1991 and 1995). In terms of gender distribution, the data indicate that scleroderma occurs much more frequently in woman than in men. Results have been reported in England and Wales during 1968-1985, where the crude female to male ratio was more than 4:1 (Silman, 1991).

Although criteria for inclusion of patients and starting times varied somewhat, cumulative survival rates using the life-table method for populations including all ages and both males and females were typically 60% to 70% at 5 years and 40% to 50% at 10 years after first physician diagnosis or after enrolling at a medical centre (Medsger, 1994).

1.2.1 Classification

1.2.1.1 Clinical criteria

The American College of Rheumatology's preliminary criteria for scleroderma were developed during the Scleroderma Criteria Cooperative Study (ACR, 1980). These criteria were defined in a multi-centre study of 264 scleroderma patients and more than 400 patients with other related disorders evaluated within the first two years of diagnosis (Tab. 1.1).

Table 1.1. ACR preliminary criteria for scleroderma

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.

1.2.1.2 Scleroderma disease spectrum

According to clinical and laboratory features, the most widely accepted subsets of scleroderma are diffuse cutaneous systemic sclerosis (DcSSc) and limited cutaneous systemic sclerosis (LcSSc), which have been summarized in an editorial co-authored by several well-recognized experts (LeRoy *et al.*, 1988) (Tab. 1.2). It is now realised that although the two subset classification system of scleroderma is of value in disease classification, it is limited by the fact that clinical outcomes within each major subset are so varied. Thus, although overall survival is worse for diffuse cutaneous scleroderma than limited cutaneous, those patients with limited disease who develop isolated pulmonary hypertension (approximately 10-15% of cases) have a 60% mortality at 5 years (Stupi *et al.*, 1986). Similarly, patients with limited cutaneous scleroderma who develop significant lung fibrosis or acute renal failure have a similarly poor prognosis to diffuse scleroderma cases with equivalent complications (Altmann *et al.*, 1990).

Table 1.2. Subsets of scleroderma (SSc)

DcSSc (40% of SSc patients)
Onset of RP within 1 year of skin changes (puffy or hidebound)
Truncal or acral skin involvement
Presence of tendon friction rubs
Early and significant incidence of interstitial lung disease, oliguric renal failure, diffuse gastrointestinal disease and/or myocardial involvement
Absence of anti-centromere antibodies
Nailfold capillary dilatation and capillary destruction
Anti-topoisomerase I antibodies (Scl-70; 30% patients)
LcSSc (60% of SSc patients)
Presence of RP for years (usually decades)
Skin involvement either limited to the hands, face, feet and forearms (acral) or absent skin changes or not present
A significant late incidence of PHT with or without interstitial lung disease, trigeminal neuralgia, skin calcifications and telengiectasiae
A high incidence of anti-centromere antibodies (70-80%)
Dilated nailfold capillary loops usually without capillary dropout

1.2.2 Aetiology

Although the basic aetiology of scleroderma is unknown, it is almost certainly multi-factorial with genetic and environmental factors playing a part (Briggs, Black and Welsh 1990). In the last few years several studies evaluating the possible participation of retroviruses in aetiology of scleroderma have also been published (Jimenez *et al.*, 1995).

1.2.2.1 Genetics

Scleroderma occurs mostly in females and gender may be the strongest genetic marker. There are however, several lines of evidence which indicate familial or genetic predisposition to scleroderma. First, although rare, there are familial clusterings of scleroderma and related diseases, particularly Raynaud's Phenomenon. Autoantibodies associated with scleroderma are found in high frequency in blood relatives of scleroderma patients, although the incidence of antinuclear antibodies (ANA) in spouses suggests an environmental component. 36% of relatives with ANA had clinical features of connective tissue disease not observed in spouses (Briggs *et al.*, 1993). Thirdly, many centres worldwide have

observed abnormal frequencies of the major histocompatibility (MHC) antigens associated with scleroderma.

Although the cause of chromosomal abnormalities in scleroderma is not known, the high rate of chromosomal instability seen in first-degree relatives of patients makes it likely that this phenomenon is partially relevant to the aetiology of scleroderma. The original work was reported by Housset *et al* (1969), but only recently have attempts been made to utilize the new DNA technology in order to re-address the question of chromosome damage in scleroderma. Artlett *et al* (1996) characterized genetic alterations at the molecular level in the scleroderma peripheral lymphocytes, fibroblasts genome using variable number tandem repeats (VNTR). There was a significant rise in the level of VNTR mutations in scleroderma patients, their siblings and offspring. The reason for the genomic changes remains unknown, but the presence of a hitherto unrecognized clastogen is a possibility.

Early MHC serological studies have showed weak associations with the Class I MHC antigen HLA Bw35 and the class II MHC antigen DR1, particularly in dcSSc (Black *et al.*, 1983). Both DR3 and DR5 genes are linked to DRw52 and Livingstone *et al* (1987) suggested that this could be the primary MHC Class II allele associated. The class III genes have been less well studied in scleroderma. Briggs *et al* (1993) reported an association between C4AQ0 and scleroderma, which was confirmed by analysis on both the C4 allotypes and C4 genes.

Recent genetic analysis aimed at identifying cytokine genetic poymorphisms have shown an association between TNF, lymphotoxin (LT-α) and IL-10 in specific scleroderma patient groups (Fanning *et al.*, 1997). Perhaps the most striking evidence of the genetic associations in scleroderma comes from the studies on scleroderma-associated lung diseases (pulmonary fibrosis), where in addition to the MHC (HLA DR3/DR52a) linkage (Briggs *et al.*, 1991), polymorphic genotypes of the fibronectin gene were clearly shown to be associated with the development of lung fibrosis (Avila *et al.*, 1997). Such polymorphisms within genes that are known (i) to have growth factor activities, (ii) be involved in the regulation of the ECM, and (iii) reported to be aberrantly

regulated during the development of fibrosis (Rannard *et al.*, 1981; Deguchi *et al.*, 1989) may explain some if not all of the genetic associations linked to disease severity and progression.

1.2.2.2 Environmental agents

Of all the major autoimmune connective-tissue disease, scleroderma alone is thought to have an important environmental component in disease susceptibility (Silman and Hochberg, 1996). A number of environmental agents have been linked with the development of scleroderma-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). In addition, exposure to organic solvents has been reported to confer an increased risk for the development of scleroderma (Nietert *et al.*, 1996). Recently, a major focus of research has been the possible association between silicone gel breast implants and scleroderma but careful retrospective studies of 235 patients with silicone gel breast implants and of 840 female scleroderma patients found no demonstrable evidence of a statistically significant association (Hochberg *et al.*, 1993; Giltay *et al.*, 1994).

1.2.2.3 Retroviruses

From the initial report describing the presence of particles with a density characteristic of a complete retrovirus and the detection of reverse transcriptase activity in bone marrow cells from a patient with scleroderma (McCabe *et al*, 1979), evidence has accumulated supporting the hypothesis of a retroviral etiology in scleroderma. The studies conducted so far have focused on two main avenues of investigation; the possibility that retroviruses or retroviral products could alter the regulation of ECM genes and the possibility that retroviral proteins are primal antigenic stimuli for the formation of autoantibodies (Jimenez *et al.*, 1995).

A pathogenetic role for autoantibodies has long been sought in scleroderma. This may have significance in disease aetiology. Evidence that molecular mimicry plays a role in the development of autoantibodies in scleroderma

patients is the homology between some scleroderma autoantibody targets and viruses (Table 1.3). This may have significance in disease aetiology.

Table 1.3. Autoantibody targets in scleroderma and homologous viruses

Autoantibody target	Homologous viruses
DNA Topoisomerase 1	p30 gag protein from feline sarcoma virus
	UL 70 protein of human cytomegalovirus
PM-Scl antigen	SV-40 large T antigen
	Human immunodeficiency virus Tat protrein
U1 snRNA	Herpes simplex virus type I ICP4 protein
Fibrillarin (U3 snRNP)	Herpes simplex virus type I P40 protein
	Epstein-Barr virus nuclear antigen 1

(Derived from the following references: White, 1996; Query and Keene, 1988; Dang *et al.*, 1991).

Research has provided information supporting the hypothesis of a retroviral aetiology in scleroderma which has been the study of the regulatory effects of retroviruses and their proteins on the expression of genes encoding ECM proteins, such as Rous sarcoma virus (RSV) (Sandmeyer *et al.*, 1981), the HIV-1 Tat gene, and the HTLV-1 Tax protein (Taylor *et al.*, 1992).

1.2.2.4 Other aetiopathogenic agents

The formation of free radicals is implicated in scleroderma as these agents can induce both vascular activation and chromosomal abnormalities. Free radicals may occur due to hypoxic episodes and as a consequence of activation polymorphonuclear cell (Czirjak *et al.*, 1987; Murrel, 1993). Evidence for increased oxidation was first demonstrated in stored plasma from scleroderma patients compared with controls by Blake *et al.*, 1985. Recently, studies in scleroderma have also revealed deficient levels of the anti-oxidants - selenium and ascorbic acid (Herrick *et al.*, 1994) and increased oxidised lipoproteins (Bruckdorfer *et al.*, 1995). Moreover, several studies have provided support for the increased production of reactive oxygen species (ROS) in patients with

scleroderma (Stein *et al.*, 1996; Casciola-Rosen *et al.*, 1997). The very short half-life of free radical species makes direct demonstration of generation of these molecules *in vivo* dependent on detection of more stable products induced by radical species (e.g. evidence of lipid peroxidation).

1.3 Pathogenesis - Introduction

The etiology and pathogenesis of scleroderma are unknown. The current hypothesis include a genetic predisposition and environmental stimuli, which result in immune activation and vascular injury. Immune activation induces immune-mediator release, further vascular injury, fibroblast proliferation, collagen synthesis and deposition, and finally end-organ damage (Figure 1.1).

1.4 Pathogenesis I: Immune aspects

Research on the role of the immune system in general has concentrated on two areas: the cellular immunity and homoral immune system. There is growing body of evidence for abnormalities in both of these systems.

1.4.1 Cellular Immunity

1.4.1.1 T cells and T cell receptors

T cells appear to be central to the development of tissue damage in scleroderma patients. In a meticulous analysis of sequential skin biopsies from scleroderma patients, Prescott *et al* (1992) observed that one of the earliest abnormalities is a collapse of vimentin intermediate filaments around the nucleus of endothelial cells. Next, both $CD4^+$ and $CD8^+$ T cells migrate into the skin, where they are scattered throughout the subcutaneous tissue and dermis or localize to areas around the vessels, nerves, and skin appendages (Prescott *et al.*, 1992; Gruschwitz *et al.*, 1992; Kahari *et al.*, 1988). Expression of HLA class II molecules indicates that the infiltrating T cells have been recently activated (Prescott *et al.*, 1992; Gruschwitz *et al.*, 1992). Coincident with T cell infiltration, additional morphologic and functional changes are noted in the endothelial cells, then tissue fibrosis follows (Prescott *et al.*, 1992). Those fibroblasts that are actively producing collagen are located next to the area of T cell infiltration (Kahari *et al.*, 1988), which suggests that activated T cells may have a causal relationship to

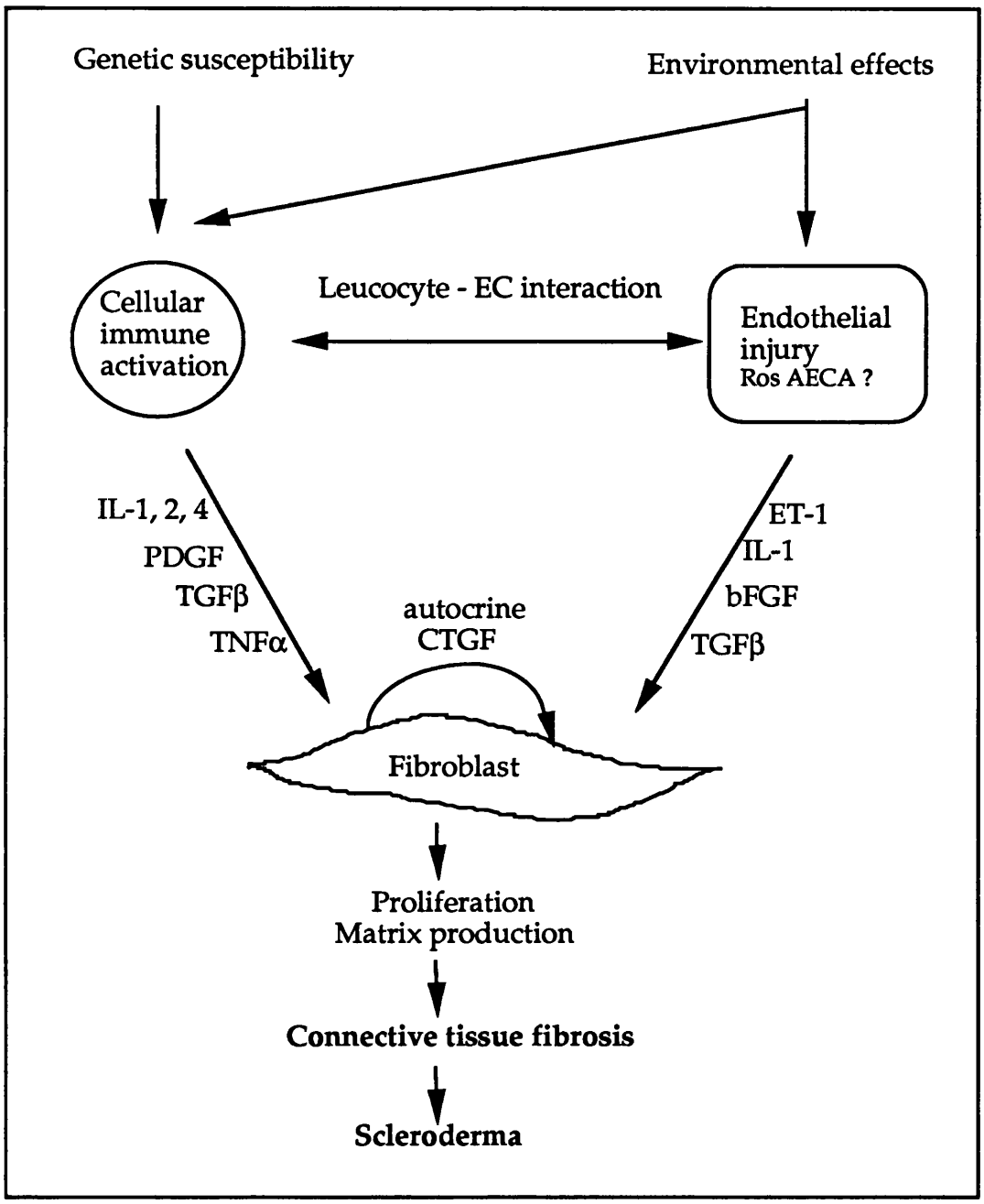


Figure 1.1 The proposed hypothesis for the pathogenesis of scleroderma. Fibroblasts in scleroderma patients synthesize increased amounts of collagen for several generations *in vitro*. Many factors influence these fibroblasts, including cytokines produced by actived immune cells and injured endothelium. Activated fibroblasts are also capable of producing cytokines and of responding to them in a paracrine fashion, resulting in a positive feed-back loop. Fibroblast properties are investigated in this thesis.
Ros -reactive oxygen species, AECA- anti-endothelial cell antibodies

fibrosis. Both $CD4^+$ and $CD8^+$ T cells are found in the skin of patients with idiopathic scleroderma, but $CD4^+$ T cells are the major subpopulation (Prescott *et al.*, 1992).

Scleroderma patients with interstitial lung disease have increased numbers and percentages of lymphocytes in the interstitium on biopsy (Rossi *et al.*, 1985) and frequently in BAL (Rossi *et al.*, 1985; Silver *et al.*, 1990). Analyses of the T cells within BAL of scleroderma patients with alveolitis show increased $CD8^+$, reduced $CD4^+$, and normal γ/δ T cells, compared to peripheral blood from the same patients or blood and BAL from normal patients (White, 1996). Relevant to the finding of increased $CD8^+$ T cells in the lungs of scleroderma patients is the observation by Kahaleh and Yin (1990) that endothelial cell cytotoxicity in scleroderma patients is mediated in part by granzyme A, which is released by cytotoxic T cells. The dominance of distinct T cell subpopulations in the skin ($CD4^+$) and lungs ($CD8^+$) of scleroderma patients suggests that different T cells may contribute to the disease process in different organs.

A change in the number or percentage of $CD4^+$, $CD8^+$, or γ/δ T cell subpopulations provides a crude indication of T cell selection. Better evidence for T cell selection comes from the finding of restriction in the T cell repertoire, as estimated through analyses of the diversity of expressed TCR. Two types of analyses of TCR diversity are commonly performed: one determines the relative use of different TCR variable (V) gene families, and the other determines the diversity of the rearranged V-diversity (D)-joining (J) junctional regions. The findings of an increased expression of the Vδ1 gene (White, 1996) in association with restricted diversity of $V\delta1^+$ junctional region sequences (Yurovsky *et al.*, 1994) suggests an antigen-driven selection of $Vd1^+$ γ/δ T cells in scleroderma patients.

1.4.1.2 Other immune cells

The skin, lung, and heart are all reported to be sites of increased mast cell numbers and degranulation in scleroderma patients. In 1985, Claman's group found that mast cells were increased only in the affected skin in early disease

(Hawkins *et al.*, 1985). These mast cells are activated and are capable of increased releasability (Claman, 1989). Mast cell granules can regulate fibroblast biologic activity and fibroblasts, in turn, support mast cell viability (White, 1996). Increased levels of nerve growth factor, a neurotrophic factor that induces an increase in mast cells and histamine release, is found in the dermis of scleroderma patients, which raises the possibility that this factor contributes to its pathogenesis (Tuveri *et al.*, 1993).

Mast cells are found in large numbers in alveolar walls and are possibly involved in pulmonary fibrosis. Mast cell numbers are increased in BAL fluids from patients with scleroderma (Chanez *et al.*, 1993). These observations, together with the known capacity of mast cells to produce TNF, IL-1 and IFNγ, suggest they may be implicated in the pathogenesis of lung fibrosis (Coker and Laurent, 1995; Bolster *et al.*, 1997).

Other cells, such as natural killer cells, eosinophils, macrophages, basophils, and neutrophils, are also reported to link with scleroderma (for review see White, 1996).

1.4.2 Humoral Immunity

1.4.2.1 B cells and auto-antibodies

Autoantibody production is an early, nearly universal finding in scleroderma patients. Identification of the antigens that are specific for scleroderma and delineation of the B cell epitopes on these targets provide clues about the aetiology of the disease. Increased plasma cells are seen within the skin of scleroderma patients (Prescott *et al.*, 1992) and in lung biopsies of scleroderma patients with alveolitis (Rossi *et al.*, 1985).

Hypergammaglobulinaemia and autoantibodies are found in the sera of scleroderma patients. Hypergammaglobulinaemia results from polyclonal B cell activation in scleroderma patients (Fleischmajer *et al.*, 1977), with increases in IgG, IgA, IgM, and IgE. IgG levels are also increased in BAL of scleroderma patients (Silver *et al.*, 1990). ANAs occur early and uniformly (90%-98%) in patients with scleroderma (Maddison *et al.*, 1993).

Multiple autoantigens which are B cell stimulatory antigens are recognized by autoantibodies from scleroderma patients. Some autoantibody targets that are quite specific for scleroderma include the topoisomerase-1 (Tan *et al.*, 1989); centromeric proteins (Fritzler *et al.*, 1980); chromosomal antigens (Saunders *et al.*, 1993); RNA polymerases I and III (Reimer *et al.*, 1988); U3-RNP (Reimer *et al.*, 1988) and the PM-Scl antigen in scleroderma overlap with polymyositis (Reichlin *et al.*, 1984).

Immune complexes are present in the sera of approximately 25% of scleroderma patients (Silver *et al.*, 1986), although they are not typically deposited in tissue (Prescott *et al.*, 1992). Another way that autoantibodies might contribute to pathology in scleroderma patients is through the inhibition of function of the antibody targets. Antibodies against centromeric proteins, topoisomerase-1, and RNA polymerases all inhibit the function of their respective targets (Bernat *et al.*, 1990).

1.4.2.2 Cytokines

Cytokines may contribute to extracellular matrix deposition and vascular activation/damage in scleroderma patients. Gonzalez-Amaro *et al* (1988) showed that mononuclear cells from scleroderma patients produce soluble factors *in vitro* that stimulate fibroblast proliferation, protein synthesis, and collagen production to a greater degree than do lymphocytes from controls. Exposure to supernatants from stimulated normal mononuclear cells can induce an scleroderma-like phenotype in normal fibroblast lines (Needleman *et al.*, 1990). Cytokines capable of altering endothelial cell function are found in scleroderma sera or tissues, including IL-1, IL-2, IL-4, IL-6, IL-8, TNFα, TGFβ, and PDGF (Needleman *et al.*, 1992), and the endothelins (Kahaleh, 1991) are other circulating factors that may damage or alter the function of fibroblasts or endothelial cells in scleroderma patients.

1.4.2.3 Adhesion Molecules

Cell surface expression of adhesion molecules is necessary for lymphocyte trafficking through the blood vessels and subsequent adherence within tissues.

Migration and adherence of lymphocytes in the skin of scleroderma patients may be facilitated by an increased expression of adhesion molecules on vascular endothelium, lymphocytes and fibroblasts.

Once T cells migrate through the endothelium into tissue, adhesion to fibroblasts is mediated in part by interactions between lymphocyte function associated antigen-1 (LFA-1) on T cells and ICAM-1 on fibroblasts (Piela and Korn, 1990; Abraham *et al.*, 1991). Serum ICAM-1 levels are elevated in scleroderma patients (Denton *et al.*, 1995). Dermal perivascular lymphocytes in scleroderma skin show increased expression of β1, β2, β3 integrins (Gruschwitz *et al.*, 1992; Sollberg *et al.*, 1992), with β2 and β3 expression occurring in acute skin (Gruschwitz *et al.*, 1992; Sollberg *et al.*, 1992). There is no difference in β4 integrin expression from normals (Sollberg *et al.*, 1992). The α1 chain expression is increased on inflammatory cell infiltrates in acute and chronic scleroderma skin, as is expression of VLA-1,-2, -3, -4, and -6 (Gruschwitz *et al.*, 1992). Scleroderma patients have reduced numbers of circulating T cells capable of adhering to endothelial cells *in vitro*, which is thought to be the result of depletion of T cells by increased adherence to vascular endothelium *in vivo* (Rudnicka *et al.*, 1992).

1.5 Pathogenesis II: Vascular biology

The evidence that generalized scleroderma is a vascular disease is compelling. Pathologists have known that microvascular lesions are associated with fibrosis of scleroderma for most of the present century (and, of course, Maurice Raynaud described episodic cold- and stress-induced vasospasm, which occurs in 90% of scleroderma patients, in 1862). The capillary lesions of scleroderma are distinctive: dilated capillary loops are interspersed between vascular areas of ECM devoid of capillaries. Endothelial cell injury and capillary injury, collapse, and obliteration by fibrosis are principal histologic microvascular features (LeRoy, 1996). The vascular abnormalities are described follow.

1.5.1 Vascular lesions

Vascular injury is critical to the pathogenesis of systemic sclerosis and may be the primary event (Campbell and LeRoy 1975). Evidence for early endothelial cell damage in scleroderma was provided by a sequential study of 60 scleroderma

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 scleroderma lung biopsies.

The vascular damage in scleroderma is widespread and can be recognised as: [i] vasomotor instability or Raynaud's Phenomenon with repeated 'transient' interruption of tissue perfusion in the digits and internal organs (systemic Raynaud's), which is often an early event in disease development (Kallenberg, 1990); [ii] microvascular abnormalities with structural changes characterized by proliferative intimal arterial lesions and obliteration of the vessels leading to chronic ischaemia. Vascular damage can be visualized in the nailfold capillaries but is also present in the small blood vessels of virtually all the viscera, muscle, subcutaneous tissue and skin (Carpentier *et al.*, 1990); [iii] intravascular pathology that is manifest by decreased red cell deformability, increased platelet activity and enhanced thrombus formation (Kahaleh, 1990).

1.5.2 Mechanisms of vascular lesions

Many factors may be important in the vascular damage but it is the endothelial cell that is thought to have a pivotal role.

Cytotoxic factor: In the last two decades, *in vitro* studies have focused on the search and identification of a cytotoxic factor involved in the disease pathogenesis. In 1979, Kahaleh and LeRoy observed the capacity of the sera of scleroderma patients to kill human umbilical vein endothelial cells (Kahaleh and LeRoy, 1979). Recently, it was found that scleroderma serum was cytotoxic to venous and arterial endothelial cells when cocultured with peripheral blood mononuclear cells, suggesting that multiple immunological mechanisms might be involved in the pathogenesis of vascular lesions (Holt *et al.*, 1989). The factor inducing endothelial cell cytotoxicity remains unidentified. There is a large body

of evidence that a circulating soluble protease may be linked to the genesis of endothelial injury, and one candidate is granzyme from activated T cell granules. Granzyme A has been identified in scleroderma sera and found to be expressed in scleroderma skin (Kahaleh and Fan, 1992). It still remains possible that there are multiple factors injurious to endothelial cells in scleroderma plasma, be they proteases, sources of free radicals (e.g. lipid peroxides) or all of these (LeRoy, 1996).

Alterations of immune mechanisms: Many investigators have focused on alterations of immune mechanisms in the genesis of vascular damage in scleroderma, particularly the interaction of activated lymphocytes with the endothelium. Alternatively, endothelial cells may function as antigen presenting cells (Jaff, 1987), presenting antigen epitopes to resting T cells and stimulating an allogeneic response, sustained largely by $CD4^+$ T cells and directed against HLA-DR. Can endothelial cells initiate and sustain the immune response? Although human endothelial cells usually do not bear class II molecules, they may be induced *in vivo* as shown on arterial endothelial cells during the vascular reaction of Kawasaki disease (Tarai *et al.*, 1990). The induction of MHC II antigens on endothelial cells may involve autologous T cells, perhaps through cytokine secretion (Pober *et al.*, 1983). An endothelial cell hypothesis would highlight the role of lymphocytes in the induction of scleroderma, placing vascular involvement and damage as a consequence of cell-mediated immunity and the interrelationship between endothelial cell and T cells as key in disease progression (Kahaleh, 1991).

Cytokines: Cytokines, such as IL-1, TNF, IFN, IL-2, IL-4, IL-6, IL-8, TGFβ, may have a role in endothelial activation and damage. It seems likely that the cytokine cascade induced by inflammatory stimuli has a role in the induction of endothelial activation and the loss of endothelial integrity. Several different mechanisms may be proposed (e.g., rearrangement of endothelial cytoskeleton, which could render endothelial cell susceptible to injury or, alternatively, poised to trigger immune activation), but the precise role of cytokines in the vascular involvement in scleroderma remains unclear.

Anti-endothelial cell antibodies: The immunoglobulin fraction of sera from 33% of scleroderma patients reveal anti-endothelial cell antibodies (AECA) which have also been reported in 74% lupus and 33% rheumatoid arthritis sera (Rosenbaum *et al.*, 1988). If these antibodies were mediating damage *in vivo*, the vascular damage seen in scleroderma would be similar to other connective tissue disorders rather than the distinct scleroderma vascular changes observed. These antibodies may therefore occur secondary to endothelial damage. Functional effects *in vitro* have now been demonstrated for these antibodies present in scleroderma sera (Del Papa *et al.*, 1996). Anti-endothelial cell antibodies may cause damage through antibody-dependent cellular cytotoxicity. These effects include upregulation of adhesion molecule expression, increased leucocyte adherence and induction of cytokine release by endothelial cells (Carvalho et al., 1996).

1.5.3 Endothelial cell products

The endothelium is now known to produce numerous molecules and to regulate many aspects of vascular stability including control of vascular tone, permeability, thrombotic potential and leucocyte trafficking (Pearson 1991). Table 1.4 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).

While endothelial cells may cause fibrosis through the increased expression of growth factors, the relationship between endothelial cells and fibroblasts in scleroderma is unclear. Factors which are released by endothelial cells that could result in increased fibrosis include TGFβ, IL-1, bFGF, ET-1 and CTGF (Kahaleh 1990, Denton *et al.*, 1996, 1997; Bradham *et al.*, 1991; Igarashi *et al.*, 1996). Recently, Denton *et al* (1996) reported that scleroderma dermal fibroblast properties are modulated when co-cultured with human endothelial cells. In addition, he demonstrated in a series of experiments using conditioned-medium on similar pattern of endothelial cell-induced modulation by bFGF and IL-1 (Denton *et al.*, 1997). TGFβ and CTGF have been extensively investigated in

scleroderma and details of these studies are given in Chapter 4. The role of ET-1, a particularly important candidate in scleroderma 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 5 and 6.

Table 1.4. Major molecules synthesised by the vascular endothelium

Vasoactive molecules	**Growth factors and cytokines**
Endothelin-1 (ET-1)	Connective tissue growth factor (CTGF)
Nitric oxide (NO)	Platelet-derived growth factors (PDGF)
Prostaglandin E2	Transforming growth factor-β (TGFβ)
Prostacyclin (PGI-2)	Basic fibroblast growth factor (bFGF)
	Interleukin-1α and β
	Interleukin-6
	Platelet activating factor
Extracellular matrix	**Adhesion proteins**
Collagen III, IV and V	E-selectin
Fibronectin	P-selectin
Thrombospondin	Intercellular adhesion molecule-1 (ICAM-1)
von Willebrand Factor	Vascular cell adhesion molecule (VCAM-1)
Anticoagulation factors	**Enzymes**
Antithrombin III	Angiotensin converting enzyme (ACE)
Thrombomodulin	Aminopeptidyl peptidase (CD13)
Protein S	Neutral endopeptidase (CD10)
	Dipeptidyl peptidase IV (CD26)

(derived and modified from Black and Denton in Oxford Textbook of Rheumatology 1997)

1.5.4 Adhesion molecules

Following injury to and activation of the vascular endothelium that adhesion molecules such as E-selectin, VCAM-1 and ICAM-1 are upregulated in response to cytokines and other factors. These endothelial adhesion molecules bind to specific ligands on T and B lymphocytes, platelets, neutrophils, monocytes and natural killer cells, facilitating their adhesion to vascular endothelium and subsequent migration through what have now become "leaky vessels" into the extracellular matrix with the ultimate potential for fibroblast activation. Therefore if endothelial change could be detected, and stabilised at an early stage, it would almost certainly influence the clinical expression and progression of the disease. Increased cell surface expression of HLA class II molecules, β_1 integrins, and endothelial cell adhesion molecule-1 (ECAM-1) indicates activation

of endothelial cells in scleroderma patients (Prescott *et al.*, 1992; Gruschwitz *et al.*, 1992; Claman *et al.*, 1991). Endothelial leukocyte adhesion molecule-1 (ELAM-1) is a member of the selectin family that appears on the endothelial cell surface during inflammation and mediates endothelial cell interactions with neutrophils, granulocytes, monocytes, and $CD4^+$ T cells (Picker *et al.*, 1991). ELAM-1 levels are elevated in scleroderma patients, but levels do not correlate with disease activity and are not scleroderma-specific (Carson *et al.*, 1993). ELAM-1 and ICAM-1 expression are both increased on endothelial cells in scleroderma patients (Gruschwitz *et al.*, 1992; Claman *et al.*, 1991), where levels correlate with the amount of mononuclear cell infiltration (Gruschwitz *et al.*, 1992). Dermal endothelial cells of scleroderma patients also have increased expression of very late activation antigens VLA-2 and VLA-4 (Gruschwitz *et al.*, 1992).

1.6 Pathogenesis III: Connective tissue fibrosis

The extracellular matrix is a complex structure composed of a large number of distinct molecules. It has become increasingly clear that ECM proteins not only provide a scaffold for cells but also influences cellular phenotype and functions, such as migration, shape, proliferation, differentiation, and biosynthesis of connective tissue macromolecules. Indeed, the ECM has been shown to play an important role in wound healing, tumour progression, and the development of fibrosis (Krieg *et al.*, 1988).

Histopathologic and biochemical methods have been used to study the ECM composition of skin in scleroderma (Fleischmajer *et al.*, 1980). While the concentration of collagen in the dermis is normal, the total amount of collagen under the surface area of affected skin is increased due to the increased thickness of the dermis. Although patients with early scleroderma exhibit increased amounts of type III collagen deposits in the lower dermis, type I collagen production dominates in later stages of the disease. This results in a normal relative proportion of type I : type III collagens (4:1) in skin (Lovell *et al.*, 1979) and lung (Coker and Laurent, 1995) of patients with established scleroderma.

In a 1974 landmark study, LeRoy demonstrated that skin fibroblasts from patients with scleroderma that were cultured *in vitro* produced increased

amounts of type I collagen compared with fibroblasts from age and sex matched healthy individuals (LeRoy, 1974). Subsequent studies have confirmed that the production of several connective tissue components (e.g. type I, III, VI, and VII collagens, fibronectin, decorin, and glcosaminoglycans) are up-regulated in scleroderma fibroblasts in tissue culture. The increased biosynthesis of these macromoleclules is accompanied by an elevation of the steady-state levels of mRNA transcripts which is largely the result of increased transcription of the corresponding genes (Jimenez *et al.*, 1986; Kahari *et al.*, 1988). In addition to the persistent up-regulation of genes encoding multiple connective tissue proteins, scleroderma dermal fibroblasts also display enhanced expression of proto-oncogenes (such as myb, myc, src and ras *et al*), which are known to participate in cell proliferation and activation (Trojanowska *et al.*, 1988). The results from studies of connective tissue metabolism by scleroderma fibroblasts in monolayer cultures were confirmed by observations from *in situ* hybridizations of scleroderma skin employing collagen cDNAs (Kahari *et al.*, 1988). These studies demonstrated elevated collagen mRNA transcripts in subpopulations of fibroblasts. In contrast to scleroderma, fibroblasts in normal skin show much lower levels of collagen gene expression.

Whereas monolayer cultures have been utilized extensively to study the biosynthetic characteristics of normal and scleroderma fibroblasts, it is recognized that cell behavior in this system may differ markedly from the *in vivo* situation. In particular, fibroblasts in monolayer cultures are not surrounded by extracellular matrix. Normal fibroblasts propagated *in vitro* in a three dimensional collagen lattice, a situation which mimics more closely the physiologic environment than monolayer cultures, cause contraction of the surrounding gel, and drastically reduces the rate of collagen production and the levels of type I collagen mRNA (Mauch *et al.*, 1992). The low level of collagen synthesis under these conditions, which resembles that of fibroblasts in the normal resting dermis, results from a combination of transcriptional and post transcripional regulatory events (Eckes *et al.*, 1993). Integrins of the β1 family on the surface of fibroblasts appear to be involved in mediating these effects. However, the mechanisms by which signals from the surrounding extracellular matrix are transmitted to fibroblasts and alter the expression of a specific set of

genes have not been investigated in detail. In striking contrast to normal fibroblasts, scleroderma fibroblasts cultured in three dimensional gel fail to down regulate collagen synthesis while maintaining their ability to contract the gel (Ivarsson *et al.*, 1993). The apparent failure of scleroderma fibroblasts to down regulate collagen biosynthesis when cultured in three dimensional matrices may reflect alterations in fibroblast matrix receptor function, and may be important in the development of fibrosis. The study of the modulation of collagen synthesis in fibroblasts propagated in three dimensional matrices has been a fruitful area for investigation.

Although human scleroderma fibroblasts may be readily grown and propagated in tissue culture there are still considerable difficulties in using these cells for experiments based on the limited numbers of cells and biopsies obtained (scleroderma is an uncommon disease), the variation in properties of cells obtained from different individuals, and their tendency to revert to the normal phenotype or senesce on prolonged passage in tissue culture. The generation of fibroblast cell lines maintaining their disease phenotype can help to overcome some of these difficulties. Indeed, these have been developed through the introduction of a number of different viral oncogenes into cells, such as in fibroblast cell line from Werner syndrome (Saito and Moses, 1991), and myogenic cultures from patients with Duchenne muscular dystrophy (Simon *et al.*, 1996). Establishing long term fibroblast cell lines from scleroderma has been described in Chapter 7.

1.6.1 Connective Tissues: composition and function

1.6.1.1 The collagen family: genes and proteins

Collagen constitutes a superfamily of related but genetically distinct proteins (Bailey and Black, 1988; Prockop and Kivirikko, 1995). According to the primary structure of a chains and their assembly into collagen molecules, Nineteen different collagen types are currently recognised (Prockop and Kivirikko, 1995). Their primary function is to maintain the structural integrity of tissues such as bone, tendon and skin.

The collagen family of proteins can be divided into 8 subgroups on the basis of the polymeric structures they form or related structural features: [1]. Collagens that form fibrils (types I, II, III, V, and XI), [2]. Collagens that form network-like structures (the type IV family, and types VIII and X), [3]. Collagens that are found on the surface of collagen fibrils and are known as fibril-associated collagens with interrupted triple helices (FACITs that include types IX, XII, XIV, XVI, and XIX), [4]. The collagen that forms beaded filaments (type VI), [5]. The collagen that forms anchoring fibrils for basement membranes (type VII), [6]. Collagens with a transmembrane domain (types XIII and XVII), and [7]. The newly discovered types XV and XVIII collagens that have been only partially characterized. An additional group [8] consists of proteins containing triple-helical domains that have not been defined as collagens (van der Rest and Garrone, 1991; Prockop and Kivirikko, 1995).

The Gene structures of the Fibril-forming collagens show a great deal of similarity (Vuorio and de Crombrugghe, 1990). One common feature of these genes is that the major triple-helical domain of each chain is coded for by 42 exons. Most of the exons are 54 bp and the others are either twice 54, three times 54, or combinations of 45 and 54 bp exons. Also, each exon begins with a complete codon for glycine, and therefore the exon codes for a discrete number of -Gly-X-Y- tripeptide units. In addition, the pattern of exon sizes is similar in all the genes and has been highly conserved throughout evolution.

Type I is the most abundant collagen and is found in a variety of tissues which belong to fibril-forming collagens. All these collagens (types I-III, V, and XI) are similar in size and in that they contain large triple-helical domains with about 1000 amino acids or 330 -Gly-X-Y- repeats per chain. In addition, they are also first synthesized as larger precursors, and the precursors need to be processed to collagens by cleavage of N-propeptides and C-propeptides by specific proteinases. Finally, they are similar in that they all assemble into cross-striated fibrils in which each molecule is displaced about one-quarter of its length relative to its nearest neighbour along the axis of the fibril.

The genes for the α2(I) chain of type I collagen and α1(III) chain of type III collagen contain alternative promoters that code for different polypeptides (Bennett and Adams, 1990). The alternative promoter of the COL1A2 gene is located within intron 2, and the transcript contains a short open reading frame that is out of frame with the collagen coding sequence (Bennett and Adams, 1990).

1.6.1.2 Collagen biosynthesis

Biosynthesis of collagen is complex and involves several post-translational modifications that are catalyzed by specific enzymes (Peltonen *et al.*, 1985). The type I and III collagens are the first synthesized as larger precursor molecules known as procollagens. The intracellular steps in the assembly of a procollagen are (Figure 1.2) cleavage of signal peptides, hydroxylation of Y-position proline and lysine residues to 4-hydroxyproline and hydroxylysine; hydroxylation of a few X-position proline residues to 3-hydroxyproline, addition of galactose or both galactose and glucose to some of the hydroxylysine residues, addition of a mannose-rich oligosaccharide to one or both of the propeptides, association of the C-terminal propeptides through a process directed by the structure of these domains, and formation of both intrachain and interchain disulfide bonds. After the C-propeptides have associated and each chain has acquired about 100 4-hydroxyproline residues, a nucleus of triple helix forms in the C-terminal region, and the triple helical conformation is then propagated to the N-terminus in a zipper-like manner (Kivirikko, 1993).

After secretion of procollagen from fibroblasts, the N-propeptides are cleaved by a procollagen N-proteinase and the C-propeptides by a separate procollagen C-proteinase. Later the collagen self assembles into fibrils that are then stabilized by intermolecular cross-link formation, which is catalyzed by lysyl oxidase.

Intracellular processing: Recently, analyses of cDNAs provided the complete amino acid sequences for the α subunit of 4-hydroxylase from human (Helaakoski *et al.*, 1989), and for the β subunit from several organisms (Kivirikko *et al.*, 1990). In addition, complete amino acid sequences have been reported for human lysyl hydroxylase (Hautala *et al.*, 1992).

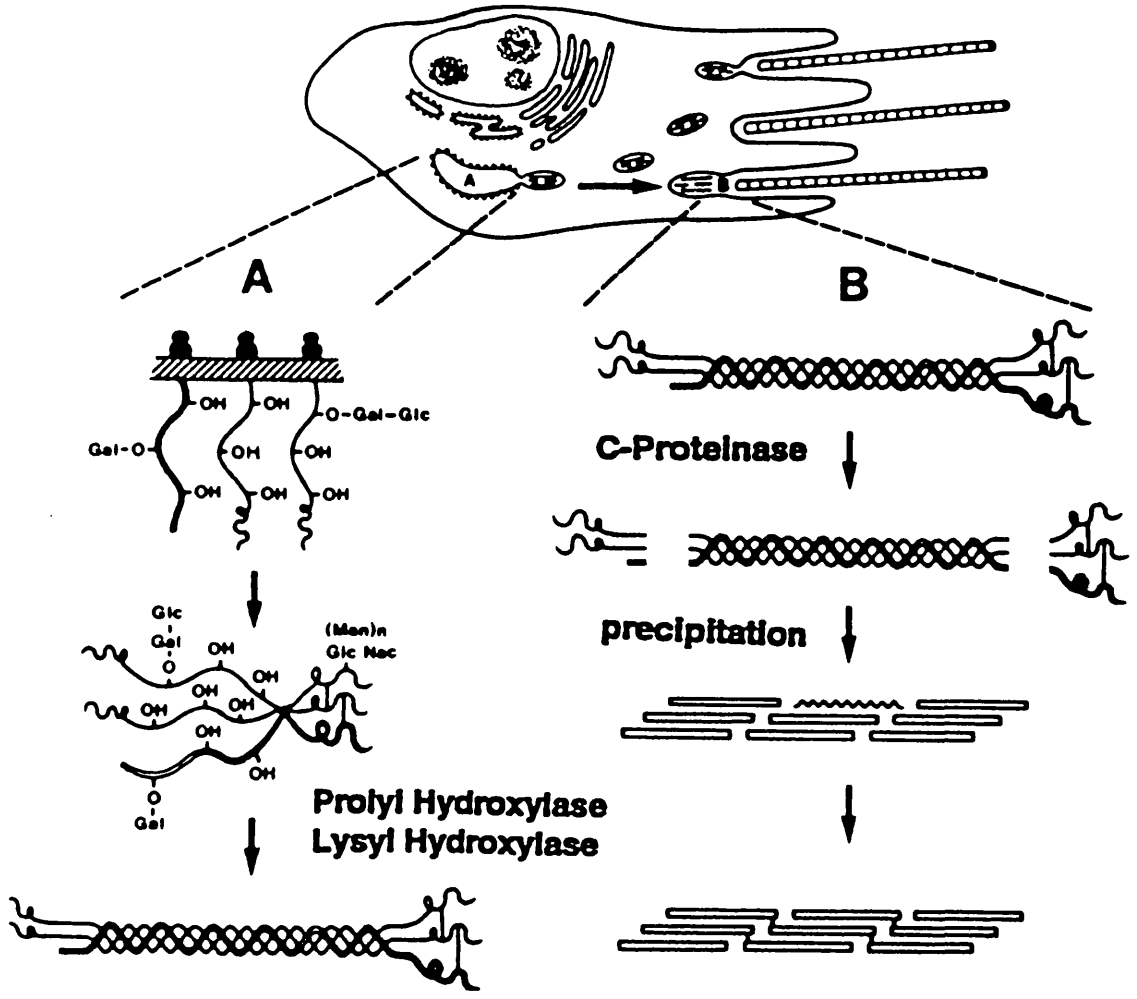


Figure 1.2. Steps in the biosynthesis and deposition of collagen. Shown at the top of the diagram is a fibroblast producing collagen. Key intracellular steps are shown under A and include synthesis of chains and their modification by prolyl hydroxylase during synthesis and assembly, leading to the formation of triple helical procollagen. Under B, the procollagen has been secreted, is converted to collagen by removal of segments at the ends of the molecule, precipitated into fibres, and crosslinked. Prolyl hydroxylase, C-proteinase, and lysyl hydroxylase are key enzymes. (Re-drawn and modified from Prockop and Kivirikko, 1995).

Recent reports also suggest that chain association and folding of type I and IV collagens may involve a specific molecular chaperone protein called Hsp47 or colligin (Nakai *et al.*, 1992; Sauk *et al.*, 1994). Hsp47 binds specifically to type I procollagen and to types I and IV *in vitro*. Cellular levels of the protein parallel the rates of synthesis of type I or type IV collagen in many experimental situations. Cross-linking studies in intact cells demonstrated association of type I procollagen with Hsp47, and this association was increased when cells were heat shocked or treated with the iron chelator a,a'-dipyridyl that effectively inhibits the hydroxylation of proline residues (Nakai *et al.*, 1992). Treatment of cells with antisense oligonucleotides to Hsp47 decreased the rate of synthesis of type I procollagen (Sauk *et al.*, 1994).

Extracellular events: Extracellular collagen fibrils are formed by secretion of a soluble procollagen that is then enzymatically processed to an insoluble collagen. Both the N- and C-propeptides of procollagens must be cleaved by specific proteinases for the proteins to self-assemble into fibrils under physiological conditions. The N- propeptides of both types I and II procollagens are cleaved by same specific procollagen N-proteinase (Hulmes, 1992). The N-propeptide of type III procollagen is probably cleaved by a different type III N-proteinase (Hulmes, 1992).

More recently, the process was studied in a system in which pCcollagen, a soluble and partially processed precursor lacking the N-propeptide, is cleaved to collagen by purified procollagen C-proteinase in a physiological buffer and at physiological temperature ranges (Prockop and Hulmes, 1994). Cleavage of pCcollagen to collagen reduces the solubility of the protein by about 1000-fold. The resulting collagen reproducibly self-assembles into tightly packed fibrils (Kadler *et al.*, 1987).

One series of experiments was carried out by isolating type I procollagen and cleaving it with C-proteinase to generate type I pNcollagen (Prockop and Hulmes, 1994). The pNcollagen assembled into thin, sheet-like structures that were cross-striated in longitudinal sections and of a uniform thickness. Mixtures of type I collagen and pNcollagen co-polymerized to form a variety of

pleomorphic fibrils. The results were consistent with the hypothesis that under some circumstances type I pNcollagen has a biological role in altering the morphology of type I collagen fibrils (Romanic *et al.*, 1992).

1.6.1.3 Collagen genes as models of genetic regulation

The human genes coding for collagen α1(I) and α2(I) chains are designated COL1A1 (chromosome 17q) and COL1A2 (chromosome 7q), respectively. COL1A1 is 18 kb long (Chu *et al.*, 1984). This complex gene contains 51 exons and intervening sequences (introns) of varying sizes. The pattern of exon structure and size is conserved among different collagen types, suggesting a common evolutionary origin. The COL1A1 gene contains multiple polyadenylation signals at the 3' end, resulting in mRNA transcripts of two sizes which differ in the lengths of their untranslated 3' regions. The functional significance of the multiple mRNA species is unknown.

The type I collagen genes are large and difficult to manipulate. De Crombrugghe's group has assumed that in these genes, as in most other genes, the regulatory sequences that determine the rate of transcription of these genes are localized at their 5'end. Several laboratories have therefore focused their attention on the regulatory elements present at the 5' end of these genes (de Crombrugghe *et al.*, 1990). To facilitate the study of regulatory elements of the type I collagen genes, they have substituted most of the structural protein of these genes with a much smaller surrogate or marker gene. One commonly used marker gene is the bacterial chloramphenicol acetyltransferase (CAT) gene which is not endogenous to eukaryotic cells (Gorman *et al.*, 1982). To study the function of the regulatory DNA segments, collagen genes can be introduced into fibroblasts by a gene transfer method called DNA transfection. In transient expression experiments fibroblasts are harvested 48 hours after DNA transfection, lysed, and assayed to determine the activity of CAT. Such experiments provided information about the activities of these regulatory the type I collagen genes in fibroblasts.

For approaches to study DNA regulatory elements, DNA transfection and *in vitro* DNA binding studies, are two of the major approaches that were used to

study some of the regulatory elements of the α1(I) and α2(I) collagen genes. To better understand the overall transcriptional activity and transcriptional controls of the type I collagen genes, a comprehensive study is needed of the various cis acting elements and their cognate DNA binding factors.

Regulatory elements of the α2(I) collagen gene are located on each side of the start of transcription. Starting from within the gene one finds a cell-specific transcriptional enhancer in the first intron (Rossi and de Crombrugghe, 1987). As in the α2(I) collagen gene, regulatory elements of the α1(I) gene are also distributed on either side of the start transcription. The first intron appears to contain both stimulatory and inhibitory segments but the precise delineation of these sequences has not yet been established (Rossouw *et al.*, 1987). The finding that the first intron of COL1A1 contains enhancer elements is based on studies involving insertion of the retrovirus Mov13 in this intron. Fibroblasts from transgenic Mov13 mice have been used as a model in which the endogenous collagen α1(I) production is greatly impaired (Dezamba *et al.*, 1993).

The ECM was once thought to have primarily structural functions and to provide the mechanical scaffold for cells and tissues. It is now clear that specific structural domains of ECM constituents are endowed with various biological activities. ECM serves as a reservoir for growth factors and as source of information for cells. Cell adhesion-promoting activity has been characterized for collagen I (Davis, 1992). Interestingly, cell adhesion to collagens is dependent on the helical conformation of the molecules. However, denatured collagen I can also induce cell adhesion. The influence of collagenous molecules on cellular activities might also be mediated by collagen peptides (Katayama *et al.*, 1993). In particular, N-propeptides of collagen were found to have feedback regulatory effects on collagen biosynthesis in fibroblasts. Degradation of collagen by bacterial collagenase results in small peptides that were found to have high chemotactic activity for fibroblasts and other cells (Postlethwaite *et al.*, 1978).

1.6.1.4 Inhibiting collagen biosynthesis

Collagen synthesis may be directly inhibited at several stages. These include procollagen gene transcription, mRNA translation, pre-collagen hydroxylation or

glycosylation, intracellular transport, cleavage of procollagen peptides or chain polymerization and cross-linking (Coker and Laurent, 1995).

Recent studies have demonstrated that synthesis of type I collagen can be specifically inhibited in cell culture by the use of antisense oligonucleotides (Laptev *et al.*, 1994). However, the degree of inhibition obtained is highly variable and rarely exceeds 50% (Colige *et al.*, 1993; Laptev *et al.*, 1994). In related experiments, an antisense gene to human type I collagen in which only the 3'-half was inverted was shown to be highly effective in inhibiting collagen synthesis in transgenic mice expressing an internally deleted human COL1A1 minigene (Khillan *et al.*, 1994). These results raised the possibility that chimeric gene constructs that contain intron sequences and in which only part of the gene is inverted may be particularly effective as antisense genes that can inhibit collagen synthesis in fibrotic conditions. However, both the antisense-oligonucleotide strategy and the antisense-gene strategy appear to present considerable problems in the delivery of the agents in ways that will be effective in inhibiting fibrosis *in vivo*. Procollagen C-proteinase is another possible target for inhibition of fibrosis. Most of the available evidence suggests that procollagen cannot participate in fibril assembly unless the C-propeptide is specifically cleaved from the precursor (Hulmes, 1992). Initial studies suggested that basic amino acids and peptides may specifically inhibit C-proteinase (Prockop and Kivirikko, 1984).

Several of the post-translational enzymes appear to be attractive targets for specific inhibition because they are unique to collagen biosynthesis. These include prolyl 4-hydroxylase, procollagen C-proteinase, and perhaps also lysyl hydroxylase and lysyl oxidase. Numerous compounds are now known that inhibit prolyl 4-hydroxylase competitively with respect to some of its cosubstrates or the peptides substrate (Kivirikko *et al.*, 1989). Several attempts have been made to develop inhibitors for lysyl hydroxylase. Minoxidil and many of its derivatives have the surprising effect of reducing both lysyl hydroxylase activity (Murad *et al.*, 1992) and the mRNA (Hautala *et al.*, 1992) in cultured cells. Their mechanism of action is unknown. Also, whether inhibition of lysine hydroxylation will in itself be effective in inhibiting fibrosis is unclear.

1.6.2 Fibrosis in scleroderma

1.6.2.1 Fibroblast adhesion molecules

The communication between cell-cell and cell-ECM is mediated by specific receptors located on the fibroblasts. There are two important adhesion molecules in scleroderma fibroblasts: ICAM-1 and collagen binding integrins. Integrins also interact with cell-surface Ig superfamily receptors, such as ICAM-1 (for review see Springer, 1990; Mojick and Shevach, 1997).

Human intercellular adhesion molecule-1 (ICAM-1, CD54) is an important cell surface molecule of the immune system mediating antigen-independent cell-cell contact between leukocytes and cells of various origins. Originally identified as an adhesion molecule that mediates the aggregation of phorbol ester-stimulated lymphocytes (Rothlein *et al.*, 1986), ICAM-1 serves as counter-receptor for the leukocyte integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) (Rothlein *et al.*, 1986; Diamond *et al.*, 1990). Isolation of cDNA revealed that ICAM-1 is a member of the immunoglobulin supergene family and contains five immunoglobulin-like domains, a single transmembrane region and a short cytoplasmic domain (Simmons *et al.*, 1988).

Although weakly expressed in non-stimulated cells, ICAM-1 expression is upregulated by inflammatory mediators such as the cytokins TNFα, IL-1β and IFNγ as well as LPS and PMA on the surface of many cell types, including fibroblasts (Myers *et al.*, 1992; Shi-wen *et al.*, 1994).

Abraham *et al* (1991) showed increased expression of ICAM-1 on scleroderma fibroblasts and both resting and activated T cells attached far more readly *in vitro* to scleroderma fibroblasts than to normal fibroblasts. These observations could be explained by increased expression of cell-cell and cell-matrix adhesion molecules, such as β1 integrins and ICAM-1 on the surface of scleroderma fibroblasts (Sollberg *et al.*, 1992; Needleman, 1990). These molecules play a major role in the "homing" of pathogenic lymphocytes to skin and their adhesion to tissue fibroblasts (Springer, 1990).

In situ hydribization studies have shown that dermal fibroblasts displaying elevated levels of collagen mRNA transcripts, indicative of on-going collagen synthesis, are frequently localized in perivascular areas, generally in the vicinity of activated inflammatory cells (Kahari *et al.*, 1988). Similarly, enhanced expression of ICAM-1 is seen on fibroblasts that are close to infiltrating inflammatory cells (Gruschwitz *et al.*, 1992). The close association of infiltrating immune cells and biosynthetically activated fibroblasts in scleroderma tissues suggests that direct immune cells-fibroblast interactions, or immune cell-derived informational molecules may be responsible for fibroblast activation in scleroderma.

Induction of ICAM-1 correlates with increased cytoplasmic levels of ICAM-1 mRNA suggesting that regulation of ICAM-1 expression occurs mainly at the transcriptional level although post-transcriptional mechanisms can also be involved. To study ICAM-1 regulation at the transcriptional level, the 5'-regulatory region of human ICAM-1 gene was cloned and analyzed in detail (Voraberger *et al.*, 1991). ICAM-1 gene expression in scleroderma and normal fibroblasts and regulation by ET-1 are investigated within this thesis (chapter six).

1.6.2.2 Oncogenes in fibrosis

Proto-oncogenes code for protein that generally act as transcription factors, such as c-fos, c-jun, c-myc, and c-myb, which have been implicated in the regulation of DNA synthesis and other events linked to cell division (Heldin and Westermark, 1990). In studying the rapid induction of c-myc gene by PDGF in BALB/c-3T3 mouse fibroblasts, it was shown that an 81-basepair sequence with the c-myc promoter function on the growth factor-responsive element (Sacca and Cochran, 1990). To probe the basis of proliferation in scleroderma fibroblasts, Trojanowska *et al* (1988) have investigated c-myc gene expression and found that under low serum condition (1% serum), scleroderma fibroblasts express a 2.5-3 times higher level of c-myc message than do normal fibroblasts. Others have reported increased expression of proto-oncogenes in scleroderma fibroblasts (Feghali *et al.*, 1993; Trojanowska *et al.*, 1996). Using the RT-PCR reaction technique, Piccinini *et*

al (1996) detected c-myb transcripts in scleroderma skin fibroblasts rendered quiescent by serum deprivation, but did not find a similar effect in normal fibroblasts. b-myb message was 1.5-5 times higher in most pathologic cells studied. These data suggest that the b-myb and c-myb genes may play a role in scleroderma fibroblast proliferation and function.

The early expression of PDGF AA, BB and AB ligands in scleroderma skin (Gay *et al.*, 1989), later to be associated with increased expression of PDGF receptors (Klareskog *et al.*, 1990) and ras proto-oncogene products (Gay *et al.*, 1992), may epitomize certain molecular interactions in the pathogenesis of scleroderma.

1.6.2.3 Alterations of collagen gene expression

The regulation collagen is crucial for the complete development of vertebrates and in the maintenance of extracellular matrix. Abnormal collagen metabolism plays a central role in the pathogenesis of a number of connective tissue diseases, and in particular excessive type I collagen deposition is a primary feature seen in multiple organs of patients with systemic sclerosis. The precise mechanism by which this over-production of structurally normal collagen occurs is uncertain.

Increased transcriptional activity of the α2(I) procollagen gene (COL1A2) in scleroderma skin fibroblasts was documented by transient transfections of constructs containing the human COL1A2 gene promoter ligated to the CAT reporter gene into normal and scleroderma skin fibroblasts (Kikuchi *et al.*, 1992). The results showed that the constructs containing the normal gene promoter displayed 3-5 fold higher transcriptional activity when transfected into scleroderma fibroblasts compared with normal cells.

Hitreya and Jimenez (1996) examined the transcriptional regulation COL1A1 gene expression in scleroderma fibroblasts and evaluated the role that intronic sequences may play in the up-regulated expression of this gene in skin fibroblasts from scleroderma patients. Their results demonstrated that a construct containing -804 bp of the COL1A1 promoter showed maximal transcriptional activity and that the activity driven by this promoter region was around 2 fold higher when transfected into scleroderma fibroblasts compared with normal

cells. Furthermore, they found that inclusion of sequences from the first intron resulted in much higher transcriptional activity in both normal and scleroderma cells, and augmented by up to 4-fold the differences in transcriptional activity between normal and scleroderma cells. The results directly demonstrated transcriptional activation of COL1A1 in skin fibroblasts from scleroderma patients.

The collagen promoters contain elements that are targets for growth factors and cytokines , such as TGFβ, that induced activation of collagen gene transcription (Jimenez *et al.*, 1994). Specific DNA sequences in the proximal 5′ region of collagen genes that appear to be necessary for TGFβ-induced transcriptional stimulation in transiently transfected cell have been localized. In human COL1A1 this region locates -170/-80 and binds Spl-like protein (Jimenez *et al.*, 1994); In human COL1A2 locates -378/-183 and binds Spl and other unidentified protein (Inagaki *et al.*, 1994). These data indicate that different DNA sequences are utilized by TGFβ in the different collagen genes to activate transcription. The various "TGFβ-response elements" of the collagen genes are potential targets for trans-acting factors which modulate transcriptional activity. Much current research is aimed at identifying DNA-binding proteins specifically interacting with the cis regulatory elements, and characterizing their modifications in TGFβ-treated cells.

1.6.2.4 Regulation of collagen synthesis and turnover

1.6.2.4.1 Metalloproteinases

The degradation of collagen is a normal event in the physiological remodeling associated with morphogenesis and growth, as well as in processes such as wound healing (Murphy and Reynolds, 1993). The triple-helical structure of the fibrillar collagens, such as types I and III, is extremely resistant to the action of most proteinases, and resistance is enhanced by their being in tightly apposed fibrils, which can become heavily cross-linked in mature collagen. The family of matrix metalloproteinases (MMPs) include an interstitial collagenase and a neutrophil collagenase, which uniquely cleave the interstitial collagens at a single locus within the native helical structure at about 3/4 of the distance from the N-

terminal end. Cleaved portions of the helix quickly lose their triple-helical conformation (Birkedal-Hansen *et al.*, 1993).

Less well studied is collagen degradation in scleroderma. Changes in collagen degradation are controversial. Early reports suggested that collagenase levels were not altered in scleroderma skin (Uitto *et al.*, 1979) but this disagrees with more recent findings which shown decreased collagenase expression in cultured scleroderma fibroblasts (Takeda *et al*, 1994). Collagenase (MMP-1) is synthesized in an inactive form and controlled by various tissue inhibitory metalloproteases (TIMPS) (Birkedal-Hausen *et al*, 1993). TIMP-1 levels are elevated in sera of scleroderma patients but not those with other autoimmune rheumatic diseases (Kikuchi *et al.*, 1995). Discordant regulation of stromelysin and TIMP-1 in scleroderma fibroblasts has been reported by our group (Bou-Gharios *et al*, 1995). This work is supported by the findings another group who have reported elevated TIMP-1 levels produced by scleroderma fibroblasts (Kirk *et al.*, 1995).

1.6.2.4.2 Integrins

It is now widely appreciated that adhesion interactions between cells and matrix proteins are mediated largely by a class of heterodimeric cell surface molecules known as integrins, which are typically classified by their alpha and beta chains (Hynes, 1992). Integrins bind to a number of ECM proteins including fibronectin, fibrinogen, laminin, collagen, thrombospondin, vitronectin and von Willebrand factor mainly through RGD sequences. Only two integrins present on fibroblasts are known to bind efferently to fibrillar collagens (Kupper and Ferguson, 1993). These integrins, $\alpha 2\beta 1$and $\alpha 1\beta 1$, appear to have different functions despite the fact that they bind to similar sequences on the $\alpha 1$(I) chain of the type I collagen molecule (Gullberg *et al.*, 1992).

It has been previously observed *in vitro* that predominant expression of $\alpha 1\beta 1$ by fibroblasts is characteristic of quiescent or steady state cells, while predominant expression of $\alpha 2\beta 1$ is characteristic of activated fibroblasts (Fingerman and Hemler, 1988). Determination of surface expression of $\beta 1$-integrins on cells grown on plastic showed that scleroderma fibroblasts had a decreased expression of the $\alpha 1$-integrin subunit, as compared to their normal counterparts, supporting

a theory that scleroderma fibroblasts have a disturbed interaction with collagen. There is evidence that α1β1 is diminished on scleroderma fibroblasts (Ivarsson *et al.*, 1993). Langholz *et al* (1995) were able to enhance downregulation of collagen pro-α1(I)mRNA in collagen gel by the combined addition of α1 and β1-integrin antibodies.

1.6.2.4.3 Cytokines and growth factors

One of the earliest studies of extrinc modulation of collagen metabolism was that of Johnson and Ziff (1976) who demonstrated that supernatants from mitogen-stimulated normal human mononuclear cells enhanced the proliferation and collagen synthesis by embryonic fibroblasts *in vitro.* This work stimulated an entire field of investigation focusing on the regulation of fibroblast function by cytokines. Later, it was shown that lymphocytes and monocytes elaborate factors that can stimulate or inhibit fibroblast (Neilsion *et al.*, 1982; Postlethwaite *et al.*, 1976). The identification and characterization of specific cytokines, and cloning of the corresponding genes, has allowed the examination of specific effects of cytokines on fibroblast function to be performed.

A growing number of cytokines and growth factors have been found to modulate collagen biosynthesis and other function by fibroblasts (Table 1.5).

1.7 Animal models of scleroderma

Animal models of scleroderma have provided valuable insights into the mechanisms and the pathogenesis of this disease, and a means of testing potentially useful therapeutic interventions. A number of experimental systems that reproduce some of the pathologic alterations of this disorder have been described. A brief account of some of the animal models used in research is given below.

1.7.1 Naturally-occurring models of scleroderma

Avian scleroderma: One of the most unique genetically transmitted animal models of scleroderma is the University of California at Davis (UCD) line 200 (L200) chickens, which were propagated from an original mutant discovered in 1942 by Bernier and first described by Gershwin *et al* (1981) and van de Water and Gershwin (1985).

Table 1.5. ***In vitro*** **modulation of fibroblast functions by cytokines**

Fibroblasts function	Stimulation	Inhibition
Collagen biosynthesis	TGFβ, IGF-1, PDGF, IL-1, ET-1, IL-4, IL-6	IFNα,γ, TNFα, FGFs, EGF IL-8, IL-10, Prostaglandins
Proliferation	IL-1α and β, TGF, PDGF, CTGF, ET-1	IFNγ
Chemotaxis	TGFβ, IL-4, TNFα, PDGF, ET-1	
Glycosaminoglycan	IL-1α and β, TGFβ, TNFα	
Fibronectin	IL-4, TGFβ, CTGF	
Collagenase	IL-1α and β, TNFα, bFGF, IFNγ	TGFβ, ET-1, IL-4
TIMP	IL-1α and β, PDGF	IL-6, TGFβ
Prostaglandin E2	TNFα, IL-1α and β	

Derived from the following references: Black, 1995; Fagundus and LeRoy, 1995; Feldman *et al.*, 1994; Frazier *et al.*, 1996; Kahaleh, 1991; Salmon-Ehr *et al.*, 1996; Vuorio and de Crombrugghe 1990.

* The biological activities of some cytokines, such as TGFβ, CTGF, ET-1, are discussed further Chapter 4, 5, 6.

Despite the remarkable similarities in immunologic and biochemical changes, there are important differences between the avian disease and human scleroderma. These include: (i). The onset is much more acute; (ii). Both genders are equally affected; (iii). The vascular involvement is characterized both by proliferation of smooth muscle and sub-endothelial fibrosis; (iv). Autoantibodies, such as anti-ssDNA, rheumatoid factor, and anti-type II collagen, are more commonly present in the L200 chickens.

The tight skin (Tsk) mouse: Another inherited model of scleroderma is the Tsk mouse, a spontaneous dominant mutation that occurred in the inbred B10.D2(58N)/Sn strain (the original referred here as Tsk1), which was identified at the Jackson Laboratories by Helen Bunker and reported in detail by Green *et al* (1976). The Tsk1/+ mice (heterozygous) display cutaneous and visceral changes that closely resemble those present in patients with scleroderma. Furthermore, the biochemical and molecular abnormalities have been demonstrated in these animals (Jimenez *et al.*, 1984), but they do not develop the inflammatory and vascular changes nor the alterations in cellular immunity postulated to play a crucial role in scleroderma pathogenesis.

TSK/NZB mouse hybrids: The new murine model of scleroderma was developed by breeding the Tsk1/+ mice and the autoimmune disease-prone NZB mice to obtain an F1 hybrid displaying the connective tissue abnormalities of Tsk1/+ parent and the autoimmune abnormalities of the NZB parent (Bocchieri *et al.*, 1993). The Tsk/NZB mouse model more closely resembles the human disease condition than does its predecessor, the Tsk1/+ mouse. The (Tsk1/NZB)F1 mice may be of value for the study of the interactions between the immune system and connective tissue cells in the pathogenesis of scleroderma.

The Tsk2 - a novel mutation resembling scleroderma: A new mutation characterised by the occurrence of a tight skin phenotype in mice was first reported in 1986 (Peters and Ball). The mutation appeared in the offspring of a male from the 101/H mouse strain as a result of administration of the mutagenic agent, ethylnitrosourea. This novel mutation has been called Tsk2 and has been localised to mouse chromosome 1 (Peters and Ball, 1986). The occurrence of a

novel and separate, chemically-induced mutation that results in the excessive accumulation of dermal collagen is of great relevance. This new mutation will allow the identification of a gene distinct from that affected by the Tsk1 mutation. The Tsk2 mutation may provide valuable information regarding the role of environmental exposures in the pathogenesis of scleroderma and other chemically-induced fibrotic diseases (Christner *et al.*, 1995),

1.7.2 Chemically-induced models of scleroderma

Homologous disease and graft-versus-host disease (GVHD): The first animal models developed that resembled scleroderma was the "homologous disease" in rats (Statsny *et al.*, 1963). In this model donor lymphoid cells from were injected into neonatal Sprague-Dawley rats to induce immunologic tolerance. This caused the immunologic attack of the recipient's tissue by the donor's cells-a "graft-versus-host" response. Animals surviving in the acute phase of the "homologous disease" evolved into a chronic stage characterised by severe dermatitis with dermal and subdermal fibrosis, prominent mononuclear cell infiltration and the accumulation of connective tissue closely resembling affected skin from patients with scleroderma of recent onset (Jaffee and Claman, 1983).

Bleomycin-induced fibrosis: The occurrence of fibrotic changes and thickening of the skin in mice was seen following treatment with the antitumor antibiotic bleomycin (Ichihashi *et al.*, 1973). The lungs of treated animals showed significant pulmonary fibrosis (Adamson and Bowden, 1974), which was also reported to be similar to the lesions in the lungs of treated rabbits (Laurent *et al.*, 1981). The pathologic alterations observed in the lungs of these animals bear striking resemblance to the histopathologic changes present in the lungs of patients with scleroderma. However, the cutaneous lesions induced by bleomycin display minimal skin inflammatory changes without lymphocytic infiltration. Furthermore, these animals do not develop an autoimmune response, such as absence of autoantibodies (Mountz *et al.*, 1983).

1.8. Objectives of this study

It is recognised that scleroderma exhibits three characteristic pathologies; immune dysfunction, abnormalities of the vascular endothelium and connective tissue fibrosis. Most if not all of the vascular obliteration, tissue and organ damage associated with the disease process can be attributed to the excessive production and deposition of ECM during the fibrotic phase of the disease. The aims of this study were therefore to investigate the biology of the fibroblast within the context of the fibrotic disease, scleroderma. In particular the work focused on the regulation of fibroblast metabolism and the changes in both phenotypic and functional activities of this cell in scleroderma. Major objectives of this study were to examine how these cells respond to their extracellular environment and to specific stimuli such as cytokines and growth factors produced by other cell types such as endothelial cells and cell from the immune system. An increased understanding of these complex interaction may provide clues to the fundamental mechanism(s) that control the fibrotic process in scleroderma.

The specifc aims of this study:

1. Examine the functional activities of normal and scleroderma fibroblasts in monolayer and 3-dimensional collagen gels, and to compare collagen biosynthesis and the transcriptional regulation of collagen gene (COL1A2) in skin and lung fibroblasts.

2. Investigation of the response of normal and scleroderma dermal fibroblast to endothelin-1 (ET-1) and connective tissue growth factor (CTGF).

3. Examination of the regulation of ICAM-1 by scleroderma and normal fibroblasts, and the influence of ET-1 on adhesion molecule expression.

4. To establish long-term scleroderma fibroblast cell lines by retroviral transduction with the temperature-sensitive SV40 large T-antigen and investigate the persistence of the fibrotic phenotype.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Patient biopsies and clinical details

2.1.1 Patients

All the scleroderma patients examined in this thesis fulfilled the American College of Rheumatology (ACR) preliminary criteria for disease (1980) and were classified into dcSSc and lcSSc groups, using internationally accepted criteria (LeRoy *et al.*, 1988).

2.1.2 Clinical details

The clinical and laboratory parameters used for the assessment of disease included duration of scleroderma, skin score, internal organ involvement, and serology. The extent of skin involvement in patients with scleroderma was assessed 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.

Lung involvement consisted of pulmonary hypertension (PHT) and scleroderma-associated fibrosing alveolitis (FASSc). PHT was diagnosed in scleroderma patients when the mean arterial pressure was greater than 25/10 mm Hg, either by right heart catheterisation or Doppler echocardiogram (Black and Denton, 1997). The diagnosis of FASSc was made on the basis of evidence of abnormalities on high resolution computed tomography (CT) scan, a restrictive pulmonary deficit and/or reduced gas transfer measurement and the exclusion of other known causes of alveolar fibrosis (Harrison *et al.*, 1991).

Scleroderma associated autoantibodies of all patients were tested in the Department of Clinical Immunology at the Royal Free Hospital. Anti-nuclear and anti-centromere antibodies were detected by indirect immunofluorescence on Hep-2 cells and anti-topoisomerase I (anti-Scl-70) antibodies were detected by immunodiffusion performed on agarose gels.

2.1.3 Biopsies

4 mm^3 punch biopsies were taken from the skin of patients and normal healthy volunteers in the Rheumatology Unit and Department of Surgery at the Royal Free Hospital. Scleroderma skin biopsies were taken from involved skin. For diffuse scleroderma patients, biopsies were usually obtained from forearm skin on the non-dominant limb. For limited scleroderma, biopsies were from the dorsal surface of the non-dominant hand. Control biopsies were obtained from healthy volunteers and surgical operation.

Lung biopsies were taken from the affected areas of scleroderma patients with FASSc and compared with normal lung biopsies taken from the unaffected areas of age matched individuals undergoing lung resection for various malignancies in the National Heart and Lung Institute, Royal Brompton Hospital.

2.2 Tissue culture

2.2.1 Culture of fibroblasts in monolayer

Fibroblasts were obtained from the biopsies by *in vitro* culture as previously described (Abraham *et al.*, 1991). Briefly, dermal and lung biopsies were cut into 1-2 mm^3 pieces and placed in sterile plastic dishes or flasks. After 15 minutes of drying at room temperature the pieces of biopsy were adherent to the tissue culture plastic and then cultured in fibroblast growth medium (FGM) consisting of Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units per ml penicillin, 100 μg per ml streptomycin, 50 μg per ml gentamycin and 2.5 μg per ml amphotericin B. After 2-3 weeks of incubation in a humidified atmosphere of 5% CO_2 in air, the fibroblast outgrowths were detached by brief trypsin treatment and recultured in FGM, but without gentamycin and amphotericin B. In experiments, fibroblasts were used between passages 2 and 5 unless otherwise stated. The fibroblast phenotype was confirmed by their typical morphology in monolayer and three-dimensional collagen gel culture (Chapter three, Figure 3.4).

2.2.2 Culture of fibroblasts in collagen gel

Three-dimensional collagen (3-D) gel: Collagen type I gels were prepared as described previously (Ivarsson *et al.*, 1993). In outline, 24-well tissue culture plates were pre-coated with sterile 2% BSA in PBS (2ml/well) incubated at 37°C overnight to prevent the gels from becoming tightly bound to the plastic. The plates were then washed three times with sterile PBS. Trypsinized skin and lung fibroblasts suspended in MCDB medium were mixed with collagen solution (one part of 0.2 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], pH8.0; four parts collagen [Vitrogen-100, 3 mg/ml] and five parts of MCDB X 2) yielding a final concentration of 80,000 cells per ml and 1.2mg/ml collagen. Collagen/cells suspension (1 ml) was added to each well, after which plates were immediately incubated at 37 °C to allow collagen gels to form. After 1 hour, 1 ml of MCDB medium was added to each well, causing detachment of the collagen gels from the tissue culture plastic.

Contraction of the gel was quantified by loss of gel weight and decrease in gel diameter (using an ocular micrometer) over a 24 hour period. Initial experiments suggested that gel weight at 24 hours, corresponding to maximum contraction, correlated with the degree of contraction at earlier time points between 2 and 22 hours.

Two-dimensional collagen (2-D) gel: To facilitate microscopic examination of morphological changes in the skin and lung fibroblasts associated with gel contraction, fibroblasts suspended in serum free DMEM (0.05ml containing $5x10^4$ cells) were placed on the surface of a polymerised collagen gel, prepared as described above but without the inclusion of cells within the gel. These fibroblasts had previously been grown to confluence in DMEM/10% FCS, as describe above. These gels were incubated for 30 minutes at 37 °C, allowing the cells to adhere to the gel surface and then covered with 1ml of medium. After 8 hours, fibroblasts were photographed using an inverted phase-contrast microscope to provide a permanent record of morphological characteristics.

2.2.3 Culture of monocytic cell line and endothelial cell lines

The human monocytic cell line U937 (Harris and Ralph, 1985) was used because it grows well in standard medium (RPMI 1640 with 5% FCS), it has an established place in the investigation of leucocyte-EC binding (Carvalho *et al.*, 1996) and can readily be identified morphologically and distinguished from fibroblasts by light microscopy when cells were counted using a haemocytometer. It is also readily labelled using tritiated thymidine.

Endothelial cell lines: The extended lifespan SV40 transfected human umbilical vein (HUVEC) derived endothelial cell line 1E-7 (Flicking *et al.*, 1992) and dermal microvascular cell line HMEC-1 (Ades *et al.*, 1992) were employed. Cultures were maintained in standard endothelial cell growth medium (M199/ECGS/ heparin/20%FCS). For experiments, both cell lines were cultured in DMEM with 10% FCS.

2.3 Measurement of fibroblast DNA synthesis and growth

2.3.1 DNA synthesis measured by [^{3}H] thymidine incorporation

Thymidine incorporation is the most commonly used assay for labelling studies of DNA synthesis. For experiments in this thesis, dermal fibroblasts were cultured in 96 well plates at a density of 5,000 cells per well. After culture for 48 hours in DMEM with 10% FCS, the medium was replaced by DMEM containing 0.5% BSA for 24 hours. Quiescent cells were then incubated for a further 48 hours containing the test substances (different concentrations of ET-1 10^{-8} to 10^{-12} M/ml or CTGF 5 to 80 ng/ml). Experiments were performed in serum free conditions and 1μCi of radiolabelled thymidine ([^{3}H]-TdR) was added during the final 18 hours of culture. At the end of this period labelled culture medium was aspirated from each well. Cell monolayers were washed three times using PBS at room temperature (200μl per well), and then followed by washes with 5% tricholoroacetic acid. After washing, cells were solubilised in 200μl of 0.1N NaOH/0.1% sodium dodecyl sulphate (SDS) for 5 minutes. The contents of each well were transferred to a b-vial, the well washed with 200μl of distilled water which was transferred to the same vial and 3.5 ml of scintillant fluid (OptiPhase safe, LKB Scintillation Products Ltd., UK) added to each vial. Radioactivity was

measured using a liquid scintillation counter, comparing each sample's counts (disintegrations per minute) with background tubes.

To inhibit the synthesis and action of endogenous ET-1, quiescent cells were preincubated with the specific ET_B receptor antagonist BQ-788 (100nM), ET_A receptor antagonist PD 156707 (100nM) (Reynolds *et al.*, 1995) or $ET_{A/B}$ receptor antagonist Ro 47-0203 (bosentan, 100nM) (Clozel et al., 1994) were used to block ET receptors. The cells were then pulsed with 1 μCi of [^{3}H] Thymidine per well for 18 hours. After washing, cells were precipitated with two hundred μl of 0.1N NaOH/0.1% sodium dodecyl sulphate (SDS). The radioactivity was measured using a liquid scintillation system.

2.3.2 Growth characteristics of control and tsT-transduced fibroblasts in normal and scleroderma

Fibroblast cultures that had been grown at 36.5°C for 24 hours were detached by trypsin treatment and 2 x 10^5 cells were seeded into 25cm^2 plastic culture flasks in complete DMEM. After a further 24 hours at 36.5°C, half of the cultures were transferred to 35°C and the other half cultured at 39.5°C. Triplicate cultures were subsequently harvested by trypsin treatment at 2 day intervals and single cells counted directly using a haemocytometer, after staining with trypan blue. Cell viability was routinely found to be greater than 98%.

The anchorage-dependence of the fibroblasts was studied by examining their ability to grow in soft agar, as previously described (Stamps *et al.*, 1994). Replicate fibroblast suspensions containing 10^5, 10^4 and 10^3 cells in agar (0.3% w/v) (Noble Agar, Difco) were placed into 6-well plastic dishes previously coated with a base layer of 0.6% agar. The top layer of agar, which contained the cells, was overlayed with complete DMEM and the cultures incubated at 35°C and 39.5°C for 3 weeks. The colony-forming efficiency of the fibroblasts was determined by visual examination of the wells under the microscope and enumeration of the number of multicellular colonies which were greater than 100 μm in diameter.

2.4. Enzyme-linked immunosorbent assays (ELISA) for measuring ICAM-1 and matrix secretion

2.4.1 Capture ELISA for measuring the levels of sICAM-1

Two-epitope "sandwich" enzyme-linked immunosorbent assays (ELISA) were used to measure soluble adhesion molecules, as previously described (Mason *et al.*, 1993). The assay to measure sICAM-1 used affinity-purified MAb 6.5B5 and MAb 8.4A6 were used for capture and detection respectively. These antibodies identify two distinct epitopes on ICAM-1, are non-competitive in cross-blocking experiments and have been used to detect circulating ICAM-1 in plasma (Mason *et al.*, 1993).

Briefly, 96-flat-well microtitre plates (type SP-3; Alpha Labs, Hampshire, UK) were pre-coated overnight with Mab at 10μg per ml (50μl per well) in 100mM carbonate buffer, pH 9.6, at 37°C. The plates were then blocked with 5% (w/v) dry milk powder in PBS for 60 min at room temperature (200μl per well). All other manipulations were performed at ambient temperature. Following blocking, the plates were washed with PBS containing 0.25% (v/v) Tween-20 in PBS (PBS-T) and the supernatant samples added for 2 hours. After binding, the plates were again washed with PBS-T and the 'captured' molecules detected by the addition of the biotinylated Mab, followed by incubation with the streptavidin-biotin-peroxidase Elite ABC complex (Vector labs, Peterborough, UK). After washing, the substrate (0.5 mg per ml o-phenylenediamine(OPD) in 50 mM citrate-phosphate buffer, pH 5.5, containing 0.003% hydrogen peroxide) was added to the plates, and colour development terminated by the addition of 50 μl of 2 M sulphuric acid. Optical density was measured at 491 nm using a Bio TeK EL310 microplate reader. For quantitation, one plasma sample with a previously determined level of circulating ICAM-1 (Mason *et al.*, 1993) was used as a standard and included in each assay plate. The results are expressed as ng per ml for soluble ICAM-1, based on absorbance relative to these standards and were adjusted to correspond to the released levels in neat culture media. The values obtained with samples which were incubated in control wells pre-coated

with an irrelevant mouse immunoglobulin (10μg per ml; Serotec, Oxford, UK), were subtracted from test measurements.

2.4.2 Cell bound ELISA technique for fibroblast ICAM-1 expression

This was measured using a cell bound ELISA technique (Pober *et al.*, 1986). Dermal fibroblasts were seeded in 96 well culture plates at a density 5000 cells per well. After culture for 48 hours in DMEM/10% FCS the medium was replaced by serum free (0.5% bovine serum albumin; BSA) DMEM, at which time ET-1 was added (100nM/well). Experiments were performed over a 48 hour period in serum free conditions. The cell layer was washed three times in PBS, fixed for 20 minutes at 4 ^{o}C in 0.1% glutaraldehyde solution and then washed thoroughly with PBS.

The ELISA was performed as follows: Non-specific protein binding was blocked using 10% fat free dried mild solution for 1 hour, murine anti-human ICAM-1 antibody was added (1:1000 dilution) for 1 hour, wells were washed for 30 minutes and anti-ICAM-1 antibody binding was quantified using horseradish peroxidase conjugated goat anti-mouse IgG (1:2000) added for 1 hour. The ELISA was developed by adding OPD/H_2O_2 solution with measurement of mean absorbance at 450 nm using an automatic plate reader. 3-6 replicate wells were used for each test condition in at least three independent experiments using different fibroblast strains.

Dose-response for ICAM-1 expression in normal fibroblasts was also investigated. After serum starvation cells were stimulated with different doses of ET-1 (0.001 to 1000 nM/ml). Time course of the effects of ET-1 on ICAM-1 production was also studied. After serum starvation at different times (0, 2, 4, 8, 12, 24 hours) cell were stimulated with 100 nM ET-1.

2.4.3 Measurement of collagen type I and III secretion by inhibition ELISA

The concentration of type I and III collagen in supernatants overlying monolayer cultures on tissue culture plastic was measured using an inhibition ELISA, as previously described (Rennard *et al.*, 1980). 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 hours. After this period, the medium was removed and replenished with DMEM and ascorbate for a further 24 hours. Throughout these incubations, the cells were kept at 37°C. After this period, the media were collected and frozen at -70°C until required. 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 in 0.05Mol sodium bicarbonate buffer (pH9.6) at 4°C (Plate 1). Standard or serum was also pre-incubated overnight at 4°C in separate 96 well plates with 1.0 μg/ml goat anti-human type I and III collagen antisera (Plate 2). Plate 1 was then thoroughly washed and the contents of plate 2 transferred to plate 1 and incubated for 1 hour at 37°C. The amount of free antibody bound to 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, Chemical Co, St Louis, MO, USA) was measured at 410nm on a Titertek ELISA plate reader. All assays were performed with 3-6 replicates and appropriate controls. Finally, collagen concentration was determined using a log linear plot of type I and III collagen concentration against absorbance, comparing the fibroblast supernatants with a standard curve for collagen type I and III. The amount of soluble secreted collagen was expressed in ng per ml per 10^6 fibroblasts.

2.4.4 Measurement of collagenase (MMP-1) secretion based on a two site ELISA 'sandwich' format

Collagense (MMP-1) concentration in culture supernatants was measured by a commmercial ELISA kit (Amersham, Buckinghamshire, UK), according to the manufacturer's instructions: Standard and samples were incubated in microtitre wells precoated with anti-MMP-1 antibody for 2 hours. After washing, antiserum (polyclonal antibody to MMP-1) was added to all wells for 2 hours. Wells were washed and the second antibody bound to the wells was detected

using donkey anti-rabbit horseradish peroxidase for 1 hour. The ELISA was developed by adding tetramethylbenzidine (TMB) substrate for 30 minutes with measurement of mean absorbance at 450 nm.

In inhibition experiments, the specific ET_B receptor antagonist BQ-788 (100nM), ET_A receptor antagonist PD 156707 (100nM) or $ET_{A/B}$ receptor antagonist Ro 47-0203 (100nM) was added. The ET receptor ligands: BQ-788 and PD156707 are gifts from Rhone-Poulenc-Rorer (RPR), Dagenham, Essex. Ro 47-0203 (Bosentan) generously donated by Dr M Clozel, Hoffman LaRoche, Basel, Switzerland (Table 2.1).

Table 2.1. Characterization of ET receptors: antagonists used in the present study (Function)

Antagonists	Structures	Receptors
PD 156707	sodium 2-benzo[1,3]dioxol-5-yl-4-(4-methoxy-phenyl)-4-oxo-3-(3,4,5-terimethoxy-benzyl)-but-2-enoate	ET_A
BQ-788	N-cis-2,6-dimethylpiperidimocarbonyl -L-γMeLeuD-Nle-ONa	ET_B
Ro 47-0203	(4-tert-butyl-N-[6-(2-hydroxy-ethoxy) -5-(2-methoxy-phenoxy)-2,2'-bipyrimidin -4-yl]-benzenesulfonamine	$ET_{A/B}$

2.5 Immunostaining of fibroblasts for flow cytometry analysis (FCM)

2.5.1 Antibodies and determination of the optimal concentrations

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: CD10, neutral endopeptidase (clone B-E3, mouse IgG2a) 1.0μg/ml; CD13, aminopeptidase N (clones B-F10, mouse IgG1) 1.0μg/ml; CD26, dipeptidyl peptidase IV (clone BA5, mouse IgG2a) 1.0μg/ml; CD29, integrin β1 (clone B-D15, mouse IgG1) 0.5μg/ml; and CD49d, 1.0μg/ml, VLA-4 a-chain (clone HP2/L, mouse IgG1) 1.0μg/ml; M-38 (mouse IgG)

recognising procollagen type I 1.0μg/ml; MAbs recognising MHC I (clone W6/32, mouse IgG2a) 0.5μg/ml, MHC II (clone L234, mouse IgG2a) 1.0μg/ml, CD54 (ICAM-1; clone RR/1, mouse IgG) 1.0μg/ml and CD58 (LFA-3; clone TS 2/9, mouse IgG1) 2.0μg/ml; MAb to CD44 1.0μg/ml (Hermes antigen, rat IgG2a) was a gift of Professor Eugene Butcher (Stanford University, CA. USA). Polyclonal antibodies to human type I and III collagens (α-coll-I and α-coll-III) 1.0μg/ml and anti-human fibronectin (α-FN) 1.0μg/ml.

2.5.2 Cytokines and cytokine treatment

Human recombinant IFN-γ and TNF-α were kindly provided by Professor Marc Feldmann (Sunley Division, Kennedy Institute of Rheumatology, London, UK) and were used at optimal concentrations of 100 and 200 units per ml, respectively. ET-1 used at optimal concentration of 100nM per ml. Cytokines were added to confluence fibroblast cultures and incubated for 48 hours, after which the monolayers were washed with fresh media and used immediately in the experiments.

2.5.3 Phenotypic characterisation of fibroblasts by FCM

The expression of cell surface molecules on fibroblasts was analysed and quantified using flow cytometry (FCM) as previously described (Abraham *et al.*, 1991). Cells were grown to confluence in triplicate 25 cm^2 tissue culture flasks and detached from the monolayers using EDTA (20mMol) for 20 min at 4°C. The cells 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 above) for 60 min at 20°C. 50μg/ml of secondary anti-mouse antibody conjugated to FITC (anti-mouse IgG for all the mAbs) 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.

2.6 Indirect immunofluoresence staining - analysis of the SV40 large T antigen in transduced fibroblasts

The expression of the tsA58 SV40 large T antigen by the fibroblasts was examined by indirect immunofluorescence microscopy. Fibroblasts were cultured at 35°C in Lab-Tek culture chambers, fixed in methanol for 15 min at 4°C, washed twice with PBS and incubated with undiluted hybridoma supernatants containing mouse antibodies specific for amino- and carboxy-terminal epitopes of the SV40 large T antigen (pab 419 and 423 respectively, kindly provided by Professor D Lane, University of Dundee), for 60 min at room temperature. After washing, the slides were incubated for 30 min at room temperature with FITC-conjugated anti-mouse IgG antibody diluted 1:50 with PBS, then washed three times with PBS, mounted in 90% glycerol in PBS containing anti-fade and examined under epi-fluorescence using a Zeiss Axioplan fluorescence microscope.

2.7 Autoradiography

2.7.1 Receptor binding assays

Preliminary binding studies were performed on confluent fibroblasts in 96-well plates. Endogenous peptide levels were reduced by washing fibroblasts in 50mM Tris HCI buffer, pH 7.4, three times at room temperature. Cells were then incubated for 2 hours in buffer containing 5mM Mg Cl_2, 100 kiu/ml Aprotinin and 1% bovine serum albumin in the presence of 0.3 to 1000 pM [^{125}I]-ET-1, [^{125}I]-PD151242 (ET_A selective) and [^{125}I]-BQ3020 (ET_B selective), non-specific binding being established in the presence of 1μM unlabelled ET-1. After incubation cells were rinsed (2 times 10 minutes) with buffer at 4°C, harvested with cell lysis buffer (0.25 M NaOH containing 0.5% sodium dodecyl sulphate) for 10 minutes and counted in a Packard gamma counter. Specific binding was determined by subtracting non-specific from total binding and receptor density

and affinity (B_{MAX} and K_D) calculated using GraphPad InPlot software (Graph Pad, San Diego, California) (Katwa *et al.*, 1993).

In order to study disease-induced changes in ET receptor binding confluent normal (n=6) and scleroderma (n=6) skin fibroblasts were incubated (in duplicate) in fixed concentrations (approximate K_D value, 150 pM) of [^{125}I]-ET-1, [^{125}I]-PD151242 and [^{125}I]-BQ3020 (Table 2.2) and binding determined in a gamma counter as above.

Table 2.2. Characterization of ET receptors: radioligand used in the present study (Binding)

Radioligand	Structures	Characterization criteria	Receptors
[^{125}I]-PD151242	N-[(hexahydro-1-azepin yl) carbonyl] L- Leu(1-Me) D-Trp-D-Tyr	Antagonist	ET_A
[^{125}I]-BQ3020	N-acetyl-[Ala]endothelin-1	Agonist	ET_B

2.7.2 *In vitro* autoradiography

Membrane binding sites were localised using high-resolution autoradiography (Dashwood *et al.*, 1991). Normal and scleroderma fibroblasts were either grown on 4 chamber slides or cytospins of cells were prepared on gelatinised microscope slides. Cells were incubated, using the above protocol, with 50µl buffer containing 150pM [^{125}I]-labelled ET-1, PD151242 and BQ3020 in humidified chambers with non-specific binding being established on paired slides incubated in the presence of unlabelled ET-1. After incubation, slides were washed in buffer, dipped in distilled water (both at 4°C) and dried in a stream of cold air. Binding sites were identified by dipping post-fixed cells (paraformaldehyde vapour, 2 hours at 80°C) in molten nuclear emulsion (LM-1, Amersham) at 50°C, and exposing for 4 to 7 days in light-proof boxes at 4°C. The slides were then processed in undiluted D19 developer (Kodak), followed by Hypam fixative (Ilford, diluted 1 to 4 in water), both for 5 min at 22°C. Cells

were then stained with Mayer's haematoxylin and eosin for histology, examined under bright-field and dark-field illumination on an Olymps Vanox microscope and photographed where appropriate.

2.8 Measurement of interactions between fibroblasts and leukocytes - U937 cell adhesion assay

The assay was performed essentially as described by Carvalho *et al* (1996). Briefly, U937 cells (2 X 10^5 cell/ml) were labeled with 0.1 μCi/ml of [^{3}H] thymidine (Amersham International, Little Chalfont, UK) for 24 hours. On the day of the experiment, the cells were washed three times and resuspended in prewarmed RPMI 1640 containing 20 mM Hepes and 0.2% BSA (adhesion medium). To investigate the effect of endothelial cell product on fibroblast cell adhesiveness, ET-1 (100nM/ml) was added to confluent fibroblasts in 96-well plates. After 48 hours culture, the fibroblasts were washed three times with the adhesion medium and 5x10^5 radiolabeled U937 cells (in 0.1ml) were added to each well and co-incubated for 1 hour at 37^{o}C. The non-adherent cells were removed by a carefully standardized washing procedure. The radioactivity associated with adherent cells was quantified by β scintillation spectrometry after lysis with cell lysis buffer (0.1N NaOH/0.1% sodium dodecyle sulfate).

In some instances, neutralising MoAb to ICAM-1 was added to fibroblast cultures for 60 minutes at 37^{o}C before the addition of radiolabeled U937 cells. The effect of the ET-1 receptor antagonist bosentan (Ro 47-0203, a mixed ET receptor ligand) was assessed by adding bosentan to fibroblasts for 60 min before adding ET-1. The final concentration of bosentan (10 μM) was, with a dose-ratio of more than 100 fold, above full receptor occupancy (Clozel *et al.*, 1994).

2.9 Western blot analysis

2.9.1 Preparation of samples

Fibroblasts were seeded (2.5 x 10^5 cells per well) in six well plates for 48h and then L-ascorbic acid phosphate (30μg/ml) (WAKO PCI, LTD, Japan) was added to the medium overnight. Cell monolayers were washed twice and replaced by serum-free medium containing one TGFβ isoform (TGFβ1, 2 and 3; all at

10ng/ml) for a further 48 hours. The conditioned media were aspirated and centrifuged at 3,000xg for 10 minutes at 4°C. The media were incubated with Heparin Sepharose beads overnight. The beads were washed with 0.1M ammonium acetate, and the bound protein eluted with 2M ammonium acetate. The heparin-bound fractions were vacuum dried and fractionated by SDS-PAGE for CTGF protein analysis. The cell monolayers were lysed and solubilized in Laemmli sample buffer. The lysed cells were scraped and DNA sheared by forcing the sample repeatedly through a 23-gauge needle. Samples were subjected to SDS-PAGE followed by western blotting.

For studying the effect of rCTGF on collagen production, fibroblasts were grown to confluence in DMEM with 10% FCS and then serum starved in DMEM with 1% BSA for 24 hours. rCTGF (80ng) was then added to the cell culture for a further 48 hours. The media were removed and adjusted to 20% (v/v) ammonium sulfate followed by incubation at 4°C with rocking overnight. The next day the samples were centrifuged (microcentrifuge at full speed (14,000g) for 30 min) at 4°C, and the pellet resuspend in Laemmli sample buffer with β-mercaptoethanol ready for SDS-PAGE.

The effects of ET-1 on pro-α1(I) collagen production over time were studied. After serum starvation at different times (0, 8, 16, 24, 48, 72 hours) cells were stimulated with 100 nM ET-1.

Protein assay

Total protein content of tissue culture supernatants and cell lysates were used to determine that equal amounts of protein were compared in each assay using the Bio-Rad protein micro-assay. Standard concentrations of bovine serum albumin (BSA) diluted in culture medium or lysis buffer, depending on the nature of the samples being tested, were used to calibrate the assay and confirm its reliability in the concentration range being measured. The assay was performed according to manufacturer's instructions (Bio-Rad, Munchen, Germany). Briefly, protein standard was prepared by several dilutions containing from 1 to 25 μg/ml BSA. 200μl of standards and diluted samples were added to replicate wells of a 96 well

flat bottomed plate. Then, 50 μl Dye Reagent Concentrate was added to each well. After mixing, the test plate was incubated for 30 minutes at room temperature. Absorbance was measured on an automatic plate reader at a wavelength of 595 nm. The absorbance for standards was plotted against protein concentration to give a standard curve. Linear regression analysis allowed the protein concentration of the test samples to be calcuated.

2.9.2 Western blot analysis for CTGF and pro-α1 (I) collagen

Electrophoresis was performed on 12% polyacrylamide gels containing SDS. Immunoblotting was performed by electroblotting the proteins to nitrocellulose filters and incubating the filters in PBS with 2.5% non-fat drymilk at 4°C overnight to block nonspecific antibody binding. The blocking solution was removed and a rabbit anti-CTGF specific antibody (FibroGen Inc. USA) added to the blocking buffer and incubated for 1 hour at 25 °C. The membranes were then washed in PBS with 0.2% Tween 20 for 10 minutes five times and then incubated with alkaline phosphatase-conjugated affinity purified goat anti-rabbit IgG (Gibco, USA) at a 1:40,000 dilution in PBS containing 2.5% milk at 25 °C for 1 hour. The filters were washed and the blot was developed using Luminol/enhancer solution plus stable peroxide solution (Pierce, Rockford, Illinois. USA).

For proα1 (I) collagen, the nitrocellulose filters were first incubated with anti-pro-α1 and pC-α1 (I) collagen antibody (a gift from Dr CL Yang, FibroGen, USA). For some experiments, pro-α1(I) collagen was determined by Coomassie staining (Novex, San Diego, USA).

2.10 Analysis of fibroblast mRNA

The mRNA analysis is most often performed by RNA blotting, but lately other methods have also been introduced, such as RNase protection assay. In this thesis, both techniques have been used.

2.10.1 Analysis of matrix mRNA by Northern Hybridization

2.10.1.1 RNA extraction

Extraction of total RNA from fibroblast monolayers and 3-D gels: RNA extraction was adapted from Chomczynski and Sacci (1987) using the acid-guanidium-phenol-chlorofom method. Culture medium was aspirated and cells were gently washed with sterile PBS at 37°C. 3 ml of solution D (7ml β-mercaptoethanol/ml guanidinium isothiocyanate solution) were added to each dish and incubated at -20°C for 10 minutes to lyse the cells. The lysate was aspirated into a 5ml syringe through a 23G needle and then transferred to a RNase free high-speed centrifuge tube. 300µl of 2M sodium acetate (pH 4.0) was added to each tube and the contents mixed thoroughly. Next, 3ml of water saturated phenol was added, the tube vortexed and then 600µl of chloroform/isoamyl alcohol (50:1) added. The tube was incubated on ice for 15 minutes. Tubes were then balanced and centrifuged at >10000g for 20 minutes at 4°C. The aqueous (upper) phase, containing RNA, was then collected and transferred to a fresh tube, into which an equal volume of isopropanol was added. After 1 hour incubation at -20 °C, RNA was pelleted by centrifugation at >10000g for 20 minutes. The supernatant was completely aspirated and the pellet resuspended in 600µl of solution D. This solution was transferred to an eppendorf tube, 600µl of isopropanol added, followed by a further 1 hour incubation at -20°C. The eppendorf tube was then centrifuged at maximum speed in an eppendorf centrifuge for 20 minutes. The supernatant was discarded and the pellet washed with ice-cold 75% ethanol, spun again at maximum speed for 10 minutes and then, after aspiration of the supernatant, air dried until no visible liquid remained. The pellet was resuspended in 50µl of diethylpyrocarbonate (DEPC) treated water.

Estimating the yield of extracted RNA: The RNA concentration and purity were measured spectrophometrically by determining the absorbance of a diluted aliquot (of 6µl of sample with 540µl DEPC treated water in a quartz cuvette) at 260nm. The RNA concentration was determined based on the absorbance of a solution of known concentration (1 OD unit for 40µg/ml pure RNA). The

absorbance of the solution at 280nm was also measured to estimated the concentration of contaminating protein in the RNA sample. A ratio of OD 260:280 was used as an index of purity. Samples with a ratio of less than 1.6 were re-extracted. Generally a confluent 10cm-diameter petri dish containing 10^6 fibroblasts yields 15μg of total RNA.

2.10.1.2 Northern blotting

Electrophoresis of RNA and transfer to Hybond membrane: RNA samples were electrophoretically separated using a denaturing formaldehyde-agarose gel. This was prepared by dissolving 1g of molecular biology grade agarose in DEPC treated water, adding 1/5 final gel volume of 5X gel running buffer (0.1M MOPS, 40mM sodium acetate, 5mM EDTA, pH7.0) and then formaldehyde to give a final gel concentration of 2.2M. Samples were prepared as follow: The sample RNA solution volume containing 5 μg of RNA was calculated, this was added to a fresh eppendorf tube and for each 4.5 μl of sample volume formaldehyde (3.5 μl), formamide (10 μl) and 5X running buffer (2 μl) were added. This sample mixture was incubated at 65°C for 15 minutes, chilled on ice and briefly centrifuged. Prior to loading, 2 μl of gel loading buffer was added to each sample (50% glycerol, 1mM EDTA, 0.25% BPB, 0.25% xylane cyanol FF). The gel was pre-run for 5 minutes at 5V/cm then samples were loaded and electrophoresis performed for 60 to 120 minutes at 3-4V/cm.

The agarose gel was rinsed in DEPC treated water to remove the formaldehyde and soaked in 20X SSC buffer (standard saline citrate) for 45 minutes. The gel was placed on thick blotting pads, pre-soaked in 20X SSC, hybond membrane placed on top of the gel and a piece of pre-soaked blotting paper placed over the membrane. Multiple (8-10) dry blotting papers were placed on top of the soaked layer, a glass plate was placed over the dry layers and transfer performed for 4-12 hours at room temperature. After transfer the RNA was fixed by applying the membrane to 3 filter papers soaked in 50 mM sodium hydroxide for 5 minutes, washed in 20X SSC and was then ready for probing.

Radiolabelling of cDNA probes: The probes for α1(I), α1(III) collagen, fibronectin, MMP-1 and GAPDH were cut from their plasmids using the appropriate restriction enzymes and purfied by electrophoretic separation in low melting point agarose gel. Labelling was performed using a Megaprime labelling kit, according to the manufacturer's instructions. In brief, approximately 25ng of template DNA (10 μl of the LMP agarose containing the DNA probe) was added to 5 μl of random primers and boiled for 5 minutes to denature the DNA. The tube was placed on ice and 10 μl labelling buffer, 5 μl of αP^{32} dCTP and 2 μl of DNA polymerase was added. The mixture was then incubated for 30 minutes. The labelling reaction was stopped by adding 50 μl of 4M ammonium sulphate (pH4.5). Prior to addition of the labelled probe mixture to the hybridisation buffer, cDNA was denatured by boiling for 5 minutes. Using this protocol, probes were labelled to a specific activity of at least 10^9 dpm/mg.

Hybridisation: The hybridisation bottle was half-filled with 2X SSPE (standard saline phosphate EDTA), the membrane was applied to the inside wall of the bottle. The SSPE was poured off and replaced with 5ml Rapid-hyb (TM) buffer. The membrane was prehybridised in a Hybard oven at 65°C for 15 minutes, the labelled probe was added and hybridisation performed at 65°C for 2 hours. The membrane was washed twice with 2X SSPE at room temperature, twice with 1X SSPE at 65°C and then rinsed in 2XSSPE. It was blotted dry and wrapped in clingfilm.

Autoradiography and quantitation: The membrance was placed against X-Ray film, and left at -70 °C between 12-72 hours exposure. The film was developed and scanned using a laser densitometer.

2.10.2 RNase protection analysis of ICAM-1 mRNA expression

Total RNA was isolated from normal and scleroderma fibroblasts (2.10.1). ICAM-1 and actin transcripts in the preparation of total RNA were determined using RNase protection analysis as previously described (Ong *et al.*, 1995). In brief, two-stage PCR using partially nested primers designed from known gene

sequences were employed to amplify short genomic fragments of ICAM-1; in each case including part of the coding sequence from exon 2 (which encodes the amino acides present in the mature peptides) together with adjacent 5' intron. The fragment was cloned into appropriate riboprobe vectors, linearized and used to produce specific labeled antisense RNA transcripts by *in vitro* transcription with T3 or SP6 polymerase in the presence of [α-^{32}P]-UTP (10^9 cpm/μg). Hybridisation was performed overnight at 60°C with $5X10^5$ cpm of the appropriate ICAM-1 probe and $5X10^5$ cpm of rat actin probe. RNase digestion was then carried out at 70°C for 30 minutes, and terminated by the addition of 60 μl of proteinase K (1 mg/ml) with 3% SDS and further incubated at 70°C for 30 minutes. Phenol-chloroform and chloroform extractions were performed and the RNA fragments precipitated with 2.5 volumes of absolute alcohol. The precipitated RNA was dissolved in 5 μl of 80% formamide loading buffer, and the reaction mixture electrophoresed on 8% denaturing polyacrylamide gels. Levels of ICAM-1 transcript were measured from signal intensities after quantitation performed by Phosphorimager analysis (Molecular Dynamics), and adjusted relative to internal actin transcripts.

2.11 Polymerase chain reaction (PCR)

The polymerase chain reaction is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA and permits the easy analysis of DNA fragments by molecular cloning.

2.11.1 PCR primers for $ET_{A/B}$ receptors and ICAM-1

PCR primers were selected from published ET_A and ET_B receptor cDNA sequences using conditions described in detail in a previous paper (O'Reilly *et al.*, 1992). The ET_A oligonucleotide primer used were 5'-CCTTTTGATCACAATGACTTT-3'(forward) and 3'-TTTGATGTGGCATTGAG CATACAG-5' (reverse). The ET_B oligonucleotide primer used were 5'-ACTGGCCATTTGGAGCTGAGAT-3' (forward) and 3'-CTGCATGCCACTTTT CTTTCTCAA-5' (reverse). Internal primer pairs yielded products of 299 base pairs for ET_A and 428 base pairs for ET_B.

PCR primer was selected and made from published ICAM-1 cDNA sequence using conditions described in detail in previous papers (Simmons *et al.*, 1988; Voraberger *et al.*, 1991). The ICAM-1 oligonucleotide primer used were 5'-CCG GTG GCG GTT ATA GAG GTA CG-3' (forward) and 3'-GGT GAC AGT GAA GTG TGA GGC CC-5' (reverse). The GAPDH oligonucleotide primer used were 5'-CTT CAC CAC CAT GGAG AAGG-3' (forward) and 3'-GGC TAC AGC AAC AGGG TGG-5' (reverse). Internal primer pairs yielded products of 563 base pairs for ICAM-1 and 604 base pairs for GAPDH (Chapter 6, Figure 6.6).

2.11.2 The PCR reaction

Total cellular RNA was isolated as described in 2.10.1, and cDNA synthesised by standard methods. Briefly, RNA concentration was determined by spectrophotometory and RNA integrity was determined by gel electrophoresis and visual inspection of ethidium bromide-stained ribosomal bands. 5 μg of total RNA was resuspended in 5 μl of DEPC treated water and denatured at 65°C for 10 minutes. For 1st strand cDNA synthesis the denatured RNA was added to the following RT-reaction mixture [1μl of oligonucleotide-dT primer (0.5 μg/ml, Amersham, UK); 7 μl of DEPC treated water; 4 μl of 5x first strand buffer (250mM Tris-HCl pH 8.3; 375mM KCl; 15mM $MgCl_2$]) (Gibco-BRL, UK); 1μl of dNTPs (10mM, Pharmacia) and 1μl of M-MLV reverse transcriptase (200 units, Gibco-BRL, UK) 1 μl of RNase Inhibitor (10 U/μl, Gibco) and 1 μl 10mM DTT]. The reactions were incubated at 41°C for 2 hours and stored at -20°C until required.

1μl of first strand cDNA solution was mixed with 5μl of 10XTAQ DNA polymerase buffer, 1μl of $MgCl_2$ (50mM), $ET_{A/B}$ and ICAM-1 primer (125 pM for each), and 2μl of 4 dNTP stock solution (5mM for each nucleotide). The volume was brought to 49.5μl with distilled water. 50μl of mineral oil was placed on the surface of the reaction solution to prevent any evaporation. The reaction solution was heated to 94°C for 3 minutes to denature first strand of cDNA and then 0.5μl (2.5U) of TAQ DNA polymerase (Boehringer Mannheim) was applied.

PCR for ET-receptors ($ET_{A/B}$) using specific sequences was performed by a 40 step-cycle programme consisting of denaturation at 94°C for 1min, annealing of ET-receptor primers at 54°C for 1 min and chain extension at 72°C for 1min. PCR for ICAM-1 was performed by a 30 step-cycle programme consisitng of denaturation at 94°C for 10 sec, annealing of ICAM-1 primers at 54°C for 1 min and chain extension at 72°C for 1min. A final elongation step for 7 min at 72°C. Amplified PCR products were then electrophoresed on a 1.8% agarose gel and stained with ethidium bromide.

2.12 Introduction of DNA into fibroblasts

2.12.1 Transient transfection: Assessment of transcriptional activation of COL1A2 gene in scleroderma dermal and lung fibroblasts

To study the transcriptional regulational of COL1A2, deletion constructs containing portions of the proα2(I) collagen promoter were transfected into randomly selected normal (n=5) and scleroderma (n=5) fibroblast strains from skin and lung. The promoter constructs were sub-cloned into pGL3 plasmid and linked to the firefly luciferase reporter gene. Constructs containing an SV40 promoter were included to determine whether differences observed with the collagen promoter construct were specific for the COL1A2 gene, and transfection efficiency assessed using a plasmid containing the HSV thymidine kinase promoter sequence linked to Renilla luciferase co-transfected as a control. Normal and scleroderma fibroblasts were plated and grown to 50-80% confluence in 12-well culture plates. Cells were transfected with 2μg of each plasmid using the Lipofectamine method, according to the manufacturers instructions.

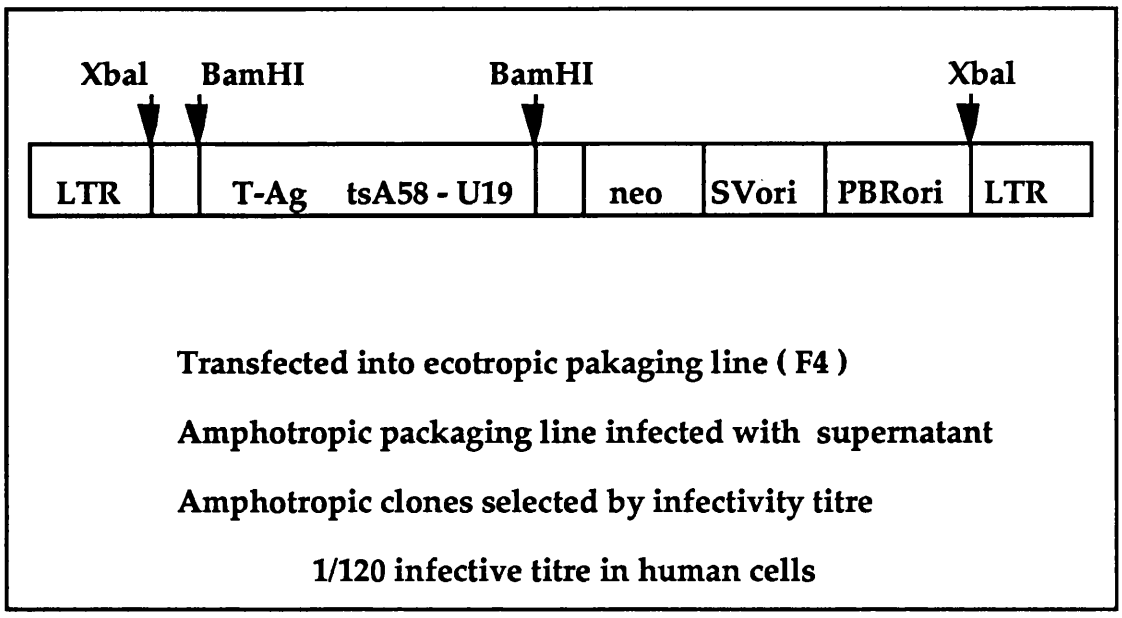
Briefly, 18 hours after seeding, cells were rinsed with serum-free medium, and 0.8ml of serum free medium containing 200μl of Lipofectamine complex suspension was overlaid above the fibroblast monolayer. After 5 hours, 0.2 ml of FCS was added and at 24 hours this was replaced by fresh serum supplemented medium. Cell extracts were assayed for reporter gene activity 72 hours after the start of transfection by luminometer (Labtech, East Sussex, UK) using the Dual-

Luciferase Reporter Assay System. COL1A2 activation was determined after standardisation of firefly luciferase activity against that of the Renilla luciferase control.

2.12.2 Stable transfection - Retroviral transduction with the SV40 tsT antigen

Infection and selection of normal and scleroderma human fibroblasts: Normal human diploid fibroblasts (NF) and fibroblasts from patients with scleroderma (SScF) were infected at passage two with a retrovirus carrying the SV40 early region-derived construct, A58-U19. The construct contains two mutations: tsA58 renders the T antigen thermolabile at 39 °C (Jat and Sharp, 1989) while the other, U19, ablates sequence-specific DNA binding and prevents autonomous viral replication (Jat *et al.*, 1986). A58-U19 was inserted into the pZipNeoSV(X)1 shuttle vector (Cepko *et al.*, 1984) (Figure 2.1), enabling infected cells to be selected on the basis of resistence to the antibiotic neomycin (G-418). An amphotropic virus-producing line was generated by infecting PA317 packaging cells (Miller and Buttimore, 1986) with supernatants from an ecotropic virus packaging line (Stamps *et al.*, 1994). Viral supernatants were stored at -80 °C and were rapidly thawed at 37 °C and filtered through a 0.45-μm-pore-size filter immediately before use.

Figure 2.1 Amphotropic retroviral vector tsAU19



Fibroblasts were cultured to 50% confluency in 75-cm^2 tissue culture flasks (approximately 10^6 cells per flask). The cells were washed with growth medium and then incubated for 3 hours with freshly thawed and filtered high titre (>10^4 cfu/ml) virus-containing supernatant in the presence of 8 μg/ml polybrene, followed by the addition of fresh medium to give a final 1 in 5 dilution of the viral supernatant. The infection was carried out at a semi-permissive temperature of 36.5°C, and was repeated three times at 24 hour intervals. Two days after the final infection, successfully transduced cells were selected by incubation at the permissive temperature of 35°C in fresh complete media containing G418 (0.5 mg/ml medium). The medium was changed every 2 to 3 days for approximately 3 weeks, after which the cells were trypsinized and one-fifth of the detached cells recultured again at 35°C. The resultant G418-resistant cells were then maintained in culture by continuous passage. At each passage one-fifth of the detached cells were re-seeded into culture flasks containing complete DMEM and expanded at 35°C. The transduced cell cultures derived from the primary NF and SScF cells, designated tsT-NF and tsT-SScF, respectively, remained resistant to G418 (<1mg/ml) and have been maintained in continuous culture for over 50 passages.

CHAPTER 3: SSc SKIN AND LUNG FIBROBLASTS EXHIBIT ELEVATED AND DYSREGULATED COLLAGEN (I) BIOSYNTHESIS

3.1 INTRODUCTION

As reviewed in Chapter one, the fibrotic process in scleroderma is believed to result from the local activation of collagen synthesis in dermal and visceral fibroblasts (Jimenez *et al*, 1986; Scharfetter *et al.*, 1988; Harrison *et al.*, 1991). In comparison with the large number of *in vitro* studies on scleroderma dermal fibroblasts, very few studies have been performed on scleroderma associated fibrosing alveolitis (FASSc) fibroblasts. The main body of evidence for abnormal fibroblast function in FASSc is derived from fibroblasts cultured from BALF in scleroderma patients (Ludwicka *et al.*, 1992) and animal models of lung fibrosis (Korn *et al.*, 1992). In this chapter, in order to delineate the abnormal characteristics of skin and lung fibroblasts, I have investigated dysregulation of collagen turnover in scleroderma fibroblasts from biopsies taken from these two sites.

Scleroderma is characterised by fibrosis of the skin and internal organs. One of the most clinically significant sites of visceral fibrosis is within the lungs, leading to FASSc. There are distinctive clinical differences between the fibrotic processes occurring in different organs in this disease. For example, in diffuse cutaneous scleroderma there is generally quite a rapid progression of skin sclerosis in the early disease, reaching a plateau within 3 to 5 years often with stabilisation or improvement (Black and Denton, 1997). In contrast, lung fibrosis is often more indolent in early disease but progresses steadily once it becomes established (Edelson *et al.*, 1985). The variation in the extent of fibrosis at different sites in scleroderma is also illustrated by those patients with limited cutaneous scleroderma who develop significant lung fibrosis despite minimal skin sclerosis.

These differences may partly reflect a genetic predisposition since both limited and diffuse disease lung fibrosis occurs at increased frequency in association

with certain class II MHC haplotypes and in association with anti-topoisomerase autoantibodies (Briggs *et al.*, 1990). The clinical differences between scleroderma associated lung and skin disease are mirrored by histological distinctions. For example, epithelial damage, both microscopic and ultrastructural, is an early feature of lung involvement in scleroderma (Harrison *et al.*, 1991) but epithelial structures (e.g. the epidermis) remain relatively unscathed in affected skin in scleroderma (Prescott *et al.*, 1992). Also leucocytic infiltrates are a more prominent component of the histology of active scleroderma lung fibrosis, with inflammatory mononuclear cells persisting even in established disease.

These contrasting features of fibrosis in the lung and skin might reflect the anatomical and histological differences between the two organs; alternatively they could be due to heterogeneity in fibroblast properties within lung and skin (Fries *et al.*, 1994) or perhaps to different pathogenetic processes operating at each site. Studies of the properties of bronchoalveolar lavage fluid from scleroderma patients have been valuable in examining the role of various potential mediators in the development of lung fibrosis (Silver *et al.*, 1990; Coker and Laurent, 1995; Bolster *et al.*, 1997). Several novel pathogenic mechanisms have been proposed including a role for thrombin as a co-mitogen (Hernandez-Rodriguez *et al.*, 1995; Bolster *et al.*, 1997), for insulin-like growth factor-1 (IGF-1) (Harrison *et al.*, 1994) and for endothelin-1 (ET-1) (Cambrey *et al.*, 1994) as a potential mediator linking vascular and fibrotic scleroderma lung pathologies. Interleukin-8 (IL-8) has also been shown to be produced in excess by alveolar mononuclear cells in scleroderma (Southcott *et al.*, 1995), emphasising the possible importance of altered cytokine release in the pathogenesis of FASSc.

Despite the clinical importance of lung fibrosis in scleroderma, previous studies of fibroblast properties in scleroderma have focused on skin disease (Jimenez *et al*, 1994; Fagundus *et al.*, 1995). In this chapter I have examined cell morphology and collagen type I biosynthesis by scleroderma and control fibroblasts in monolayer and collagen gel matrix culture to determine

whether the aberrant properties observed for dermal scleroderma fibroblasts might extrapolate to cells from lung, thereby suggesting that some of these abnormal characteristics may reflect a general scleroderma fibroblast phenotype (Ivarsson *et al.*, 1993).

3.2 PATIENTS AND STATISTICS

3.2.1 Samples

Punch biopsies (4mm^3) were obtained from clinically affected areas of the skin of scleroderma patients at the Royal Free Hospital and also from age and sex matched healthy volunteer controls.

Open lung biopsy specimens were taken from histological staging of lung fibrosis (fulfilling the ACR preliminary criteria for classification, 1980) and from tissue samples from lobectomy specimens of patients undergoing surgery for lung neoplasms. The clinical and demographic details for the scleroderma and control subjects are given in Table 3.1. Control samples were from sites away from those involved macroscopically or histologically in the lung neoplasm. For experiments cells were used between 2nd and 5th passage.

Table 3.1. Characteristics of the scleroderma patients and control subjects

	Skin		Lung	
	SSc patients (n =17)	Control subjects (n =16)	SSc patients (n =16)	Control subjects (n =10)
Sex, no.				
Males	4	5	4	7
Females	13	11	12	3
Age, years				
Mean	44	48	45	57
Range	29 - 61	35 - 59	31 - 64	35 - 76
SSc subset				
Limited	5	-	6	-
Diffuse	12	-	10	-
SSc duration, years				
Mean	6	-	5	-
Range	2 - 14	-	2 - 12	-

3.2.2 Statistical analysis

Our analytical goals were to determine whether there were differences between scleroderma and control fibroblasts in monolayer and collagen gel cultures for the measured variables. Summary statistics are expressed as mean (± SEM), indicating the number (n) in each sample in parentheses. Each data point represents a mean obtained from replicate analysis of a series of independent experiments. Paired and unpaired comparisons were made using non-parametric tests: the Wilcoxon-Mann-Whitney test for unpaired data and Wilcoxon matched pairs signed rank test for paired comparisons. Analyses were performed by the Astute (TM) statistics package within the Microsoft Excel (TM) personal computer program. The pre-selected level of significance for all statistical tests was $p<0.05$.

3.3 RESULTS

3.3.1 Skin and lung fibroblast collagen (I) protein and mRNA

The amount of type I collagen secretion was assessed by competitive ELISA in culture supernatant over a 24 hour period in skin and lung fibroblast cultures. The secretion of collagen type I by scleroderma fibroblast strains was significantly higher than mean collagen secretion of the control fibroblast strains ($p<0.01$ for skin, $p<0.05$ for lung, Wilcoxon-Mann-Whitney test, Figure 3.1, Table 3.2). The scleroderma strains showed a substantial variation in collagen (I) secretion. There were no apparent clinical or demographic differences between the high secreting strains and those with normal collagen (I) secretion.

Table 3.2: Collagen (I) secretion by skin and lung fibroblasts

	Collagen type I ng/ml/10^6 cells		
	SSc	Control	P-values
Skin	351.92 ± 110.46	185.09 ± 31.77	<0.01
Lung	90.91 ± 56.00	40.2 ± 17.50	<0.05

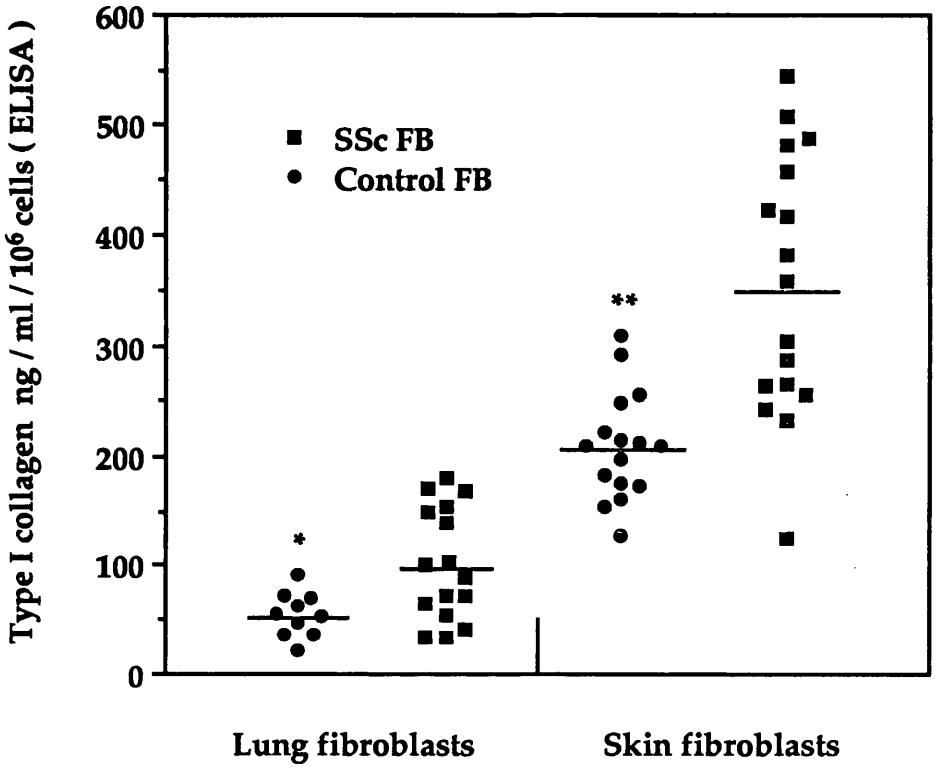


Figure 3.1. Increased type I collagen secretion by SSc skin and lung fibroblasts. The mean value of replicate experiments are shown for control lung fibroblasts (n=10), SSc lung fibroblasts (n=16), control skin fibroblasts (n=16) and SSc skin fibroblasts (n=17). Horizontal bars indicate the mean collagen secretion for each group. Collagen production in patients with SSc is significantly increased. *$p<0.05$, ** $p<0.01$ compared to SSc fibroblast strains (by Wilcoxon-Mann-Whitney test).

The results were extended by measuring the levels of mRNA specific for pro-α1(I) collagen in skin and lung fibroblasts. mRNA levels were determined by Northern hybridisation and quantified by PhosphorImager analysis. Steady state pro-α1(I) collagen mRNA levels were consistently and significantly elevated for scleroderma fibroblasts in monolayer culture (Figure 3.2, Table 3.3) compared with the control strains ($p<0.05$, Wilcoxon-Mann-Whitney test). For control fibroblasts pro-α1(I) collagen:GAPDH band density in 3-D gel was significantly lower in gel culture than in monolayer culture ($p<0.05$, Wilcoxon matched pairs signed rank test). In contrast, collagen (I):GAPDH ratio for the scleroderma fibroblast strains was not significantly different from that in monolayer culture ($p=0.24$ for skin, $p=0.35$ for lung, Figure 3.3, Table 3.3). Thus, collagen (I) mRNA levels for scleroderma fibroblast strains in 3-D gels were also significantly different from those of control fibroblast strains, reflecting aberrant down-regulation in the scleroderma cells ($p<0.01$, Wilcoxon-Mann-Whitney test). This aberrant pattern of down-regulation was observed in scleroderma strains with normal, as well as those with elevated, collagen (I) secretion.

Table 3.3: Collagen (I) : GAPDH in skin and lung fibroblasts

	SSc			Control		
	Monolayer	3-D gel	P-values	Monolayer	3-D gel	P-values
Skin	7.8±0.9	6.5±1.3	=0.24	3.9±0.7	2.4±0.6	<0.05
Lung	3.7±0.9	3.3±0.6	=0.35	1.9±0.8	1.3±0.3	<0.05

3.3.2 Skin and lung fibroblast morphology in monolayer and gel matrix culture

Both scleroderma and control fibroblasts readily grew to confluence on tissue culture plastic, exhibiting typical fibroblastic morphology (Figure 3.4A) but generally attaining at least twice the density at confluence (up to 2.5×10^6 cells per 25 cm^2 flask) compared with equivalent passage dermal fibroblast

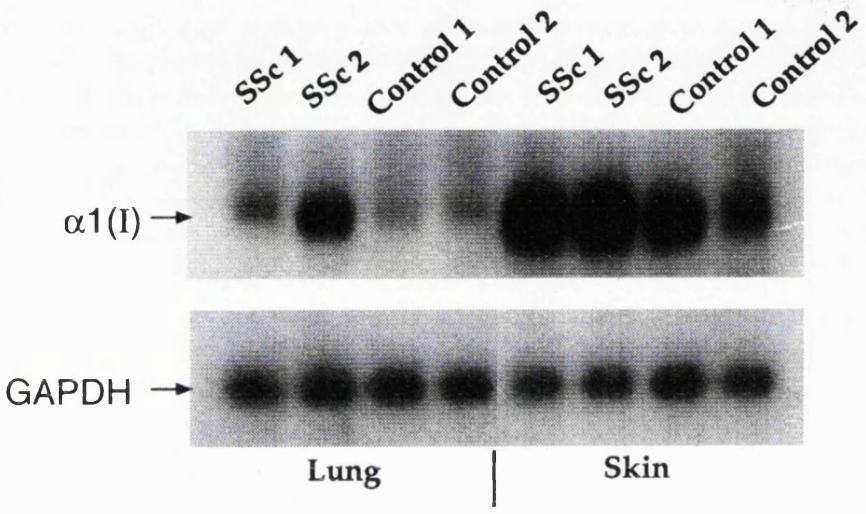


Figure 3.2. Northern hybridisation demonstrating elevated pro-α1 (I) collagen mRNA levels in SSc skin and lung fibroblasts. Representative Northern Blotting experimental results confirming higher steady state mRNA levels in SSc skin and lung fibroblast strains in monolayer culture. After transfer to hybond membrane, transcripts for pro-α1(I)collagen and GAPDH were localised using a radiolabelled cDNA probe. Following autoradiography the ratio of pro-α1(I) collagen:GAPDH band density was determined by PhosphorImager analysis.

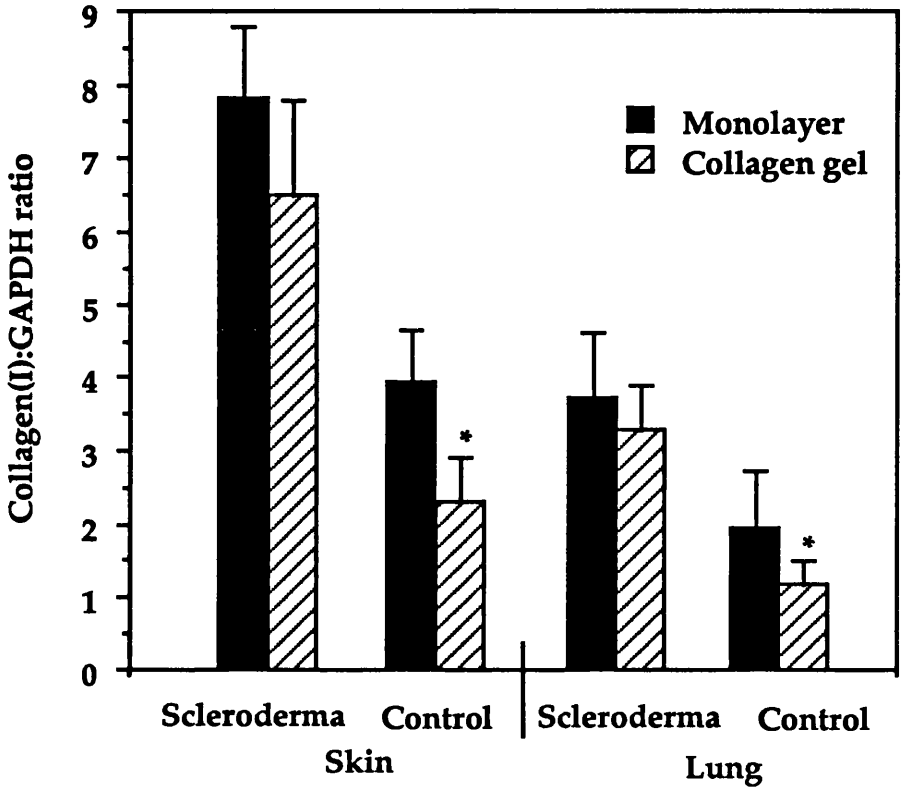


Figure 3.3. Failure of SSc skin and lung fibroblasts to down-regulate pro-α1 (I) collagen mRNA transcription in 3-D matrix. Mean (±sem) results for collagen I:GAPDH staining intensity for control and SSc fibroblast strains in monolayer and 3-dimensional collagen gel culture. Probabilities are those obtained by analysis using non-parametric testing. * $p < 0.05$.

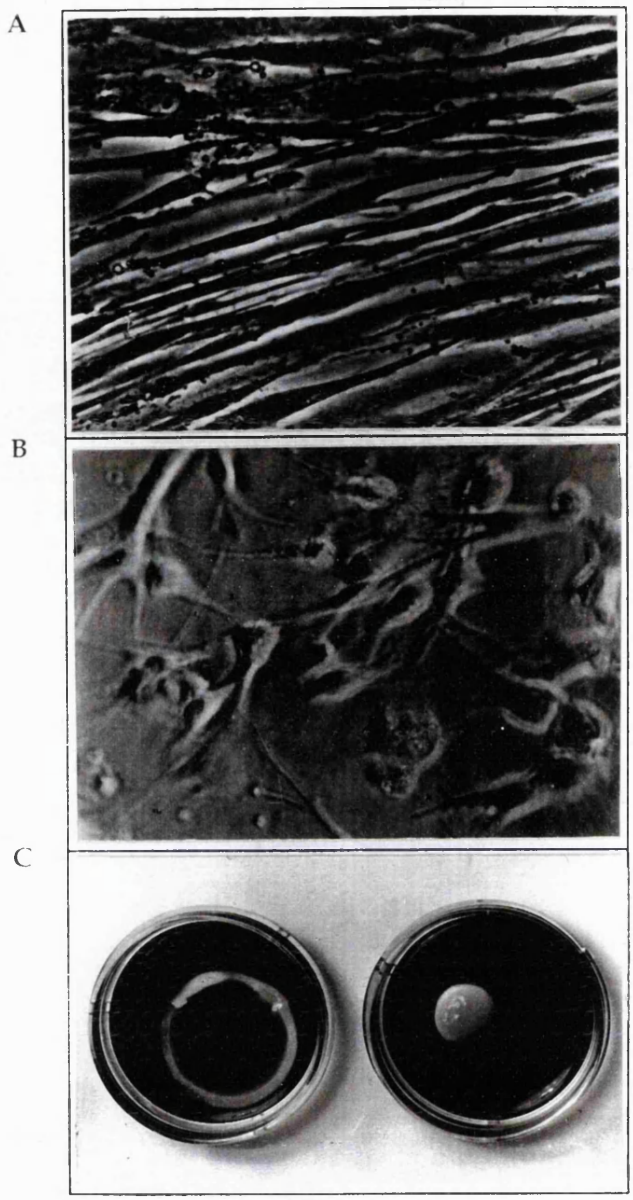


Figure 3.4. SSc lung fibroblasts cultured in monolayer and in 3-dimensional collagen gel matrix. Morphologic appearance of SSc lung fibroblasts cultured in monolayer and in 3-dimensional collagen gel matrix. A. Phase contrast micrograph depicting spindle-shaped morphology of fibroblasts cultured in monolayer (original magnification x 200). B. Phase-contrast micrograph depicting fibroblast appearance when grown in a 3-D collagen gel (original magnification x 200). C. Collagen gel appearance at 4 hours (left) and 24 hours (right) after seeding fibroblasts, showing marked contraction of the gel matrix. Similar morphology in skin fibroblasts.

(typically 0.7 to 1.0 x 10^6 per 25 cm^2 flask). When seeded within collagen type I gels both scleroderma and control fibroblasts caused contraction of the gel over a period of 24 hours forming a dense connective tissue matrix. Fibroblasts within these gels assumed an elongated bipolar shape with typical spindle shaped filopodia at both cell poles. There was no apparent difference between scleroderma and control fibroblast morphology in the 3-D gels (Figure 3.4B).

Examination of cellular morphology of fibroblasts grown on the surface of polymerised gels were similar for both control and scleroderma cells but consistent differences were observed between cells seeded in serum free culture medium and those grown in the presence of FCS. Both of these stimulated the formation of large cytoplasmic processes with extensive cytoplasmic spreading over the gels compared with cultures using serum free medium.

3.3.3 Contraction of collagen gels

Dermal fibroblasts grown in 3-D gels have been shown initiate gel contraction, and our data confirms that both scleroderma and control fibroblasts have a similar effect, as illustrated in figure 3.4C. There is no significant difference in contraction between scleroderma and control fibroblasts at time points between 0 and 24 hours. At 24 hours contraction was maximum, with mean gel weight of 162.9 ($\pm$ 24.2) mg for control lung fibroblasts and 140.6 ($\pm$ 22.7) mg for scleroderma fibroblasts, and with mean gel weight of 142.3 ($\pm$ 31.9) mg for control skin fibroblasts and 135.2 ($\pm$ 35.3) mg for scleroderma fibroblasts (Figure 3.5). The similar gel contraction between skin and lung fibroblasts was interesting, particularly in view of the difference in type I collagen protein and mRNA.

3.3.4 Transcriptional activation of COL1A2

All three COL1A2 constructs were efficiently transcribed following transient transfection in both control and scleroderma fibroblasts (Figure 3.6). For all three constructs the scleroderma strains tested showed a consistenly greater

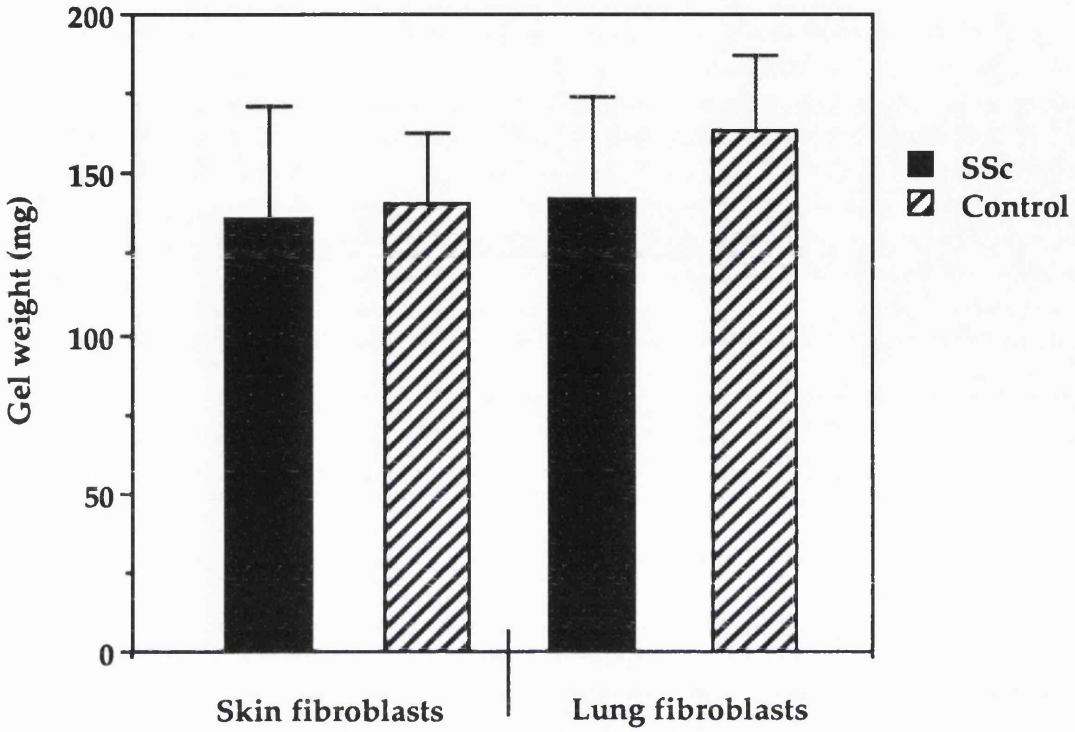


Figure 3.5. Contraction of collagen gels by skin and lung fibroblasts. Data shown that both dermal and lung fibroblasts had a similar effect to contract 3-dimensional collagen gel matrix. Control (n=6) and SSc (n=6) fibroblasts seeded in 3-D gels contained serum free medium. After 24 hours the gels were maximally contracted an no further change in gel weight occurred up to 72 hours. There are no significantly differences either SSc with normal or skin with lung fibroblasts by gel weight (mean±sem) (Student's unparied t-test).

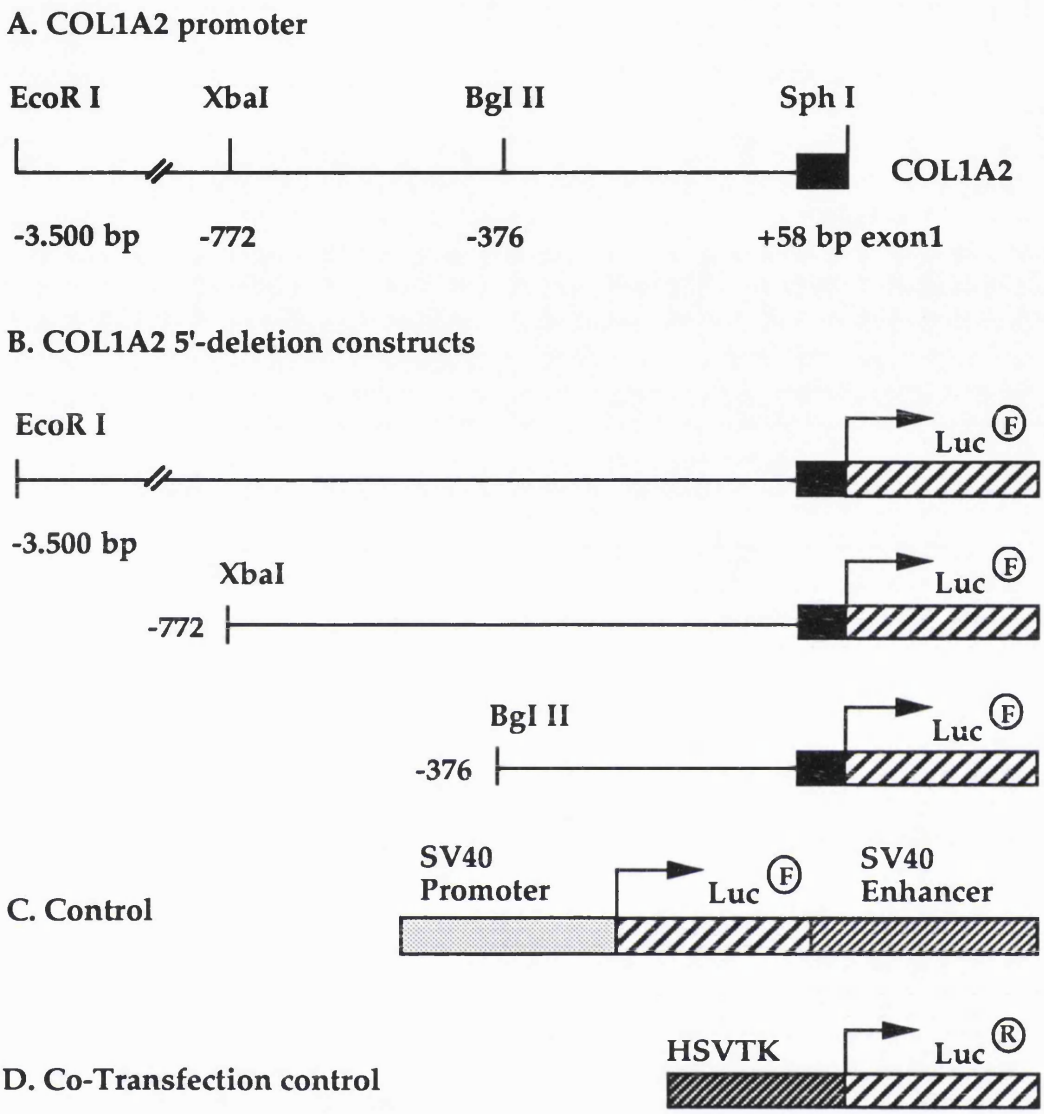


Figure 3.6. COL1A2 promoter 5'-deletion constructs used in transient transfection studies for SSc and control fibroblasts. Schematic representations of COL1A2 promoter including the first 58 bp of exon 1 of the COL1A2 gene (A), and the 3 COL1A2 5' deletion constructs used for generating the luciferase reporter plasmids employed in transient expression experiments to assess transcriptional activation of collagen α2(I) genes in SSc and control fibroblasts (B), Control Simian virus (SV40) promoter and enhancer (C) and Co-transfection control herpes simplex virus (HSV) Thymidine Kinase (D).

transcriptional activation than the control strains. This difference was seen most clearly for the -376bp and -772bp constructs which gave a three-fold increase in transcriptional activity. The difference between scleroderma and control strains was less marked for the -3.5kb promoter construct, with scleroderma strains demonstrating around a 70% greater activity of the firefly luciferase compared with control lung fibroblasts (Figure 3.7). To ensure that the observed effects were not due simply to a generalized metabolic activation of scleroderma fibroblasts in comparision with normal fibroblasts, we set out to show that increased transcription was specifically driven by the collagen promoter. For this purpose, control plasmids containing SV40 and HSV thymidine kinase strong constitutive promoters were transiently transfected into normal and scleroderma fibroblasts. The results did not reveal any differences in the transcriptional activity driven by these 2 control plasmids in normal and scleroderma fibroblasts.

3.4 DISCUSSION

This study provides the first demonstration that some of the hallmark abnormalities of scleroderma skin fibroblasts in tissue culture may be shared by fibroblasts cultured from other organs. In some respects scleroderma lung fibroblasts appear to resemble those cultured from biopsies taken from cryptogenic fibrosing alveolitis (Narayanan *et al.*, 1992). However, it is noteworthy that although the levels of collagen production per cell in monolayer culture are significantly elevated for scleroderma lung fibroblasts compared with control lung strains, they are still below the level of collagen secretion by normal dermal fibroblasts under similar conditions and much lower than that of many dermal scleroderma strains (Shi-Wen *et al.*, 1995), presumably reflecting organ specific differences between fibroblast phenotype. It would be interesting to investigate matrix biosynthesis and contraction of collagen gels in fibroblasts cultured from simultaneous skin and lung biopsies in individual patients, particularly in view of the temporal differences between the development of fibrosis at these two sites. The results obtained in such experiments would require careful interpretation.

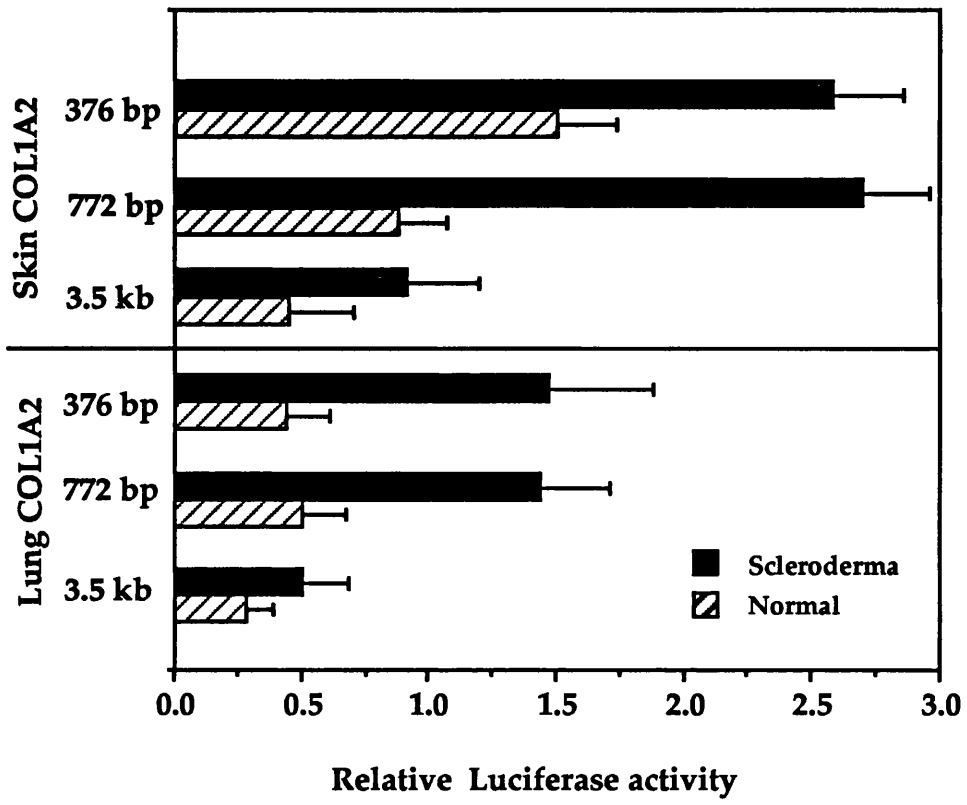


Figure 3.7. Quantitative analysis of COL1A2 transcription activity in normal and scleroderma skin and lung fibroblasts. Ralative luciferase activity (mean±sem), expressed in arbitrary units and corrected for transfection efficiency, for SSc and control fibroblast strains following transfection of COL1A2 promoter deletion constructs. Transient expression experiments were performed using Lipofectamine, according to the manufacturers instructions. Test contruct was linked to the firefly luciferase reporter gene and the control plasmid contained HSV-TK promoter construct linked to the renilla luciferase gene.

The direct study of lung fibroblasts grown by explant culture from open lung biopsies taken from scleroderma patients is complementary to studies of the cellular and cytokine composition of bronchoalveolar lavage (BAL) fluid from these patients. Thus, although fibroblast-like cells have been cultured from BAL fluid from scleroderma patients (Ludwicka *et al.*, 1992), such cells may be only indirectly related to fibrotic events in the disease, which are predominantly interstitial. Although the mean collagen production for lung scleroderma strains is greater than for control strains this is due to the 10 strains with markedly raised secretion. It is interesting that 6 strains had collagen secretion within the normal range. Drawing analogy from published data for dermal scleroderma strains it may be that even those strains with normal collagen secretion may contain an over-representation of high collagen producing strains (Fries *et al.*, 1994). That these strains with normal collagen (I) secretion do not exhibit normally regulated collagen synthesis is supported by our observation that they fail to down-regulate pro-α1(I)collagen mRNA levels in collagen matrix culture.

It has been shown that in skin fibroblasts in scleroderma there is transcriptional activation of genes encoding both the α1 and α2 constituent chains of collagen type I. Indeed the studies of Kikuchi *et al* (1992) employed similar COL1A2 promoter sequences to those in my experiments. The data extend these observations by providing evidence for increased activation of the COL1A2 gene in scleroderma skin and lung fibroblast strains compared with controls. Recent studies of the COL1A1 gene in scleroderma skin fibroblasts suggest that an enhancer element within the first intron may be important in mediating high level expression in dermal fibroblasts (Hitraya and Jimenez, 1996) but importance of such an element in activation of the COL1A2 gene is uncertain (Boast *et al.*, 1990). These results suggest that the elevated collagen (I) mRNA levels found in scleroderma fibroblasts are at least partly due to transcriptional activation.

In addition to overproduction of collagen (I) protein and mRNA, there also appears to be an important dysregulation in the down-regulation of steady

state collagen (I) mRNA levels for scleroderma skin and lung fibroblasts in collagen gel culture, in a manner similar to that previously reported by several groups studying scleroderma skin fibroblasts (Ivarsson *et al.*, 1993; Mauch *et al.*, 1992). The reduced steady state collagen (I) mRNA levels found in 3-D gel culture appear to arise through both transcriptional and post-transcriptional mechanisms (Eckes *et al.*, 1993). The impairment of down-regulation of collagen (I) synthesis reported for dermal scleroderma fibroblasts is associated with altered cell surface β1 integrin expression (Ivarsson *et al.*, 1993) and also correlates with increased mRNA transcript stability (Eckes *et al.*, 1996). Whether the postulated abnormalities in collagen binding integrin expression and mRNA stability are also present in scleroderma lung fibroblasts should be investigated in future studies.

It is interesting that lung fibroblasts appear to contract collagen gel matrices at least as well as dermal fibroblasts, and that the cells show similar morphological changes to those reported for dermal fibroblasts. These collagen gel cultures represent a more physiological environment than conventional monolayer cultures (Bell *et al.*, 1979), allowing cells to interact three dimensionally with extracellular matrix products. In view of the typical histological appearance of fibrotic lung in FASSc, in which fibroblasts are embedded within a dense collagen rich matrix (Harrison *et al.*, 1991), in marked contrast with the sparse interstitial matrix of normal lung parenchyma, collagen gels may be a particularly appropriate substrate in which to culture Scleroderma lung cells for functional analysis. For dermal fibroblasts the changes in matrix biosynthesis accompanying collagen gel contraction appear to be dependent on expression and function of cell surface integrins within the β family (Nusgens *et al.*, 1984).

The data support the view that scleroderma fibroblasts contract 3-D collagen matrices in a similar manner to normal fibroblasts (Ivarsson *et al.*, 1993; March *et al.*, 1992), although one group of investigators has suggested that the ability to cause gel contraction may be increased in scleroderma (Gillery *et al.*, 1991). Previously published studies of both skin and lung fibroblasts in

monolayer culture have suggested that α-smooth muscle actin is expressed in activated scleroderma fibroblasts, and that they might be more correctly termed myofibroblasts (Ludwicka *et al.*, 1992; Sappino *et al.*, 1990). It would be interesting to study the effect of gel matrix culture on α-actin expression in these cells, since modulation of the synthesis of cytoskeletal elements has been reported in such culture systems. If actin biosynthesis were diminished, this would support the view that the myofibroblast phenotype is not essential for matrix overproduction, but may be important in other aspects of the pathogenesis of fibrosis.

In conclusion, the data presented in this chapter suggested substantial similarity between the abnormal *in vitro* properties of scleroderma lung and skin fibroblasts. Further comparative studies between skin and lung fibroblasts in scleroderma may reveal other differences between these cell types, but these results support the concept of organ-independent phenotypic abnormalities for fibroblasts in scleroderma.

CHAPTER 4: IS THE REGULATION OF COLLAGEN BIOSYNTHESIS BY TGFβ MEDIATED VIA CTGF?

4.1 INTRODUCTION

The data shown in the previous chapter demonstrate that there are substantial similarities between elevated collagen synthesis and transcription of scleroderma skin and lung fibroblasts. One explanation for this is that the initiating stimulus, probably cytokine or growth factor, in both organs may lead to a sustained autocrine activation of the fibroblasts (Mauch *et al.*, 1993; Shiwen *et al.*, 1997). In this chapter I will explore expression of CTGF, which is a novel autocrine growth factor selectively induced by TGFβ, in scleroderma fibroblasts *in vitro*.

Human connective tissue growth factor (CTGF), a 349-amino acid polypeptide, was first discovered by Bradham *et al* in 1991 while screening human umbilical vein endothelial cell cDNA expression libaray by using a polyclonal anti-PDGF antibody (Bradham *et al.*, 1991). The gene for human CTGF has been localized to chromsome 6q23.1 (Martinerie *et al.*, 1992). The deduced molecular weight of CTGF is 38 KD. In adult tissue, CTGF mRNA is expressed in skin, heart, brain, lung, liver, muscle, and kidney (Igarashi *et al.*, 1996; Oemar *et al.*, 1997). Thus far, the physiological function of CTGF both *in vitro* and *in vivo* is not clear. However, Bradham *et al* found that HUVEC-conditioned medium containing CTGF is mitogenic to NRK cells *in vitro* and that protein extracts of Xenopus oocytes injected with CTGF mRNA are chemotactic to NIH 3T3 cells (Bradham *et al.*, 1991). Subsequently, the same research group found that in human skin fibroblasts, CTGF mRNA is induced specifically by TGFβ but not by PDGF, epidermal growth factor, or basic fibroblast growth factor (Igarashi *et al.*, 1993).

Transforming growth factor-β (TGFβ) has been shown to act as a potent stimulatory signal for connective tissue formation during wound repair and in fibrotic conditions. TGFβ is particularly effective in inducing major quantitative pro-fibrotic effects both *in vivo* and *in vitro* (Sporn *et al.*, 1987; LeRoy *et al.*, 1989). Of the cytokines capable of regulating the synthesis of the extracellular matrix,

TGFβ has undoubtedly received the most attention in scleroderma (LeRoy *et al.*, 1989). Elevated TGFβ has been reported more recently in the serum of patients with both diffuse and limited scleroderma (Snowden *et al.*, 1994). Several studies have reported TGFβ immunoreactivity in lesional skin of scleroderma patients (Higley *et al.*, 1994), with most reports suggesting that it is present in early inflammatory lesion before dense fibrosis had occurred (Pablos *et al.*, 1995). Indeed, maximal extracellular TGFβ1 immunoreactivity appears to be in the non-lesional skin taken from scleroderma patients with minimal staining in the established lesional areas (Higley *et al.*, 1994). These results suggest that TGFβ may be important in the early stages of scleroderma but less so at later stages. *In vitro* studies of TGFβ have also proven fibrotic activities. TGFβ is secreted from macrophages and lymphocytes (Assoian *et al.*, 1987; Kehrl *et al.*, 1986) and increases the production of collagen and fibronectin by fibroblasts *in vitro* (Varga *et al.*, 1987; Raghu *et al.*, 1989). TGFβ is also known to increase fibroblast proliferation (Soma and Grotendorst, 1989; Battegay *et al.*, 1990). Furthermore, TGFβ decreases degradation of extracellular matrix due to a direct inhibition of protease activity and stimulation of the synthesis of protease inhibitors (Laiho *et al.*, 1986; Lund *et al.*, 1987; Kerr *et al.*, 1990).

A potential role in a number of fibrosing diseases has been suggested by increased expression of CTGF in scleroderma skin as well as skin from other conditions such as keloid scars (Igarashi *et al.*, 1996). In human mammary carcinomas, in which there was marked connective tissue involvement, CTGF message was observed only in the fibroblasts (Frazier and Grotendorst, 1997). CTGF is also expressed at very high levels in atherosclerotic lesions but not in normal human blood vessels. High level expression of CTGF in intimal smooth muscle cells is found predominately in areas with extracellular matrix accumulation and fibrosis (Oemar *et al.*, 1997). Therefore, CTGF is thought to have a regulatory function for extracellular matrix production in fibroblasts. Recently reported experiments suggest that CTGF injected subcutaneously into the skin of new-born mice causes a marked fibroproliferative response that provides additional evidence for its fibrogenic potential (Frazier *et al.*, 1996). In addition, TGFβ and CTGF mRNA are coordinately overexpressed during wound

repair in an *in vivo* model for wound healing in the rat, indicating that CTGF may be one of the downstream effectors of TGFβ (Igarashi *et al.*, 1993). Indeed, Grotendorst *et al* have recently found a novel TGFβ-responsive element with the sequence 5'-GTGTCAAGGGGTC-3' located between positions -126 and -128 of the CTGF promoter sequence TGFβ induced a 25-fold to 30-fold increase in luciferase activity in NIH-3T3 fibroblasts that had been transfected with a promoter construct containing the 5 flanking region of the human CTGF gene linked to the luciferase reporter gene (Grotenderst *et al.*, 1996). *In vitro* experiments have suggested that CTGF release by fibroblasts is readily induced by TGFβ and it may have an important role as a TGFβ induced autocrine fibroblast growth factor (Igarashi *et al.*, 1993).

Work described in this thesis by investigates scleroderma and normal fibroblast expression of CTGF and the effects of TGFβ1, 2 and 3 on this expression. Furthermore the role of CTGF as an autocrine growth stimulator in the TGFβ treated fibroblasts will be determined.

4.2 PATIENTS AND STATISTICS

4.2.1 Samples

Punch biopsies (4mm^3) were obtained from clinically affected areas of the skin of patients with active diffuse cutaneous scleroderma and from healthy volunteer controls. The group of 8 scleroderma patients was all fulfilled the criteria of the American College of Rheumatology for the diagnosis of scleroderma. The mean age of the patients with scleroderma was 48.0 years (range 43-57) and of control subjects 45.5 years (range 41-52). The sex ratio was 7F:1M. In experiments, fibroblasts were used between passages 2 and 5 unless otherwise stated.

4.2.2 Statistical analysis: All results are expressed as means ± SEM unless otherwise stated. Student's unpaired-test was used for statistical analyses. P-values less than 0.05 considered statistically significant.

4.3 RESULTS:

4.3.1 The CTGF protein is expressed only in scleroderma cell layer

Previously, Grotendorst's group has shown that TGFβ stimulates fibroblast proliferation through induction of CTGF (Kothapalli *et al.*, 1997). The experiments described below aimed to determine whether CTGF expression or secretion was increased in early passage scleroderma fibroblasts. These studies show CTGF protein is expressed only in scleroderma cell layer (Figure 4.1A). In contrast, no CTGF was observed in the media of scleroderma or normal fibroblasts (Figure 4.1B).

4.3.2 Detection of CTGF protein in TGFβ-treated normal and scleroderma fibroblasts

After pre-incubation with TGFβ, the cell lysates of both scleroderma and normal fibroblasts contain CTGF protein, as shown by immunoblotting analysis using an anti-CTGF specific antibody. TGFβ1, 2 and 3 all stimulated normal and scleroderma fibroblast production of CTGF, with TGFβ1 being the most potent stimulator (Figure 4.2). After TGFβ1 stimulation, CTGF protein levels in scleroderma fibroblasts were about 2 fold greater than those in normal cells (Figure 4.3). In contrast, after TGFβ treatment CTGF protein was readily detectable in media from scleroderma fibroblasts but only very low, barely detectable levels of CTGF could be found in the media of normal fibroblasts, when the media was concentrated with Heparin Sepharose (Figure 4.4). CTGF expression was normalised to the same total protein levels. Quantitation of the levels of CTGF have been adjusted to take into account any differences in cell number.

4.3.3 Protein

The Bio-Rad protein micro assay was used to determine total protein content of tissue culture supernatants and cell lysates, including fibroblasts stimulated by TGFβ and CTGF. A representative standard curve is shown in Figure 4.5. The results show that normal and scleroderma fibroblasts secreted the same amount

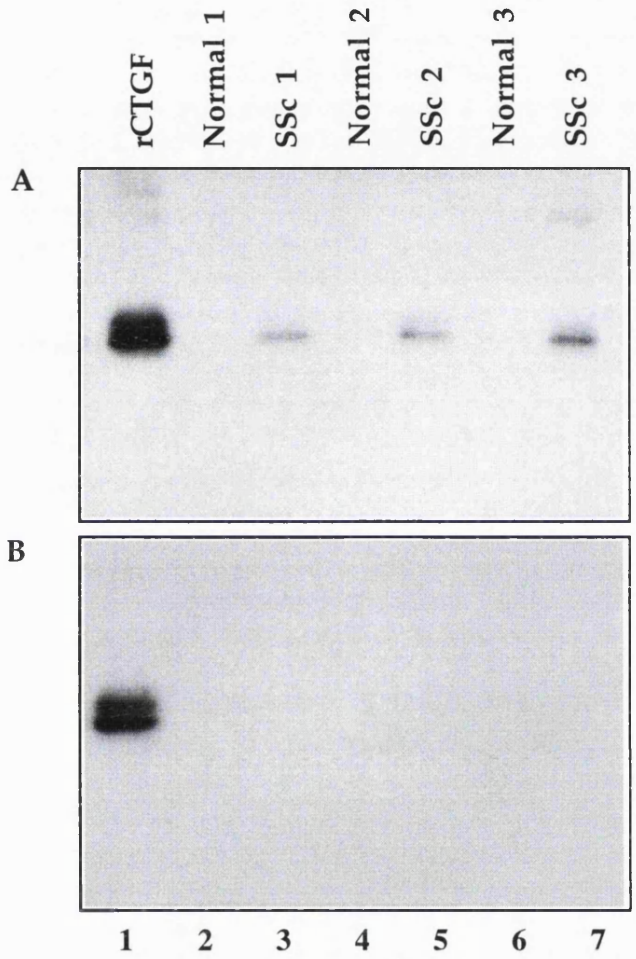


Figure 4.1. CTGF is only expressed by SSc fibroblasts. Normal (n=3) and SSc (n=3) fibroblasts were grown to confluence in 6-well plates. The growth media was removed, cells were washed with PBS, and 1ml of serum-free media was added to each well. The media was removed after conditioning for 48 hours. Cell extracts and conditioned media then run on 12% SDS gels, electroblotted to nitrocellulose, and visualized with rabbit antibody. A. cell extracts. B. media. Recombinant CTGF (5ng) shown in lane one.

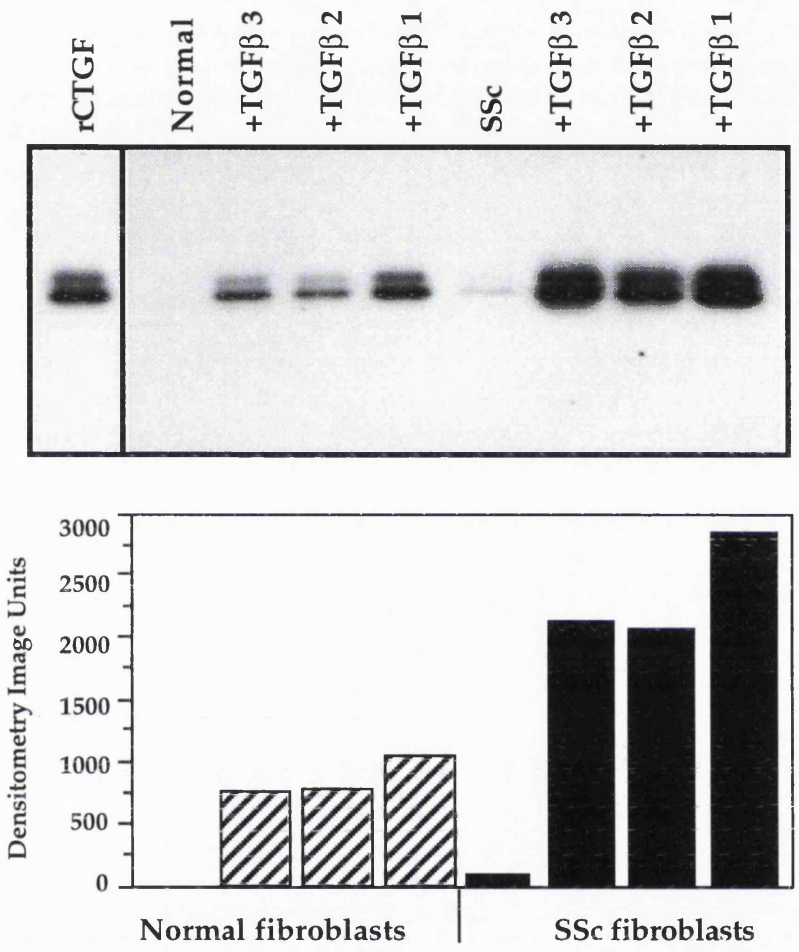


Figure 4.2. The effect of TGFβ1, 2, 3 on CTGF expression in normal and SSc fibroblasts. Comparison of CTGF protein in cell monolayer following induction by TGFβ1, 2, or 3. Confluent SSc and normal fibroblasts were incubated in serum free media with 10 ng of the respective TGFβ isoforms for 48 hours.

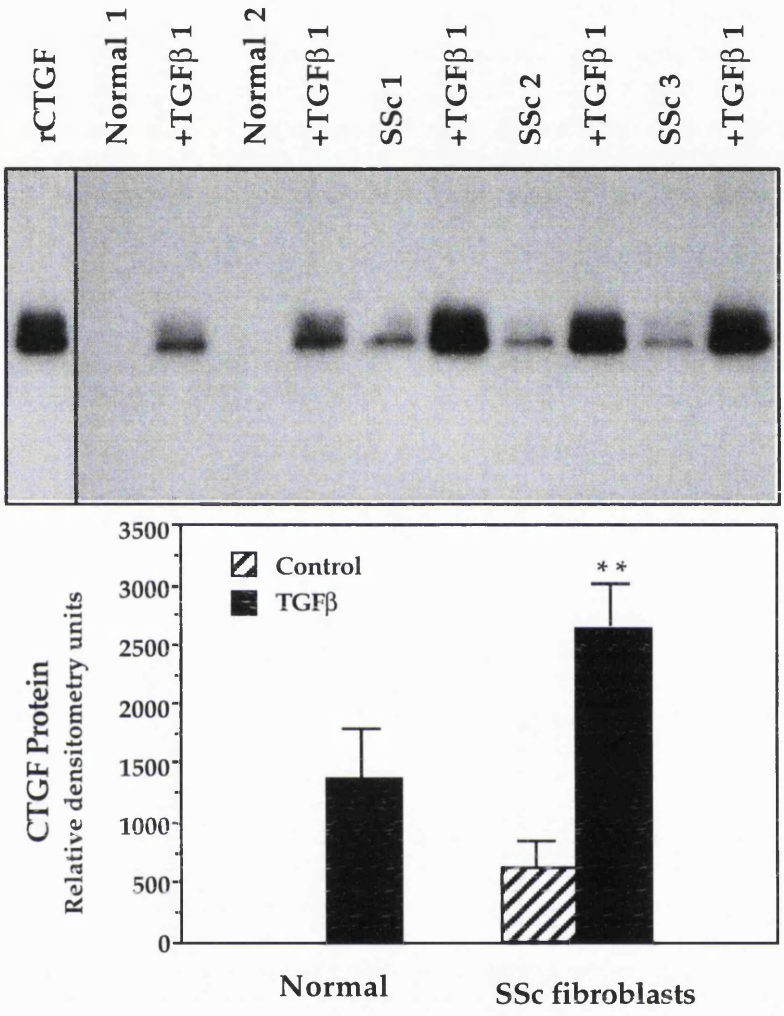


Figure 4.3. TGFβ1 stimulation of CTGF differs between normal and SSc fibroblasts. Confluent cells were stimulated with or without TGFβ1 (10ng) for 48 hours. Cell lysates were subjected to 12% polyacrylamide gels and analyzed by Western blot. The band density of film was determined by densitometry. Similar results were obtained in two additional experiments. ** $p<0.01$ for the difference between control and TGFβ. n=8 for each group.

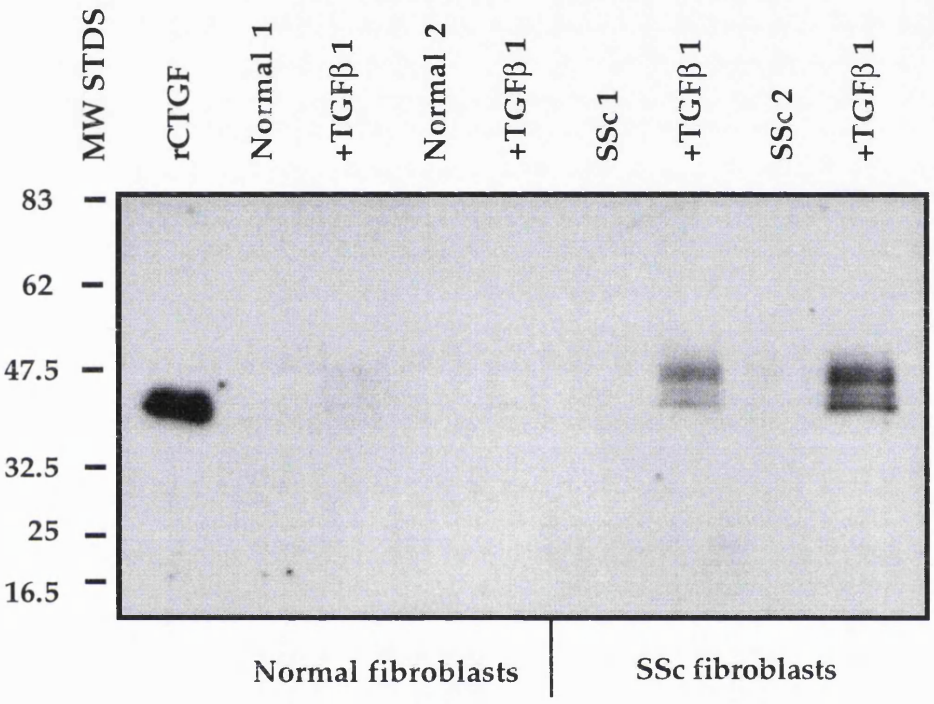


Figure 4.4 The TGFβ1 stimulation of CTGF secretion by normal and SSc fibroblasts. Western blot of fibroblasts-secreted CTGF protein. Conditioned media was incubated with Heparin Sepharose to concentrate CTGF prior to analysis.

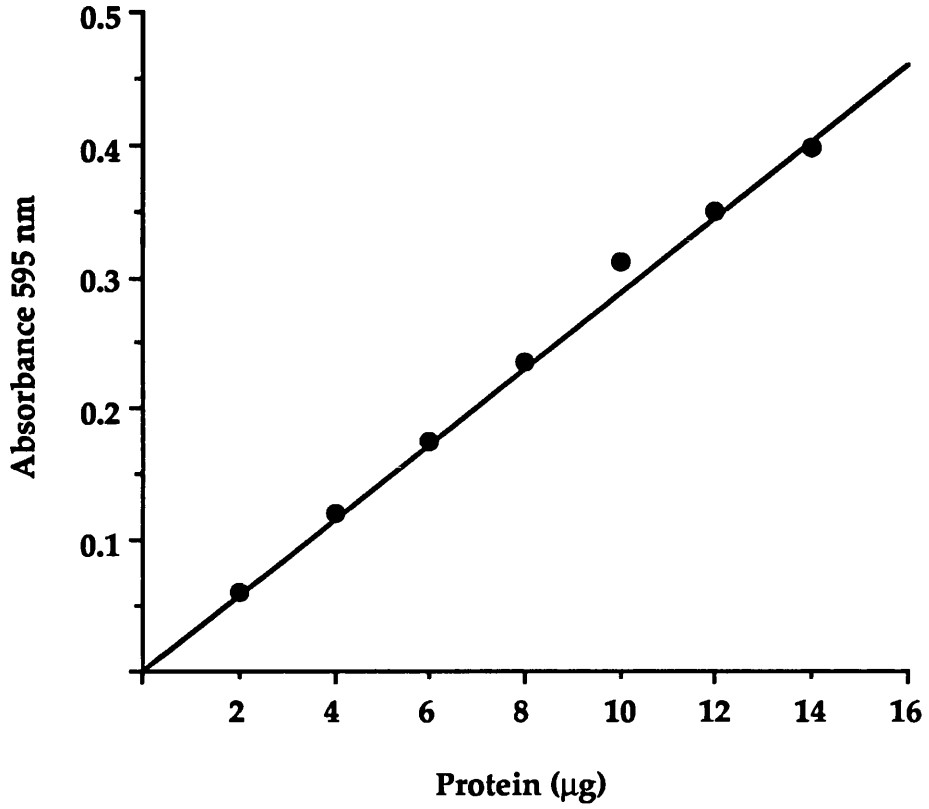


Figure 4.5. Representative standard curve for the protein micro-assay. Protein concentration in cell lysates and culture supernatants was measured using the Bio-Rad protein kid. Absorbance at 595 nm was measured in replicate samples using an automatic plate reader. This standard curve used known concentrations of bovine serum albumin and confirms the linear relationship between protein concentration and absorbance, over a range between 2-20 μg protein.

of total proteins. Furthermore, total protein synthesis after exposure of fibroblasts to TGFβ and CTGF (48 hours) was not significantly changed.

4.3.4 Scleroderma fibroblasts show increased mitogenic response to rCTGF

Increasing concentrations (20-80ng/ml) of rCTGF induced a marked elevation of thymidine incorporation in a dose-dependent manner. The difference in the mitogenic effects of rCTGF between normal and scleroderma fibroblasts was significant at concentrations higher than 20ng/ml with scleroderma fibroblasts being more responsive than control normal cells ($p<0.05$) (Figure 4.6).

4.3.5 TGFβ induced collagen secretion correlates with CTGF expression

TGFβ1, 2 and 3 enhanced collagen release by control and scleroderma fibroblasts, and TGFβ1 was shown to be the most potent isoform. Basal and stimulated pro-α1(I) collagen secretion was higher for scleroderma than for normal strains. Pro-α1(I) collagen was stained with Coomassie Blue R-250 (Figure 4.7).

4.3.6 Elevation of collagen protein synthesis by CTGF and TGFβ

To compare the ability of rCTGF with TGFβ to stimulate synthesis of collagen in normal and scleroderma fibroblasts, the rate of synthesis of collagen was examined in both TGFβ and CTGF treated monolayers of fibroblasts. The results of this study demonstrate that pro-α1(I) collagen synthesis is stimulated by rCTGF and TGFβ, with quantitative densitometric analysis of films indicating, based on the average of the three experiments, CTGF and TGFβ significantly enhanced pro-α1(I) collagen synthesis by normal and scleroderma fibroblasts (Figure 4.8). CTGF stimulated a 3-fold increase in collagen synthesis. TGFβ stimulated a 4-fold increase in collagen synthesis. Thus, with respect to the induction of collagen production, CTGF has activity similar to TGFβ.

4.4 DISCUSSION

The results presented in this chapter clearly demonstrate that scleroderma fibroblasts cultured alone express significant levels of CTGF protein *in vitro*. In contrast, normal fibroblasts were shown not to express detectable amounts of

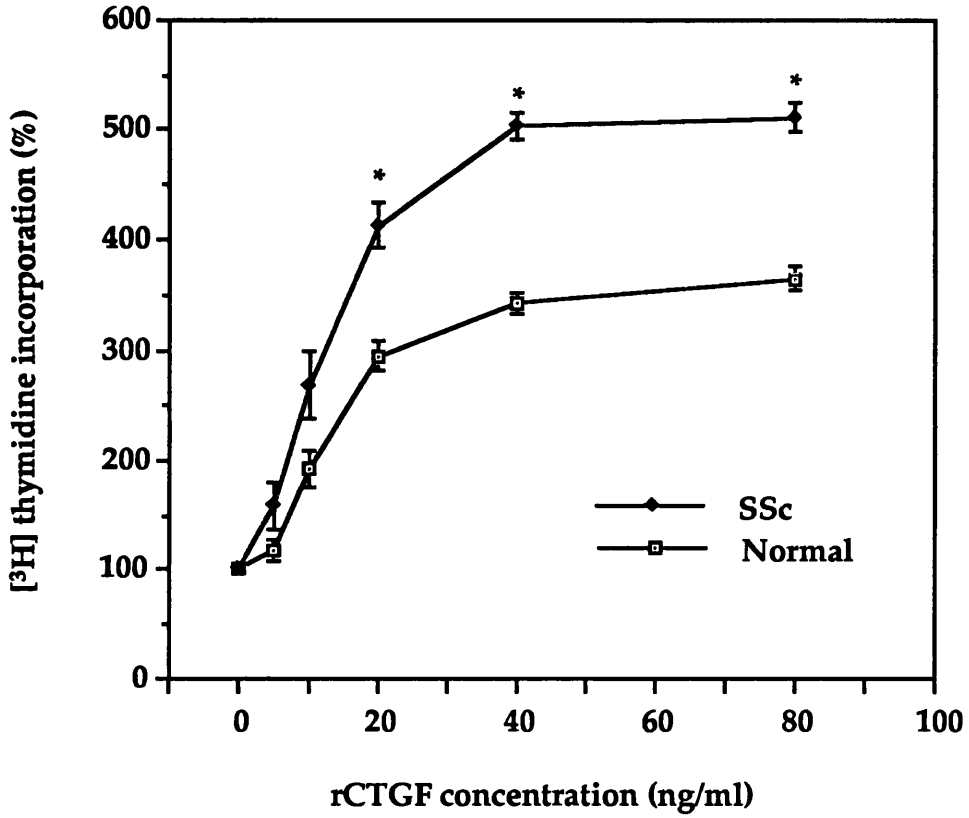


Figure 4.6. Increased mitogenic effect of rCTGF on SSc fibroblasts compared with normal fibroblasts. Confluent cells were growth arrested by serum deprivation and stimulated with specific concentrations of rCTGF for 24 hours. Mean data for three independent experiments using three SSc and three normal fibroblast strains. Mean (±SEM) basal incorporation was 18,400 (± 2955) for SSc and 16,033 (± 2237) dpm for normal strains.
* indicates significance $p<0.05$, unpaired Student's test.

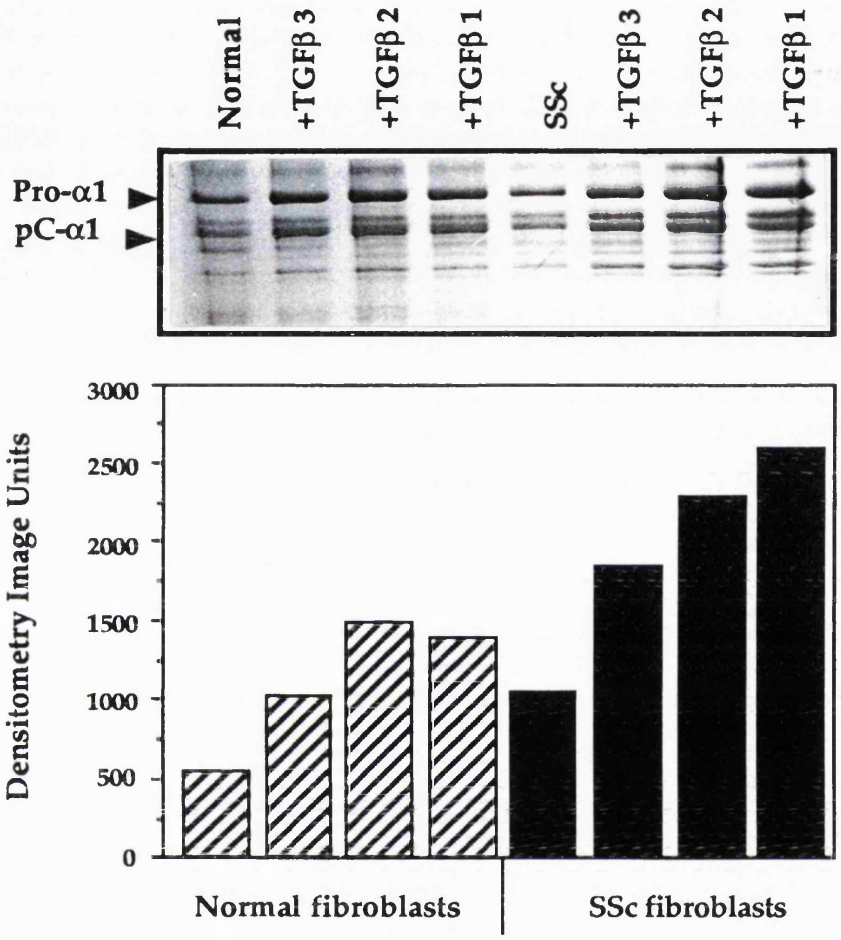


Figure 4.7. The effect of TGFβ1, 2, 3 on type I collagen synthesis in normal and SSc fibroblasts. Confluent monolayer cultures of normal and SSc fibroblasts were stimulated for 24 h with TGFβ1, 2, and 3 (10 ng per ml). Secreted pro-α1(I) collagen was determined by Coomassie blue staining of SDS gels after precipitation from media with ammonium sulphate.

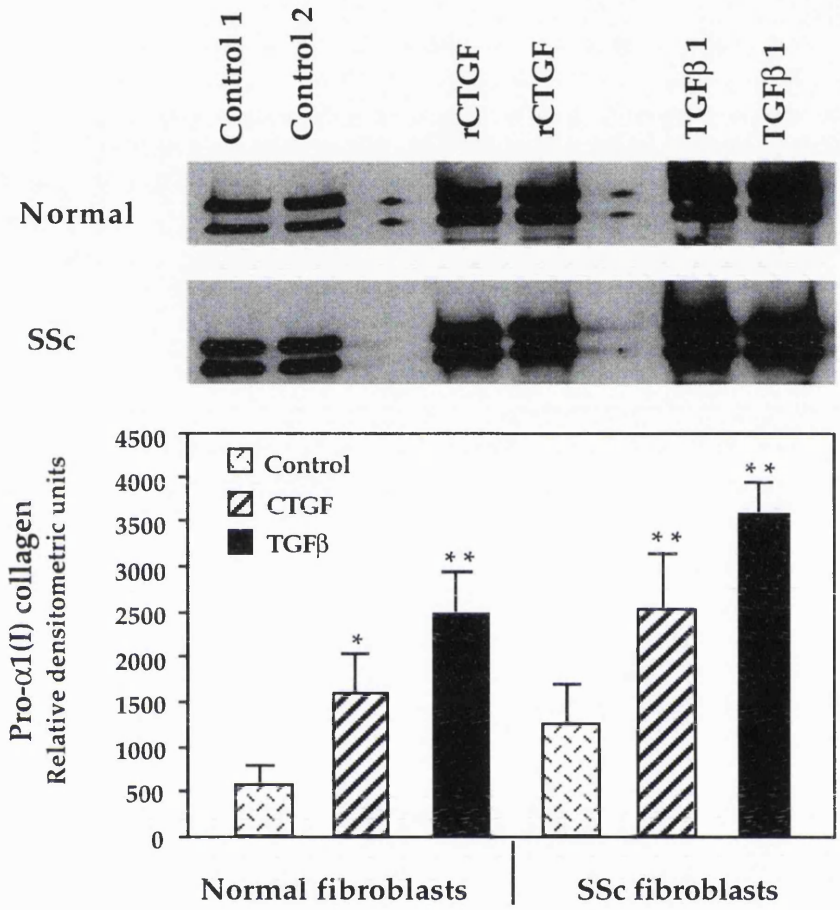


Figure 4.8. Stimulation of pro-α1(I) collagen synthesis in normal and SSc fibroblasts by rCTGF and TGFβ. Confluent monolayer cultures of normal and SSc fibroblasts were stimulated for 24 hours with either rCTGF (40 ng per ml) or TGFβ1 (10 ng per ml). Proα1(I) collagen synthesis was determined by western blot.
* $p<0.05$ and ** $p<0.01$, unparied Student's t-test compared with control cells. n=8 for each group

CTGF. The production of CTGF in sclerodema fibroblasts was enhanced in the presence of TGFβ1, and in addition normal fibroblasts were induced to express CTGF, by TGFβ exposure. In un-stimulated cells, CTGF was not detected in culture media either from normal or scleroderma fibroblasts in my experiments. However, after TGFβ treatment CTGF protein was readily detectable in media from scleroderma fibroblasts, but only very low, barely detectable levels of CTGF could be found in the media from normal cells (Figure 4.4).

It is not known how or by which mechanism(s) CTGF is released into the culture media by fibroblasts. The most plausible explanation for CTGF secretion is that TGFβ directly stimulates CTGF production (the CTGF promoter is known to contain an TGFβ responsive element) and a proportion of the growth factor is directly secreted or transported to the cell surface by specific receptors. In additon it is possible that inview of the fact that CTGF contains an heparin binding motif and thus exhibits a high affinity towards the heparin, CTGF may bind to heparin sulphate containing proteoglycans at the cell surface acting as an depot for the growth factor. Stimulation by TGFβ may result in the production of specific proteases that may enhance the release or shedd the CTGF from the cell surface. Another possibility is that CTGF acts as an autocrine factor that once expressed is able to bind to cell surface receptors, and may be associated with the TGFβ-mediated signalling pathway (Kothapalli *et al.*, 1997), and thereby may stimulate albeit indirectly its own expression. The CTGF protein has previously been reported to be efficiently secreted into the culture medium after stimulation of quiescent NIH 3T3 cells, and to have a relatively short half-life of about one hour (Ryseck *et al.*, 1991). These results would suggest that the most likely influence of TGFβ on CTGF secretion is at the biosynthetic stage.

The potential role for CTGF in scleroderma and other fibrotic disease is therefore intriguing. TGFβ expression has been shown to be increased in scleroderma lesions, particularly in the earlier stages suggesting an early involvement of TGFβ in the progression of scleroderma following the cascade of other growth factors (Higley *et al.*, 1994). Igarashi *et al* (1995) also demonstrated CTGF mRNA expression in skin fibroblasts located in the sclerotic lesions from patients with scleroderma, whereas no CTGF gene expression was observed in sections

without histological evidence of sclerosis. These results show a correlation between skin fibrosis and CTGF gene expression, supporting the hypothesis that TGFβ play an important role in the pathogenesis of scleroderma since CTGF is induced by TGFβ. Perhaps the most compelling evidence for a link between TGFβ activity and CTGF the studies performed by Kothapalli *et al* 1997, who showed that the presence of antisense sequences to CTGF abolished TGFβ-mediated anchorage dependent responses in mammalian cell lines.

Since there is a co-ordinate expression of TGFβ and CTGF in scleroderma and selective induction of CTGF by TGFβ in fibroblasts, the biological activities of recombinant CTGF and TGFβ should been compared. Unfortunately, for several years, the investigation of the physiological functions of the CTGF gene family has been hampered by the lack of purified or recombinant protein. Due to the high content of cysteine in CTGF protein family members (11% of the total amino acid content), CTGF protein production is relatively difficult (Kireeva *et al.*, 1997; Oemar *et al.*, 1997). Recently, a research group at FibroGen Inc. has successfully produced recombinant CTGF (rCTGF), using the baculovirus expression system. I have used rCTGF and these studies indicate that rCTGF can induce scleroderma fibroblast proliferation and extracellular matrix synthesis. Thus, CTGF appears to mimic some of the actions of TGFβ with regard to the stimulation of connective tissue cell growth and synthesis of type I collagen. There is also a significant difference to the response to CTGF between scleroderma and normal fibroblasts which provides indirect support for the hypothesis that CTGF may be involved in the pathogenesis of scleroderma.

As in scleroderma, there is evidence that CTGF expression may be closely linked to the expression of TGFβ; CTGF is overexpressed in dermal fibroblasts of scleroderma patients (Igarashi *et al.*, 1995), and the over expression of TGFβ has also been reported in scleroderma lesions (Smith and LeRoy 1990). These observations suggest a causative association between TGFβ, CTGF and the increased deposition of collagen. In a rat animal model for wound healing, TGFβ and CTGF mRNA are also co-ordinately overexpressed during wound repair (Frazier *et al.*, 1996). Furthermore, cancer stroma formation involves induction of similar fibroproliferative growth factor (TGFβ and CTGF) as wound repair

(Frazier and Grotendorst, 1997). TGFβ is now recognised as a ubiquitous cytokine with diverse effects on many cell types. It is dimeric and secreted as an inactive precursor molecule, a large latent complex, which requires activation into a functional ligand (Border and Noble, 1994). Most of the TGFβ in tissue and that produced by cells in culture is in the latent form (Flaumenhaft *et al.*, 1993). Consequently, amount of RNA transcript does not always reflect the level of active TGFβ present (Flanders, 1989). Because the methods employed to extract growth factors from tissue may convert latent TGFβ to its active form, it is difficult to determine how much active TGFβ is present in tissues. The fibroblast expression of CTGF in culture is dependent on the presence of active TGFβ (Frazier *et al.*, 1996). Factors other than TGFβ have not been shown to induce a significant level of expression of the CTGF gene (Igarashi *et al.*, 1993). These studies suggest that the presence of CTGF gene activity may be useful as a marker for the presence of active TGFβ in tissues.

When dermal fibroblasts from scleroderma patients are cultured *in vitro*, they exhibit basal intracellular CTGF expression, perhaps cells have been stimulated by TGFβ *in vivo*. Interestly, the expression of CTGF in scleroderma fibroblasts was greater than that in normal fibroblasts in the presence of TGFβ, suggesting increased response of scleroderma fibroblasts to TGFβ. The results demonstrate that CTGF stimulates fibroblast proliferation and pro-α1 (I) collagen synthesis in a manner similar to TGFβ. Although not yet isolated, it is likely that CTGF exerts its activity through binding to a cell surface receptor that subsequently transduce signals intracellularly. Further work to examine the receptor binding profiles, and molecules involved in the signalling pathway in addition to the isolation and characterisation of the receptor will further our hypothesis that CTGF is a major autocrine growth factor involved in fibrotic conditions. While CTGF has similar pro-fibrotic properties to TGFβ (indeed it may be downstream mediator of TGFβ effects), it appears to have a more limited range of biological activity, i.e. CTGF is only expressed during the fibrotic process. Therefore it is likely that this growth factor may represent a more appropriate candidate for diagnostic purposes during disease progression and to test potential anti-fibrotic therapeutic strategies.

CHAPTER 5: ENDOTHELIN: ROLE IN FIBROBLAST PROLIFERATION AND MATRIX BIOSYNTHESIS

5.1 INTRODUCTION

The results presented in Chapter 4 showed that scleroderma fibroblasts demonstrate increased responsiveness to CTGF. In contrast, data which I present below shows reduced responses for scleroderma fibroblast modulation of synthesis of collagen and other matrix molecules by ET-1. The impaired response to ET-1 was associated with reduced expression of ET_A receptors on the cell surface. This is interesting since it suggests that different cytokines may modulate fibroblast properties independently.

The endothelins (ETs) are a family of 21 amino acid residue peptides that were originally isolated from the culture medium of porcine vascular endothelial cells (Yanagisawa *et al.*, 1988; for review see Rubanyi and Polokoff, 1994). The three isoforms so far identified exhibit significant homology, and are synthesised as larger prepropolypeptides which are processed by endopeptidase activity (endothelin converting enzyme, ECE) through pro(big)endothelins into the mature peptides (Levin, 1995). They are produced by a variety of tissues and cell types, and exhibit tissue-specific patterns of expression (Rubanyi and Polokoff, 1994; Kahaleh, 1991). As all endothelins exert their biological activities via specific cell surface receptors, the distribution and functional activities of these receptors are likely to have significant impact on a variety of disease processes. Three endothelin receptors have been identified (ET_A, ET_B and ET_C), and these are found widely expressed in the cells of many organs including the heart, kidney, liver, lung and skin (Rubanyi and Polokoff, 1994; Levin, 1995). The ET_A and ET_C receptors have a higher affinity for ET-1 and ET-3 respectively whereas the ET_B receptor subtype is equipotent for all three isoforms. The receptors are members of the G-protein-coupled receptor family, and signal transduction follows ligand binding, resulting in intracellular events that include mobilisation and influx of cytosolic Ca^{2+}, G-protein-mediated activation of phosphlipase C, activation of protein kinase C and increase in arachidonic metabolism (Rubanyi

and Polokoff, 1994; Levin, 1995; Pollock *et al.*, 1995). The ET_A receptor predominates on vascular smooth muscle and mediates vasoconstriction (Martin and Brenner, 1990; Lin *et al.*, 1991) whereas, the ET_B subtype receptor, when present on vascular endothelium, mediates vasodilatation through nitric oxide release (Takayanagi *et al.*, 1991). Pharmacological data have suggested that the ET_B receptor can be further divided into ET_{B1} and ET_{B2} based on differential vasoactivity (Sokolovsky *et al.*, 1992). Moreover, antagonists for the ET receptor subtypes have been developed, which provide new and potentially effective tools to interfere with vascular disease (Clozel *et al.*, 1994; Reynolds *et al.*, 1995; Levin, 1995).

As reviewed in chapter 1, scleroderma is a disease characterised by progressive vascular damage, autoantibody production, increased fibrosis and extracellular matrix deposition in the dermis and visceral organs. The pathogenesis of this condition is unknown. Considerable evidence suggests that ET-1 may play a significant role in scleroderma pathogenesis (Kahaleh, 1991; Knock *et al.*, 1993). For example, studies have shown increased plasma ET levels in patients with Raynaud's phenomenon and scleroderma (Kahaleh, 1991; Vancheeswaran *et al.*, 1994). ET-1 levels in patients with diffuse scleroderma appear to be higher than in the limited disease (Yamane, 1992), perhaps reflecting the extent of endothelial damage in two major disease subsets. Quantitation of ET binding sites by micro-autoradiography has suggested significantly higher levels in scleroderma than in control tissues (Abraham *et al.*, 1997). Since endothelial and epithelial cells produce ET-1 *in vitro* (Rubanyi and Polokoff, 1994; Levin, 1995), it is likely that these cells represent the major source of ET-1 in scleroderma, although other cells, notably macrophages, polymorphonuclear cells, smooth muscle cells and fibroblasts also synthesise ET-1 (Haynes and Webb, 1993; Ehrenreich *et al.*, 1990; Sessa *et al.*, 1991), the level of which can be modulated in response to a range of stimuli including hypoxia, vasoactive hormones, and cytokines (Kourembana *et al.*, 1991). As inflammatory cell infiltrates and aberrant cytokine regulation are features of scleroderma these factors are also likely to influence the disease process.

In addition to being potent vasoconstrictors, the ETs exhibit biological effects *in vitro* and *in vivo* on both vascular and non-vascular smooth muscle (Kahaleh, 1991). They are also known to be mitogenic peptides exerting significant proliferative activity on smooth muscle, endothelial cells (Rubanyi and Polokoff, 1994; Levin, 1995), and fibroblasts (Kahaleh, 1991; Kikuchi *et al.*, 1995). In addition ETs cause the release of proinflammatory cytokines, and have considerable fibrogenic activity, influencing the maintenance of the extracellular matrix (ECM) by altering fibroblast metabolism, in particular the deposition of ECM by regulating the production and turnover of matrix components (Kahaleh, 1991; Guarda *et al.*, 1993).

Experiments described in this chapter examine the hypothesis that scleroderma fibroblasts may show an altered pattern of matrix synthesis in response to ET-1, reflecting their increased exposure to ET-1 *in vivo*, and further that ET-1 can induce a fibrogenic phenotype into non-scleroderma fibroblasts in tissue culture. I have also examined the possible role of cell-specific expression of endothelin receptor sub-types on fibroblasts from skin fibrosis by utilizing receptor blocking agents to target specific ET_A or ET_B endothelin receptors.

5.2 PATIENTS AND STATISTICS

5.2.1 Samples

Punch biopsies ($4mm^3$) were obtained from clinically affected areas of the skin of patients with active diffuse cutaneous scleroderma and from healthy volunteer controls. The group of 6 scleroderma patients all fulfilled the criteria of the American College of Rheumatology for the diagnosis of scleroderma. The mean age of the patients with scleroderma was 45 years (range 40-52) and of control subjects 43 years (range 37-51). The sex ratio was 5F:1M.

5.2.2 Statistical analysis

All results are expressed as means $\pm$ SEM unless otherwise stated. Student's unpaired T-test was used for statistical analyses. P-values less than 0.05 were considered statistically significant.

5.3 RESULTS

5.3.1 Scleroderma fibroblasts show reduced mitogenic response to ET-1

There was a significant difference in the mitogenic effects of ET-1 for normal and scleroderma fibroblasts, at concentrations higher then 10^{-11} M. I found that increasing concentrations (10^{-11} to 10^{-9} M) of ET-1 induced a marked elevation of thymidine incorporation in a dose-dependent manner in normal fibroblasts with 0.5 % FCS. The effect was much diminished for scleroderma fibroblasts (Figure 5.1). To determine which of the two ET receptor types mediate ET-1-stimulated proliferation, growth studies were performed in the presence of the selective ET_A receptor antagonist, PD 156707 and Ro 47-0203 (a mixed antagonist) which significantly attenuated the increase in mitogenic activity stimulated by 10^{-9} M ET-1 in normal fibroblasts ($P<0.01$). In contrast, BQ-788, a specific ET_B receptor antagonist, used at the same concentration was ineffective at antagonising ET-1 induced thymidine incorporation (Figure 5.2).

5.3.2 ET-1 enhances type I and type III collagen secretion but diminishes secretion of MMP-1

Type I and III collagen synthesis in normal fibroblasts was increased after incubation with ET-1. The incremental increase in collagen synthesis reached statistical significance ($p<0.01$) (Figure 5.3). However, the concentrations of endothelin needed to demonstrate this effect (ET 10^{-7} M) were higher than those needed to produce the mitogenic effect (ET 10^{-9} M). Although basal collagen synthesis by scleroderma fibroblasts was higher than control cells, this difference disappeared after treatment of the cells with ET-1. When normal fibroblasts secreted comparably high levels to scleroderma strains, the scleroderma strains did not show increased levels of secretion as high as normal ($p<0.05$) (Figure 5.3). The effect of ET-1 on collagen synthesis in normal fibroblasts was not blocked by PD 156707 or BQ-788, but by Ro 47-0203 ($p<0.01$) (Figure 5.4). Decreased basal levels of MMP-1 production in scleroderma fibroblasts compared to normal cells ($p<0.01$) was also observed. ET-1 decreased MMP-1 in normal fibroblasts ($p<0.01$) (Figure 5.5), to similar levels as those seen in non-treated scleroderma cells.

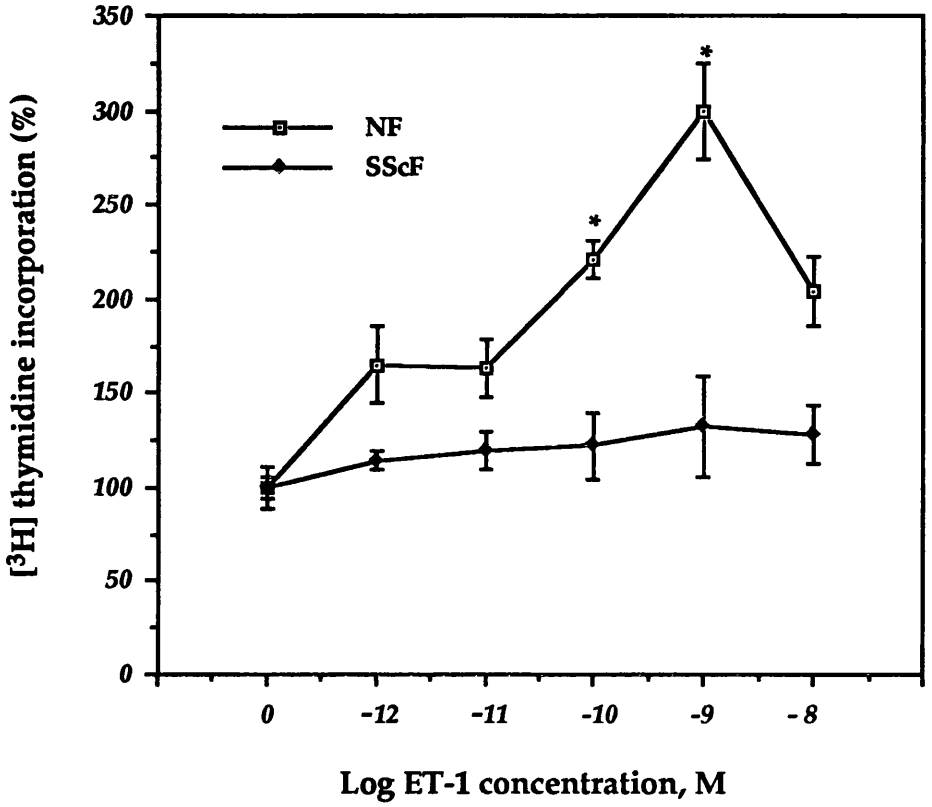


Figure 5.1. Fibroblast proliferation in response to endothelin-1. ET-1 promotes dermal fibroblast DNA synthesis, measured by incorporation of tritiated thymidine over a 16h labelling period, but SSc strains are significantly less responsive than normal fibroblasts. Data shown are mean (±sem) % basal (control) dpm based on 6 replicate wells for three independent experiments using different fibroblast strains. For control wells the mean radioactivity was 21465±2064 dpm for normal and 23761±1475 dpm for the SSc strains. *$p<0.05$ SSc compared with normal by Student's unpaired t-test.

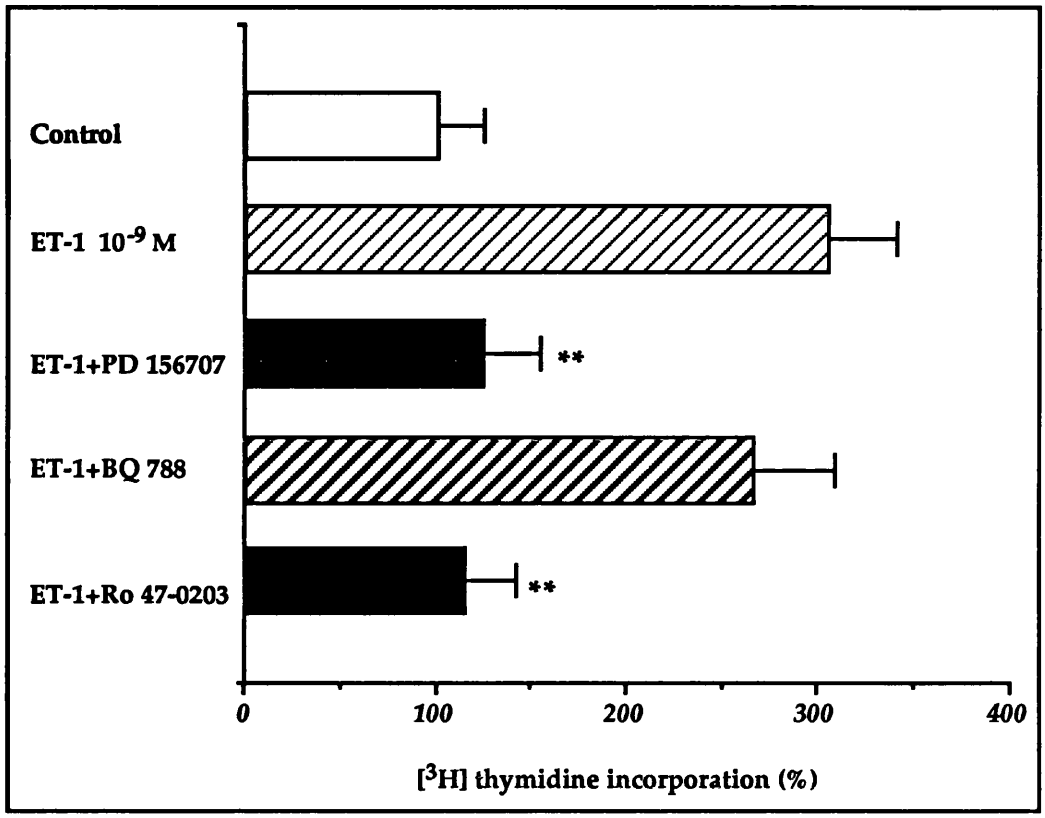


Figure 5.2. Inhibitory effect of ET receptor antagonists on the mitogenic effect of ET-1. Tritiated thymidine incorporation stimulated by exogenous ET-1 (10^{-9}M) in normal fibroblasts. Effect of PD 156707, BQ 788 and Ro 47-0203 (100nM) on basal DNA synthesis from a representative experiment (N = 4). Results are expressed as a percentage of control cells culture alone; each bar represents means $\pm$ SD. ** $p < 0.01$ compared to controls which were not added with antagonists.

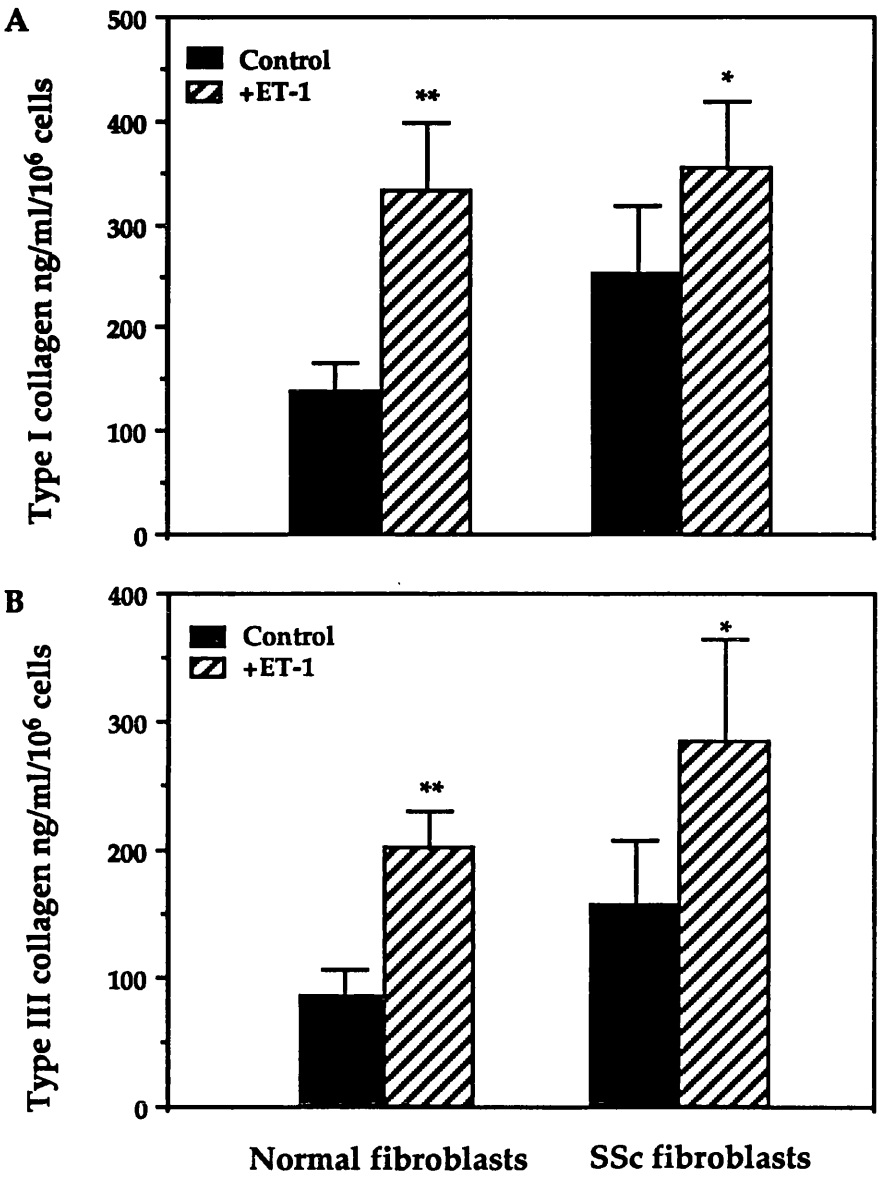


Figure 5.3. Endothelin-1 increases collagen type I and III secretion by dermal fibroblasts. Collagen type I (A) and type III (B) secretion by both normal (n=6) and SSc (n=6) fibroblasts is increased by incubation with ET-1 (10^{-7}M) for 48 hours. Aliquots (100ul) of conditioned medium were tested with an ELISA method. The SSc cells show a reduced response compared with normal fibroblasts. Data are mean (±sem) for 3 independent experiments. * indicates significantly (* $p<0.05$, ** $p< 0.01$, Student's unpaired t-test) above control collagen concentration.

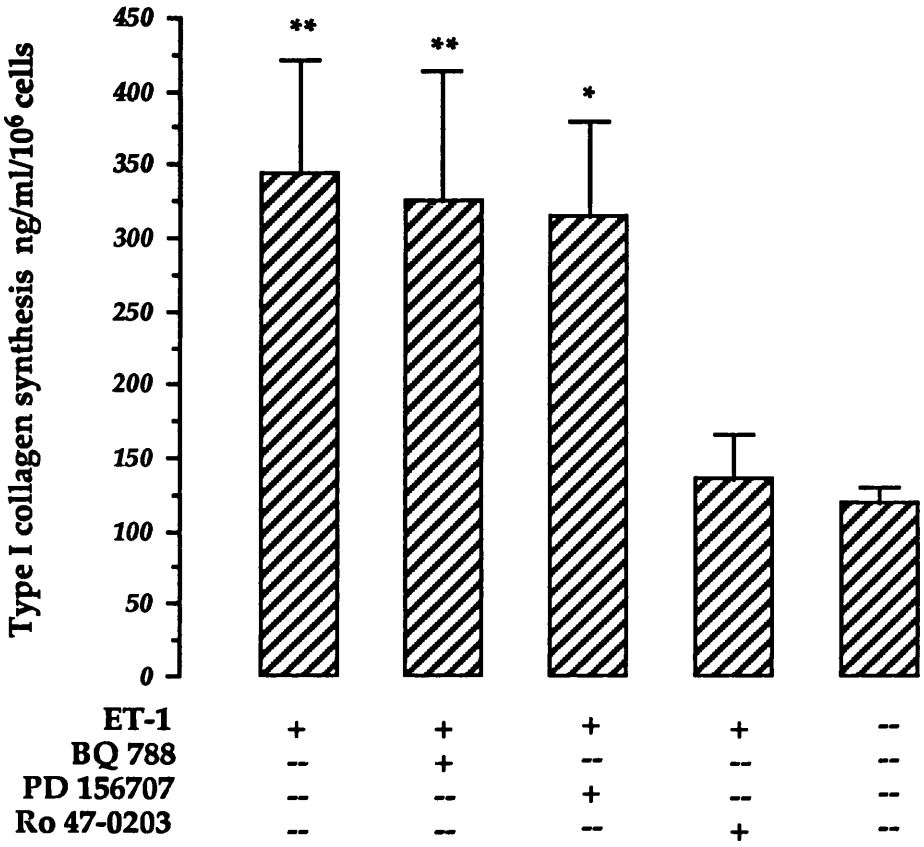


Figure 5.4. Both ET_A and ET_B receptor subtypes mediate ET-1-induced collagen synthesis. Collagen secretion by normal fibroblasts (n=5) is increased by incubation with ET-1 (10^{-7}M) for 48 hours. PD 156707 and BQ 788 (100 nM) did not prevent the increase in collagen type I synthesis by ET-1. This effect can be completely abolished by co-incubation with the mixed $ET_{A/B}$ receptor antagonist bosentan (100nM). * indicates significantly (* $p<0.05$, ** $p< 0.01$ Student's unpaired t-test) above control collagen concentration.

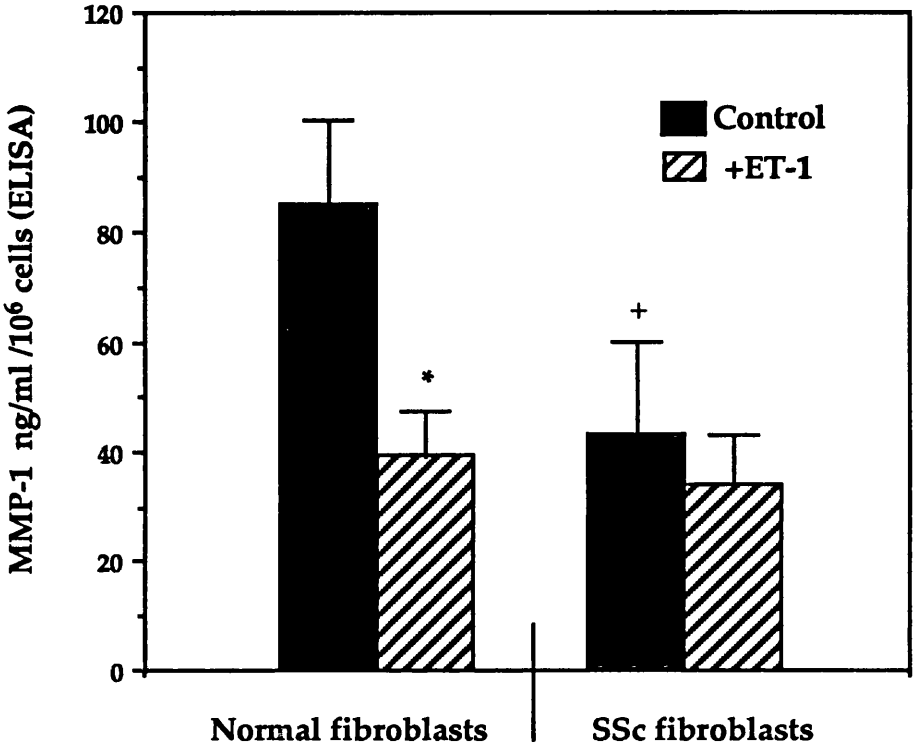


Figure 5.5. Endothelin-1 reduces secretion of MMP-1 by normal, but not SSc fibroblasts. Aliquots (100ul) of conditioned medium were tested with an ELISA method. There is decreased collagenase (MMP-1) secretion in cultured SSc fibroblasts (n=6). MMP-1 secretion by normal cells (n=6), but not by SSc cells, is reduced by incubation with ET-1 (10^{-7}M) for 48 hours. Data are mean (±sem) for 3 independent experiments. + indicates significantly ($^{+}$ $p<0.05$, Student's paired t-test) below normal fibroblasts. *indicates significantly (* $p<0.05$, Student's unpaired t-test) below normal control fibroblasts of MMP-1 concentration.

Time courses for pro-α1(I) collagen secretion in response to ET-1 (100nM) in normal fibroblasts with and without ET-1 are shown in Figure 5.6. These show elevated secretion of pro-α1(I) collagen was as early as 8 hours after addition of ET-1, with maximal levels of collagen synthesis were reached 72 hours.

5.3.3 Changes in matrix component mRNA reflect those of secreted proteins

Compared with normal fibroblasts, scleroderma fibroblasts exhibit increased production of transcripts of pro-α1(I), pro-α1(III) collagen, slightly increased fibronectin, and substantially reduced levels of MMP-1 mRNA (Figure 5.7). Since the data (section 5.3.2) suggest that the effects of ET-1 on collagen synthesis in dermal fibroblasts appear to be mediated through both ET_A and ET_B receptors. Ro 47-0203 was used to study differential regulation of transcription of matrix in scleroderma and normal fibroblasts. Incubation of normal fibroblasts treated with ET-1 in the presence Ro 47-0203 blocked the effect of ET-1 on pro-α1(I) and (III) collagen mRNA (Figure 5.8).

5.3.4 ET receptor are expressed differently in normal and scleroderma fibroblasts

Five separate samples of total RNA extracted from normal and scleroderma fibroblasts, in comparison with human endothelial cell lines HMEC-1 and IE-7, were examined for ET_A and ET_B receptor gene expression. Two bands of 299 base pairs and 428 base pairs, corresponding to the predicted amplified sequences for ET_A and ET_B, were found in all 5 samples (Figure 5.9).

K_D values derived from binding studies using labelled ligands [^{125}I]-ET-1, [^{125}I]-PD151242 and [^{125}I]-BQ3020 on cells were calculated to be 107, 149 and 168 pM respectively (Table 5.1) (Figure 5.10). Normal and scleroderma cells were incubated in 150 pM radioligand for subsequent experiments. The level of ET_A binding sites was significantly higher in normal (1475.0$\pm$73.8 dpm) compared with scleroderma fibroblasts (818.2$\pm$25.6) ($p<0.05$), while ET_B binding sites were not significantly different. Interestingly ET_B binding sites were present on both cell types, but at very low levels (5-10% ET-1 binding) (Table 5.2).

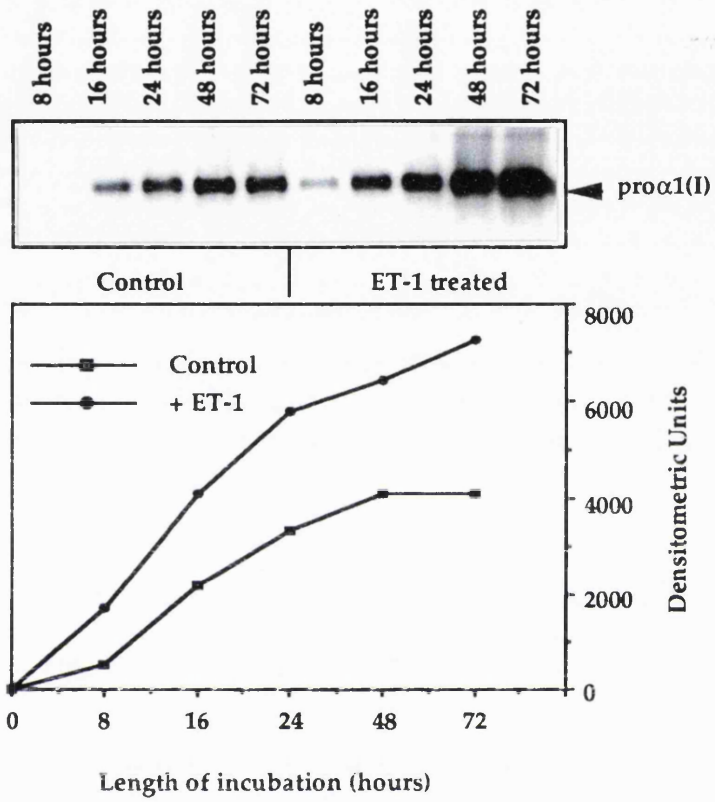


Figure 5.6. Kinetics of collagen production on unstimulated fibroblasts and in response to ET-1. Time course of the effects of ET-1 on up-regulation of proα1(I) collagen production in normal fibroblasts, as determined by Western blot. At different time (8, 16, 48, 72 hours) fibroblasts were incubated without or with ET-1 (100nM).

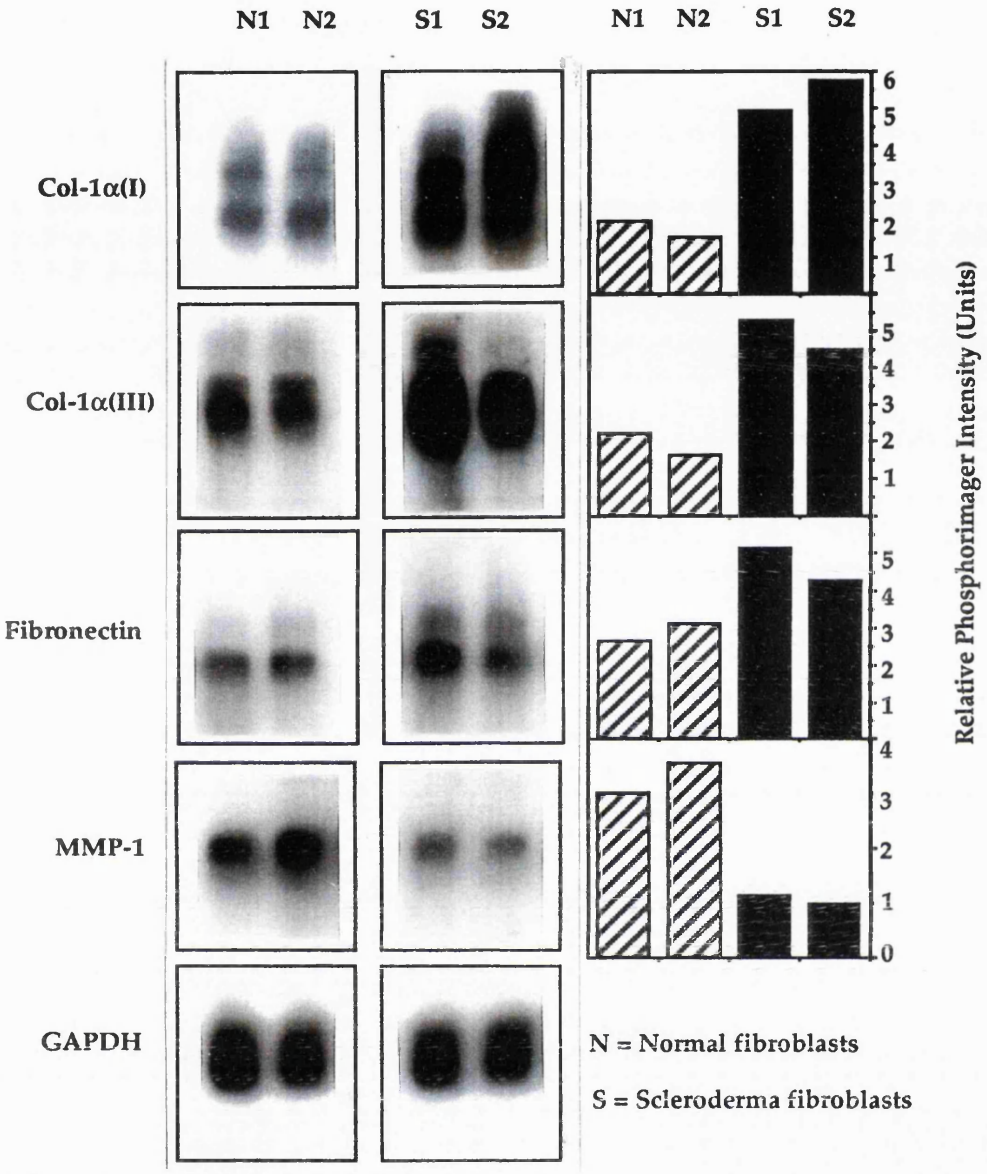


Figure 5.7. Northern hybridisation showing the constitutive expression of mRNA for matrix proteins in SSc and normal fibroblasts. Total cellular RNA was extracted from two normal and SSc fibroblast strains for analysis by Northern hybridisation. Following electophoresis in a denaturing gel and transfer to hybond membrane, transcripts for pro-α1(I)collagen and GAPDH were localised using a radiolabelled cDNA probe. Following autoradiography (left) the ratio of pro-α1(I)collagen :GAPDH band density was determined using the PhosphorImager (right).

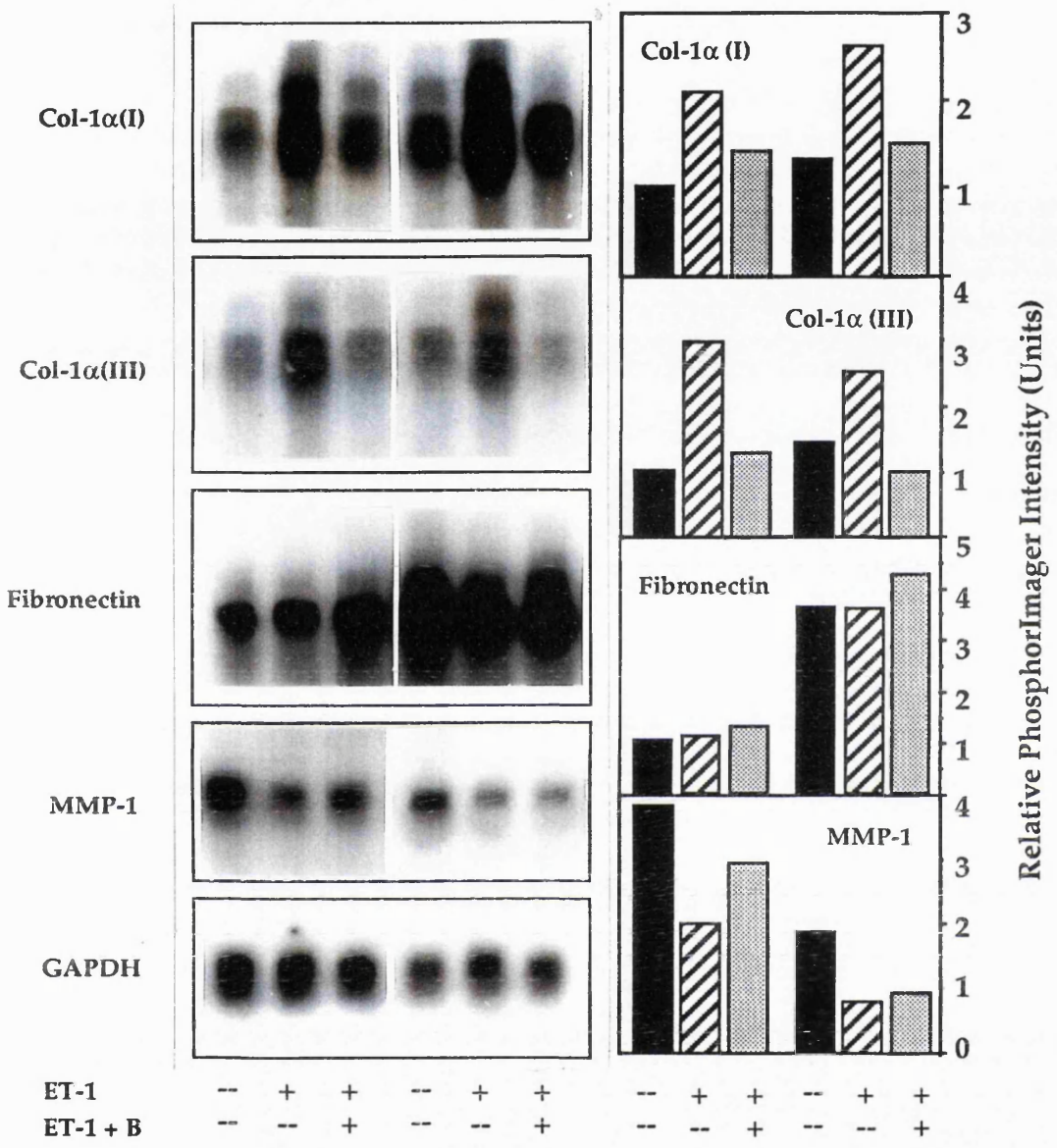


Figure 5.8. Northern blot analysis of endothelin-1 mediated regulation of matrix protein mRNA expression in normal dermal fibroblasts. Confluent cultures of normal skin fibroblasts were treated with ET-1, or plus mixed ETA/B receptor antagonist bosentan, and total RNA was analyzed by Northern hybridization. B= bosentan.

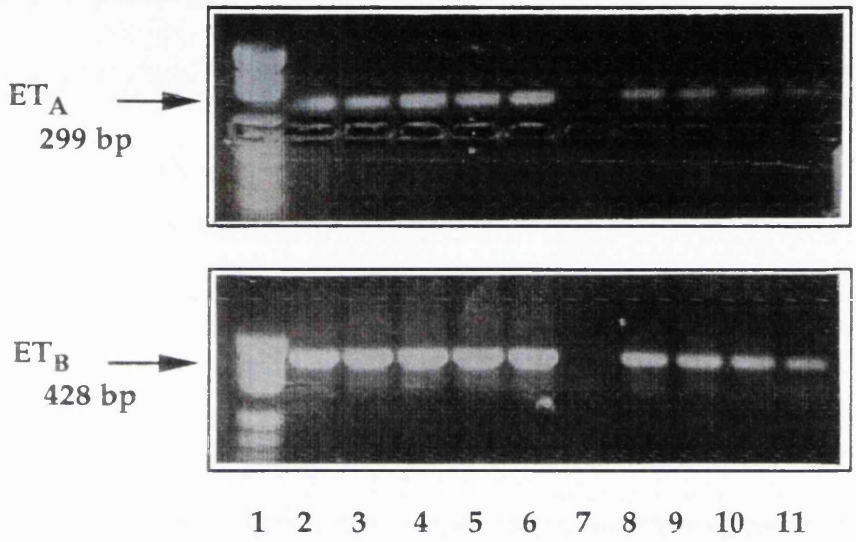


Figure 5.9. Analysis of ET_A and ET_B receptor mRNA expression by normal and SSc fibroblasts using RT-PCR. Agarose gel showing products of PCR amplification of ET_A (299bp) and ET_B (428bp) cDNA from five separate cultures. Lanes: pUC18 (Msp I digest) (line 1), normal fibroblasts (lines 2-4), SSc fibroblasts (lines 5 and 6), control No RT (line 7), HMEC-1 (lines 8 and 9), IE-7 (lines 10 and 11).

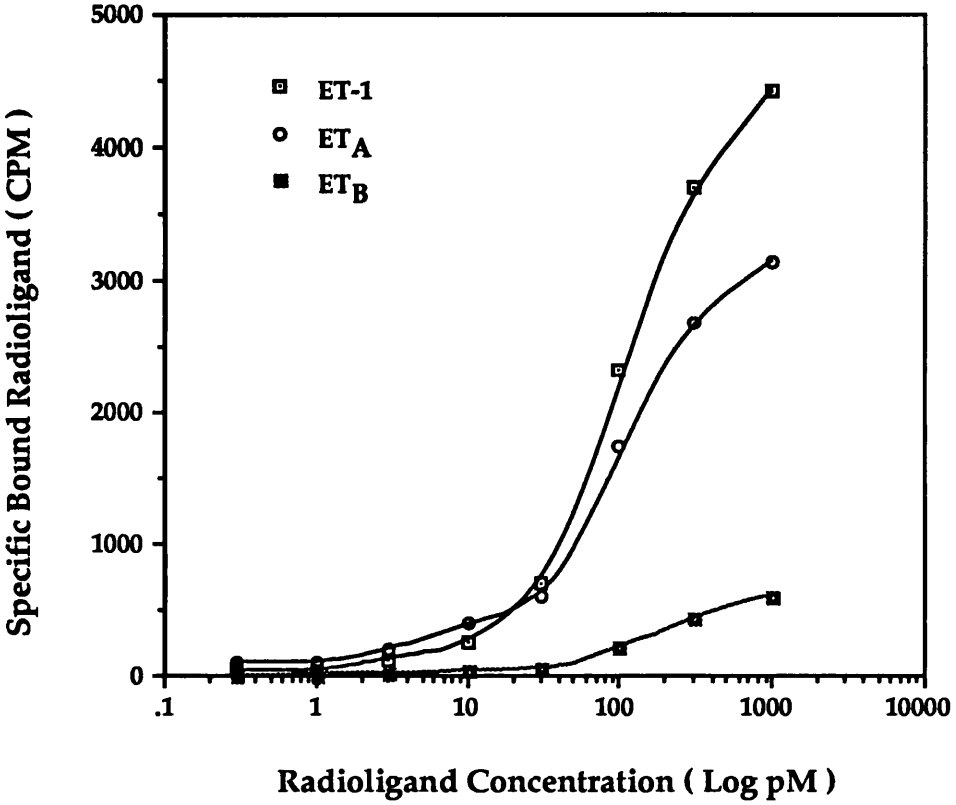


Figure 5.10. Semilogarithmic saturation plot for normal dermal fibroblasts. Fibroblasts were seeded (5000 cells per well of a 12 mm diameter culture well). Specific binding was plotted as function of radioligand concentrations 0.3 to 1000 pM [^{125}I] ET-1, [^{125}I] PD 151242 (ET_A) and [^{125}I] BQ 3020 (ET_B). Each point indicates the mean of duplicate samples and is representative of four independent pairs.

In vitro autoradiography has shown that ET_A receptor binding on scleroderma fibroblasts (Figure 5.11 C) is much less than that on normal fibroblasts (Figure 5.11 A). Microscopic examination of [^{125}I]-ET-1 binding to cells indicated that there was a marked heterogeneity of binding to fibroblasts (data not show).

Table 5.1. Dissociation constants (Kd) and maximal density of receptors (Bmax) derived from saturation binding experiments with normal dermal fibroblasts

Radioligand	Kd (pM)	B_{Max} (sites/cell)
[^{125}I]-ET-1	107 ± 19	4734 ± 181
[^{125}I]-PD151242	149 ± 23	4145 ± 126
[^{125}I]-BQ3020	168 ± 38	602 ± 15 *

^ Data were derived by non-linear curve-fitting software (Graph Pad InPlot) and saturation binding data obtained as described in Methods.
+Values represent mean ± SD.
* $P < 0.05$ compared to [^{125}I]-ET-1.

Table 5.2. Quantitative examination of ET_A and ET_B receptors binding sites on normal and scleroderma fibroblasts

	Receptor binding sites	
Fibroblasts	ET_A receptor	ET_B receptor
Normal (n=6)	1475.0 ±73.8	75.7 ±20.2
SSc (n=6)	818.2 ±25.6 *	81.2 ±29.2

* $P < 0.05$ compared to normal fibroblast strains.

Receptor selective ligands:

[^{125}I] PD 151242, specificET_A receptor binding;
[^{125}I] BQ 3020, specific ET_B receptor binding.

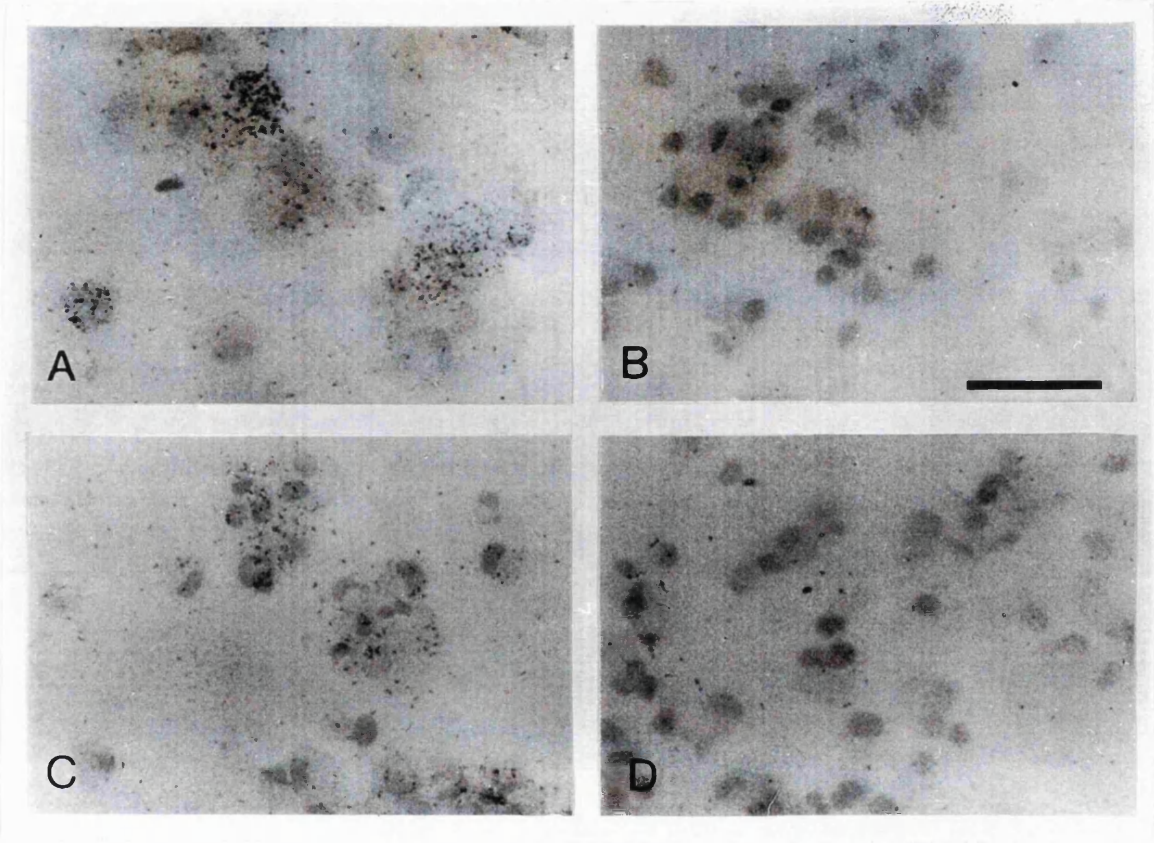


Figure 5.11. ET_A receptor binding to SSc and control fibroblasts. Bright-field illumination autoradiographs of slides incubated in 150 pM [^{125}I]-PD151242 alone (total binding). A, normal fibroblasts; C, SSc fibroblasts. Same cells (right, B and D) illustrate non-specific binding determined by co-incubating in the presence of 1μM unlabelled ET-1. Note accumulation of grains in A and C. Scale bar is 50 μm.

5.4 DISCUSSION

The involvement of vascular injury in the pathology of scleroderma has been suggested by the presence of early vascular changes associated with endothelial cell damage preceding the onset of tissue fibrosis (Campbell and LeRoy, 1975), and the relationship between disease outcome and the severity of vascular lesions (Kahaleh, 1990). Although a number of cell types involved in the development of scleroderma fibrosis have been studied, the cellular and molecular mechanisms responsible for scleroderma fibrosis are not clear. In the present study, I have addressed these issues by examining scleroderma, and in particular the possible role of the endothelin-1 and endothelin receptors by comparing scleroderma fibroblasts with normal cell strains.

The results of these experiments showed that exogenous ET-1 caused a consistent and dose-dependent increase in DNA synthesis in normal fibroblasts, in agreement with data described by Kahaleh *et al* (1991) and Kikuchi *et al* (1995), and this stimulatory action was completely blocked by the ET_A receptor antagonist PD 156707. In comparison with normal fibroblasts, scleroderma fibroblasts showed a reduced growth response. Since the proliferation of normal fibroblasts that is induced by ET-1 is reversed by the specific ET_A receptor antagonist PD 156707, but not by the ET_B receptor antagonist BQ-788, it can be concluded that the mitogenic effects of ET-1 in normal and scleroderma fibroblasts are mediated through the ET_A receptor subtype. Down-regulation of ET_A receptors may be associated with the decreased mitogenic response to ET-1 in scleroderma fibroblasts. The mechanism of this down-regulation of ET_A receptor expression is unknown. One possible explanation for the reduced effect of exogenous ET-1 on scleroderma fibroblasts *in vitro* is that these cells have been exposed to elevated endogenous levels of this mediator *in vivo*.

Previous reports with the [^{3}H]-proline incorporation method have shown that ET-1 increases collagen synthesis in cultured human dermal fibroblasts (Kahaleh, 1991). Using a similar experimental approach, I found that ET-1 increased type I and III collagen synthesis in normal fibroblasts using an inhibition ELISA. I have

also shown that scleroderma fibroblasts exhibit a significantly reduced collagen synthesis response to ET-1 compared with normal fibroblasts. It thus appears that intrinsic factors (possibly endogenous ET-1) consequently stimulate collagen synthesis *in vivo* in scleroderma and that exogenous ET-1 could play no further role in augmenting collagen synthesis in scleroderma *in vitro*.

So far, I have shown that ET-1 stimulates fibroblast replication and collagen synthesis *in vitro*. Our group and others have also shown the presence of raised levels of circulating ET-1 that correlates with increased skin fibrosis, and with the increase in dermal ET-1 synthesis and its binding sites *in vivo* (Knock *et al*, 1993; Vancheeswaran *et al*, 1994; Yamane *et al*, 1992). Taken together, there is now ample evidence that endothelins, especially ET-1, are pro-fibrotic cytokines. It is possible that a combination of several other cytokines, such as IL-1, TGF-β, PDGF and CTGF, play a major role in scleroderma fibrogenesis (Sporn *et al*, 1987; Walsh *et al*, 1989; Frazier *et al*., 1996; Denton *et al*, 1997).

It is well documented that ET-1 mediates varied biological effects through different ET receptors, therefore it is likely that an altered expression of ET receptors may be associated with different functional responses. The cellular responses to endothelins are mediated by the interaction of endothelins with specific receptor subtypes ($ET_{A/B}$ receptor). ET-1-enhanced collagen synthesis was not antagonized by PD 156707 alone or by BQ 788 alone but was achieved with the mixed $ET_{A/B}$ receptor antagonist bosentan (Ro 47-0203, $p<0.01$). These data suggest that dual blockade of $ET_{A/B}$ receptors is necessary to abrogate ET-1 induced collagen synthesis, perhaps due to cross-talk between the receptors as there signally pathways.

Both $ET_{A/B}$ receptor subtype expression and mRNA transcript are demonstrated in tissue culture fibroblasts. Kikuchi *et al* showed ET_A receptors on normal and scleroderma dermal fibroblasts, but ET_B receptors were not detected, suggesting that the ET_A receptor was the only subtype expressed by human dermal fibroblasts (Kikuchi *et al*, 1995). In contrast, my experiments demonstrate ET_B receptor gene expression by fibroblasts from normal and scleroderma skin, and

the binding studies suggest both $ET_{A/B}$ receptor subtypes exist in cultured normal and scleroderma fibroblasts, perhaps offering greater support to the hypothesis that stimulation of ET receptors may lead to skin fibrosis *in vivo*. The reduction in ET_A binding sites on scleroderma fibroblasts is somewhat paradoxical but does correlate with the reduced growth and collagen synthesis in response to endothelin-1.

In summary, I have presented evidence that normal and scleroderma dermal fibroblasts express ET receptor subtypes *in vitro,* and demonstrated modulation of fibroblast properties by exogenous ET-1. The mitogenic effects of ET-1 in dermal fibroblasts appear to be mainly mediated through the ET_A receptor subtype, whereas collagen synthesis appears to be mediated through both $ET_{A/B}$ receptors. Furthermore, whilst scleroderma fibroblasts show a reduced growth and collagen synthesis response to ET-1, normal fibroblast properties are substantially modulated by ET-1 to give a fibrogenic phenotype. Overall, these data support the hypothesis that endothelin-1 may be important in relation to the perivascular fibrosis associated with various pathological states of scleroderma and that there may be new approaches and therapeutic potential of certain ET antagonists in treating this condition.

CHAPTER 6: ET-1 MEDIATED REGULATION OF ICAM-1 EXPRESSION IN NORMAL AND SCLERODERMA FIBROBLASTS

6.1 INTRODUCTION

In addition to matrix over-production and altered responsiveness to cytokines, scleroderma fibroblasts exhibit altered adhesion molecule expression. Previous studies have shown increased surface ICAM-1 expression on scleroderma fibroblasts. In this chapter, I have confirmed and extended this observation by examining soluble ICAM-1 compared with controls and showing that this is increased from cultured scleroderma fibroblasts (Shiwen *et al.*, 1994). The association of elevated expression and shedding of ICAM-1 with increased ET-1 levels in serum and BALF led to the hypothesis that ET-1 may modulates ICAM-1 expression. The data presented in this chapter support this hypothesis. These observations further suggest that ET-1 may function as a proinflammatory mediator to regulate interactions between immune cells and fibroblasts in scleroderma.

Intercellular adhesion molecule-1 (ICAM-1, CD54) is a cell surface adhesion molecule belonging to the immunoglobulin supergene family (Springer, 1990; Mojock and Shevach, 1997). It is expressed on a number of different cell types, including fibroblasts, epithelial and endothelial cells, and is the ligand involved in adhesive interactions with various types of leukocytes (Springer *et al.*, 1987). The identification and tissue localisation of ICAM-1 have provided evidence for involvement in a number of inflammatory conditions, and it is now recognised as the primary ligand mediating T cell attachment to dermal fibroblasts from patients with scleroderma (Needleman, 1990; Piela and Korn, 1990; Abraham *et al.*, 1991).

The expression of ICAM-1, the counter-receptor for the integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), is up-regulated by cytokines such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), although cell-specific variations in responsiveness have been found to these cytokines

(Dustin and Springer, 1988; Mojock and Shevach, 1997). ICAM-1 is also important for leukocyte trans-migration through the endothelium, and migration into inflammatory sites (Smith *et al.*, 1989), cellular interaction during the immune response (Dougherty *et al.*, 1988), and as the major receptor for the group of rhinoviruses responsible for the common cold (Marlin *et al.*, 1990).

The presence of mononuclear cell infiltrates (Degiannis *et al.*, 1990; Majewski *et al.*, 1991; Sollberg *et al.*, 1992) and increased levels of certain cytokines (Degiannis *et al.*, 1990) in the skin of patients at early stages of the disease (scleroderma) process have highlighted the importance of adhesive interactions with leukocytes and the control of adhesion molecule expression in scleroderma. Furthermore, previous data from our laboratory, as well as others, have shown increased plasma ET-1 and sICAM-1 levels in patients with scleroderma similar to those which are also found in other diseases, such as chronic congestive heart failure (Tsutamoto *et al.*,1995). Soluble forms of ICAM-1 have been detected in plasma and serum of patients with scleroderma, providing potentially useful clinical markers (Denton *et al.*, 1995; Gruschwitz *et al.*, 1995). Circulating ET-1 levels are also raised in scleroderma patients with widespread skin fibrosis (Yamane *et al*, 1992; Vancheeswaran *et al.*, 1994). Interestingly, it has been demonstrated that fibroblasts from patients with dcSSc produce significantly higher levels of sICAM-1 (Shi-wen *et al.*, 1994) and ET-1 (Kawaguchi *et al.*, 1994) than fibroblasts from normal individuals. There is further evidence of increased levels of ET-1 and sICAM-1 in bronchoalveolar lavage fluid (BALF) from scleroderma patients (Du Bois *et al.*, 1992; Cambery *et al.*, 1994). These results provide further evidence for a link between endothelial and fibroblast dysfunction in this disease.

McCarron *et al* (1993) have shown that ET-1 can up-regulate the expression of ICAM-1 and VCAM-1, and induce the expression of E-selectin on human microvascular endothelial cells. Other recent reports have shown that the expression of adhesion molecules on fibroblast-like synovial cells can be modulated by ET-1, such as ICAM-1, VCAM-1 and CD44 (Schwarting *et al.*, 1996). Hayasaki *et al* (1996) also reported ET-1 increased ICAM-1 expression on rat cardiac myocytes and rat aortic endothelial cells. These observations indicate

that vasoactive peptides may function as potent proinflammatory mediators by regulating interactions between circulating leukocytes and the vascular endothelium. However, the effect of ET-1 on scleroderma fibroblast ICAM-1 expression remains unexplored.

Work described below measures the surface-associated, soluble ICAM-1 protein and mRNA transcript levels in normal and scleroderma fibroblast cultures and examines the effects of ET-1 on ICAM-1 expression and release. Additional functional binding, ET-1 induced changes in U937 cells binding to fibroblasts has also been studied.

6.2 PATIENTS AND STATISTICS

6.2.1 Samples

Punch biopsies ($4mm^3$) were obtained from clinically affected areas of the skin of scleroderma patients at the Royal Free Hospital and also from age and sex matched healthy volunteer controls. The group of 20 scleroderma patients were all women, with active, diffuse disease (dcSSc) of between 2-4 year duration (average 3.2 years) at the time of biopsy. Their modified Rodnan skin scores were between 18 and 48 (average 26).

6.2.2 Statistical analysis

Statistical comparisons were made using the Student's t - test as employed in the Stat Works statistical program. Correlation between variables were analysed using the Spearman's rank correlation coefficient, and linear regression analysis was used to determine the association between 2 independent variables. The number of replicates of each experiment is noted in each figure, the data expressed as mean ± SEM. The level of significance was taken as p values less than 0.05.

6.3 RESULTS

6.3.1 FACS analysis of basal and ET-1 induced ICAM-1 expression on fibroblasts

Representative flow-cytometric analyses (FACS) of the surface expression of ICAM-1 on early passage normal and scleroderma fibroblasts are shown in Figure 6.1A. Visual appraisal of the FACS profiles revealed a normal distribution of these molecules on the surface of both types of fibroblasts with half-height widths spanning approximately one log fluorescence.

Quantitative measurement of ICAM-1 on the surface of these cells was carried out as previously reported (Abraham *et al.*, 1991) to examine their level of expression. As shown in Figure 1B, fibroblasts from normal skin had ICAM-1 levels corresponding to average fluorescence intensities (AFI) between 12.5 and 38.0 (average ± SEM, 29.7 ± 4.1) (Table 6.1). However, the mean level of surface ICAM-1 expression was significantly higher on scleroderma fibroblasts (AFI 52.9 ± 7.2; $p < 0.05$), being nearly twice that of the normal cells. It was notable that even after long periods in culture and extended passage (up to passage 12), the scleroderma fibroblasts retained their phenotypic characteristic of raised ICAM-1 expression (Figure 6.1B). Incubation with ET-1 significantly increased ICAM-1 expression by cells from both normal individuals and scleroderma patients (Table 6.1).

Table 6.1. Effects of ET-1 on fibroblast surface ICAM-1 expression

	Normal (AFI±SEM)	SSc (AFI±SEM)	t test
Control	29.7 (± 4.1)	52.9 (± 7.2)	$p<0.05$
ET-1 (100nM)	81.3(± 12.5)	83.4 (± 13.1)	NS
t test	$p<0.01$	$p<0.05$	

Mean data for three independent experiments using different normal and SSc fibroblast strains.
Unpaired Student's t test.

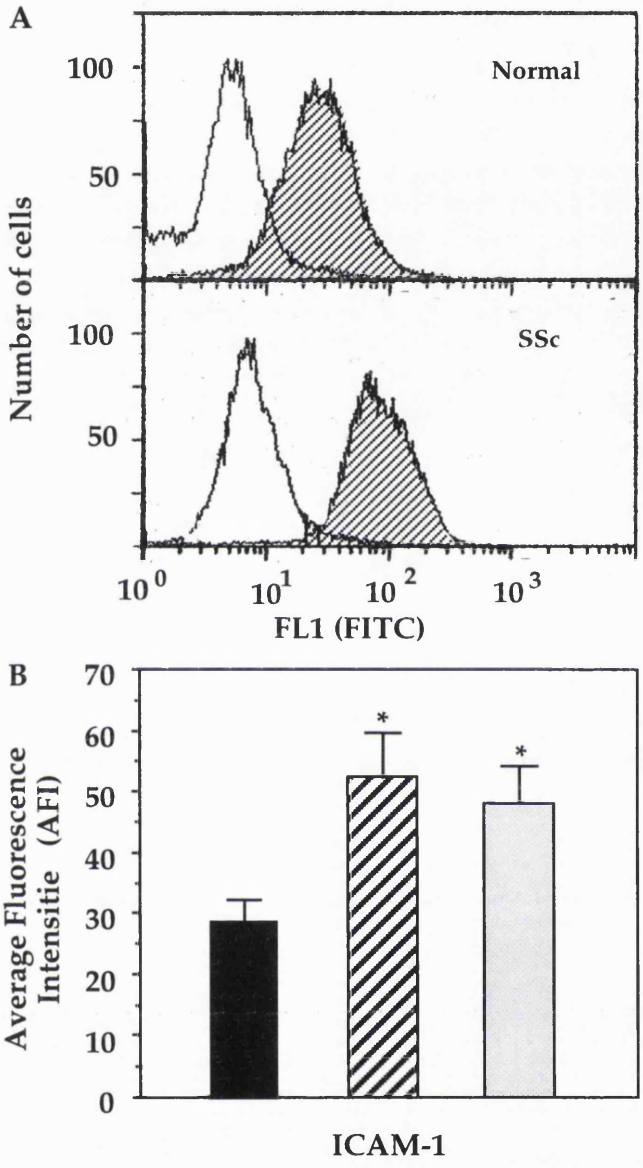


Figure 6.1. Surface expression of ICAM-1 A, Representative flow cytometric profiles of ICAM-1 expression on normal and SSc dermal fibroblasts. Hatched profiles of normal and SSc cells, stained with ICAM-1 respectively, are shown in comparison with control cells (unhatched profiles) stained with isotype-matched Mabs. FL1 (FITC) = log fluorescence with fluorescein staining. B, The cell surface density of ICAM-1 on fibroblasts from 7 different normal individuals (solid bars) and 7 different patients with SSc (hatched bars) between passage 2 and 5, and SSc cells at passage 12 (grey bar). *$p<0.05$.

6.3.2 ICAM-1 mRNA levels

RNase protection assay revealed that ICAM-1 transcript was demonstrated in all normal and scleroderma fibroblast strains examined (Figure 6.2). Quantitative examination by phosphorimager analysis using an internal control transcript (actin) showed that the ICAM-1 transcript was significantly increased within scleroderma fibroblasts.

6.3.3 sICAM-1 released into culture supernatants of normal and scleroderma fibroblasts and effects of ET-1 on the level of sICAM-1

The supernatant media of confluent monolayer cultures of normal and scleroderma fibroblasts contained readily detectable amounts of soluble forms of ICAM-1 (sICAM-1), as shown in Figure 6.3. The mean level of sICAM-1 was significantly higher in the culture supernatant of scleroderma cells (1.04 U per ml) than in comparable media of normal cells (0.37 U per ml) (Figure 6.3) (Table 6.2). Among the scleroderma fibroblasts, 11 of the 20 samples contained levels of sICAM-1 which were more than 2 SD higher than the average level in the media of normal fibroblasts (i.e., greater than 0.75 U per ml).

Table 6.2. Levels of sICAM-1 released by normal and scleroderma fibroblasts

	sICAM-1 (mean±SEM nuits per ml)		t test
	Normal fibroblasts	SSc fibroblasts	
Control	0.37 ± 0.05	1.04 ± 0.26	p<0.001
ET-1 (100nM)	0.42 ± 0.07	0.98 ± 0.21	p<0.01
t test	NS	NS	

Mean data for three independent experiments using different normal and SSc fibroblast strains.
Unpaired Student's t test

The observation that ET-1 markedly increased the cell surface expression of ICAM-1 by both scleroderma and normal fibroblasts, prompted me to examine the effect of ET-1 on the levels of the soluble forms of ICAM-1 released by normal

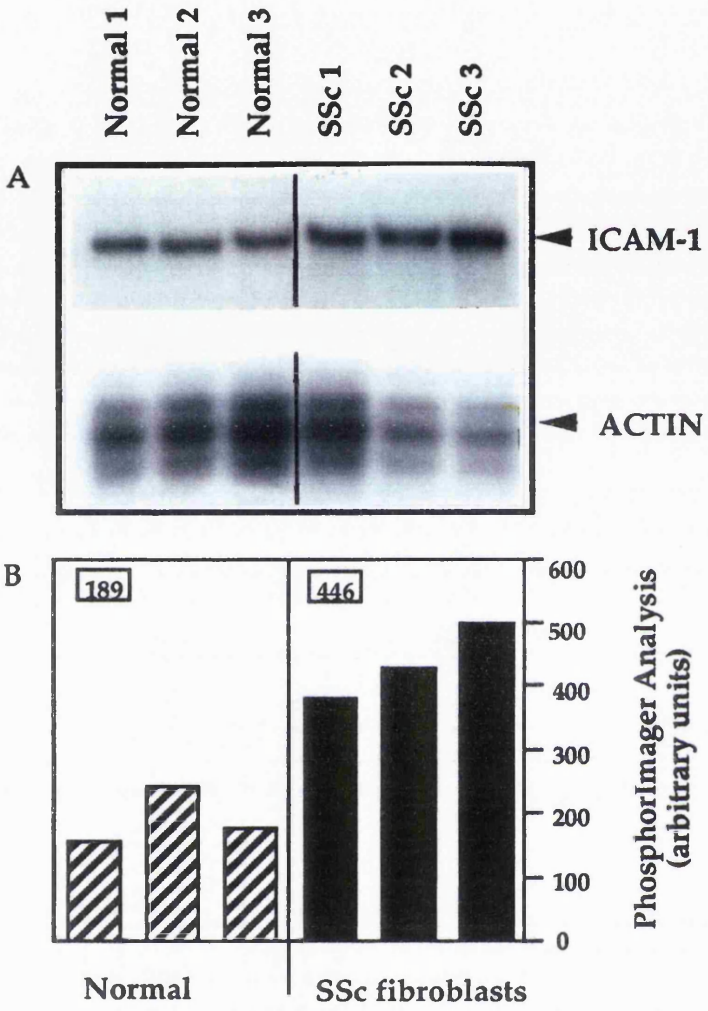


Figure 6.2. RNase protection assay for expression of ICAM-1 mRNA in normal and SSc fibroblasts. (A) Total RNA was extracted from three normal and three SSc fibroblast lines, and hybridised to uniformly labelled cRNA probes specific for ICAM-1. Transcript levels were compared relative to an actin control mRNA analysed simultaneously. (B) Quantitation was performed using phosphorimager analysis (Molecular Dynamics). PhosphorImager values are shown as average intensities (boxed) and are calculated relative to the control expression of human actin.

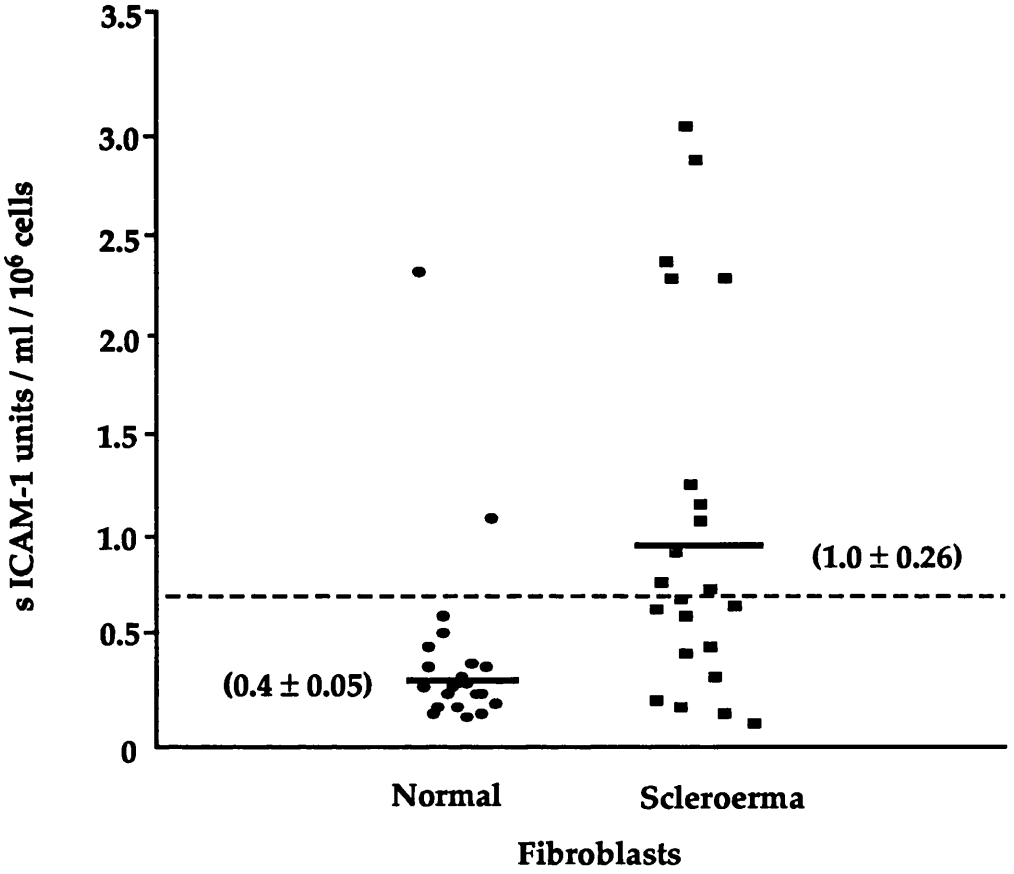


Figure 6.3. Level of soluble ICAM-1 released by normal and SSc fibroblasts. Capture ELISA's were used to measure the levels of soluble adhesion molecules released by 20 normal and 20 SSc fibroblast lines in culture. Horizontal bars represent the mean + 2SD of the control normal cells. The numbers in parentheses are the average values ± the SEM, n=20.

and scleroderma fibroblasts. However, the presence of ET-1 was shown to have no significant effect on the levels of sICAM-1 released by either normal or scleroderma fibroblasts (Table 6.2).

6.3.4 Surface ICAM-1 expression (ELISA)

The upregulation ICAM-1 by ET-1 was concentration-dependent. As shown in Figure 6.4(A) maximal levels were reached at 100nM. Higher concentrations of ET-1 had no additional effect on ICAM-1 expression. The time course of ET-1 induced ICAM-1 expression for normal fibroblasts is shown in Figure 6.4(B). Increased ICAM-1 expression was as early as 4 hours after addition of ET-1, with maximal levels of ICAM-1 were reached 24 hours. Furthermore, the basal ICAM-1 expression was significantly different between scleroderma and normal fibroblasts ($p<0.05$). ET-1 (100nM) increased ICAM-1 expression in fibroblast strains but with consistently greater responses for normal strains ($p<0.01$) (Figure 6.5) (Table 6.3).

Table 6.3. ET-1 promoted ICAM-1 expression on normal and scleroderma fibroblasts

ICAM-1	basal	ET-1 (100nM)	paired t-test
Control	0.05±0.01	0.19±0.10	$p<0.01$
SSc	0.10±0.02	0.17±0.04	$p<0.05$

* mean± sem absorbance at 450 nm by cell bound ELISA

6.3.5 ET-1 increases ICAM-1 gene expression by fibroblasts

Five separate samples of total RNA extracted from normal fibroblasts were examined for ICAM-1 mRNA expression by RT-PCR. ICAM-1 mRNA was barely detectable in all 5 fibroblast cell lines cultured in the control media. In contrast, incubation with ET-1 upregulated ICAM-1 mRNA expression (Figure 6.7). No significant change in mRNA levels for GAPDH control probe was found after ET-1 treatment (Figure 6.7, top) confirming that the differences noted above were not due to mRNA loading, transfer, or nonspecific stimulation.

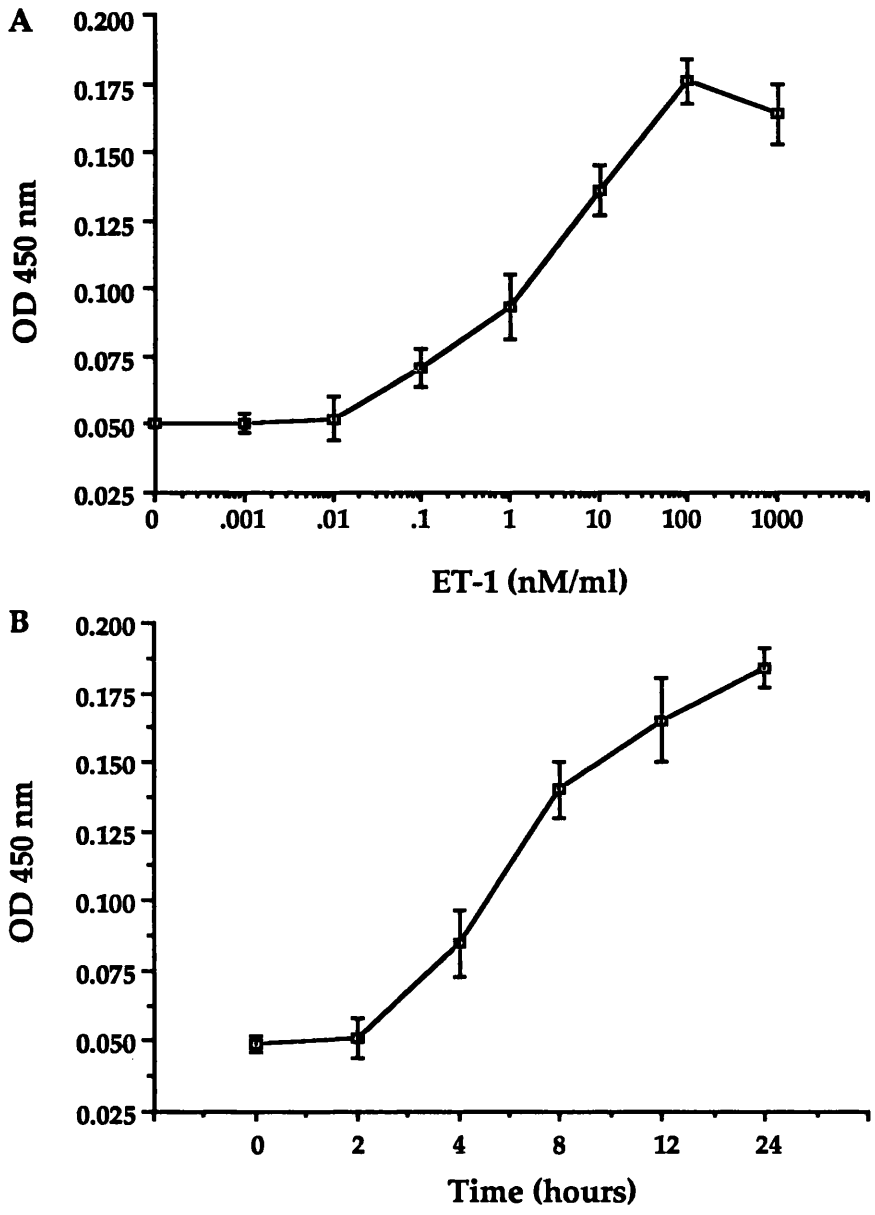


Figure 6.4. Dose-response curve (A) and Time course (B) for ET-1 induced ICAM-1 expression in normal fibroblasts. (A) Does-response curve for ICAM-1 expression by normal fibroblasts. Data are for ET-1. (B) Kinetics of the expression of ICAM-1 on normal fibroblasts in response to ET-1 (100nM). At different times (0, 2, 4, 8, 12 and 24 hours) cells were incubated with ET-1. Expression of ICAM-1 was determined in a cell bound ELISA by absorbance at 450nm and each data point respresents mean $\pm$sd for 6 replicate wells.

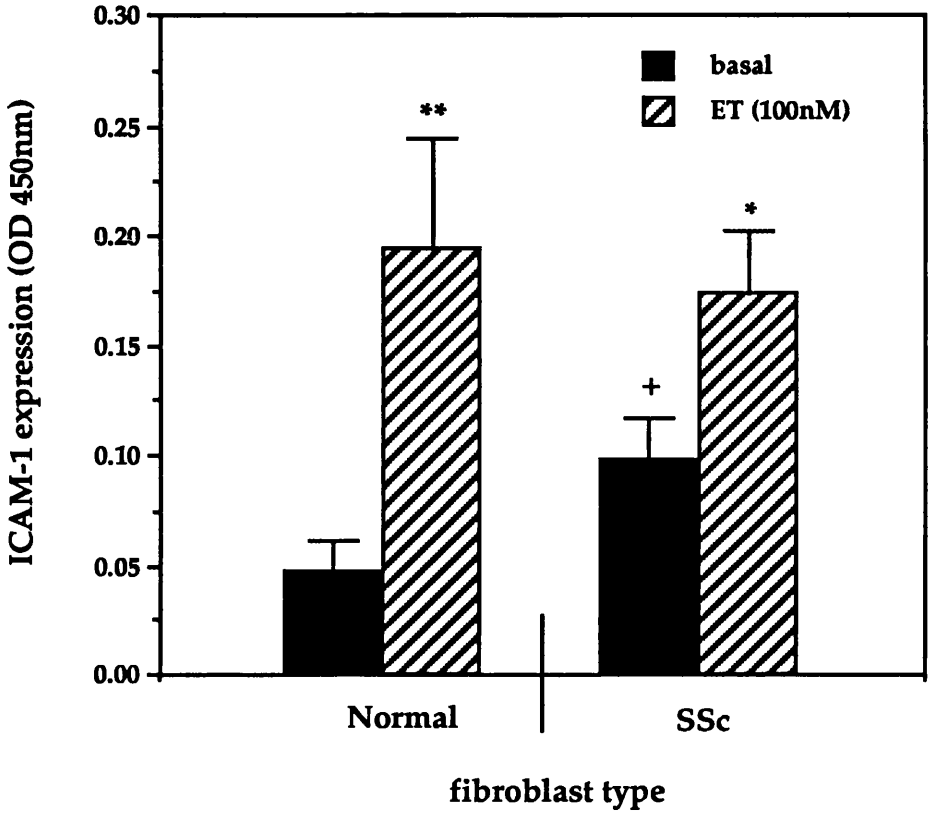


Figure 6.5. Effect of ET-1 on normal and SSc fibroblast surface expression of ICAM-1 by ELISA. Constitutive expression of ICAM-1 by normal and SSc dermal fibroblasts was significantly upregulated after incubation with ET-1 for 48 hours. Normal strains showed a greater response than SSc fibroblasts. Results are mean (±SEM) absorbance for 3-6 replicates in 3 independent experiments. Analysis by Student's unpaired t-test, * indicates significantly (*$p<0.05$; **$p<0.01$) above basal control; + indicates significantly ($p<0.05$) above basal normal fibroblasts.

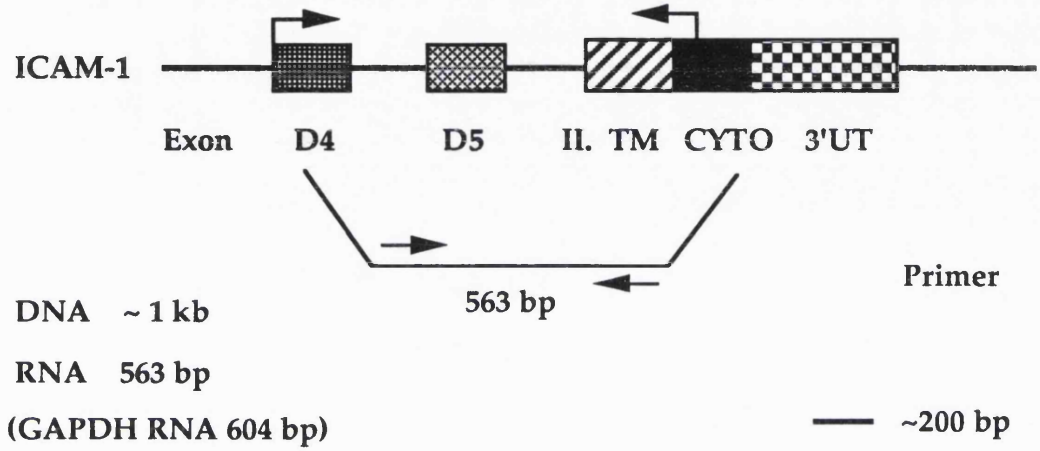


Figure 6.6. Structural organization of the ICAM-1 gene. In ICAM-1 gene, the position of the exon-intron boundaries correlates with the domain structure of the protein. Exons are indicated by boxes. Introns as well as 5' and 3' flanking sequences are represented by lines. TM, transmembrane region; CYTO, cytoplasmic domain; UT, untranslated region.

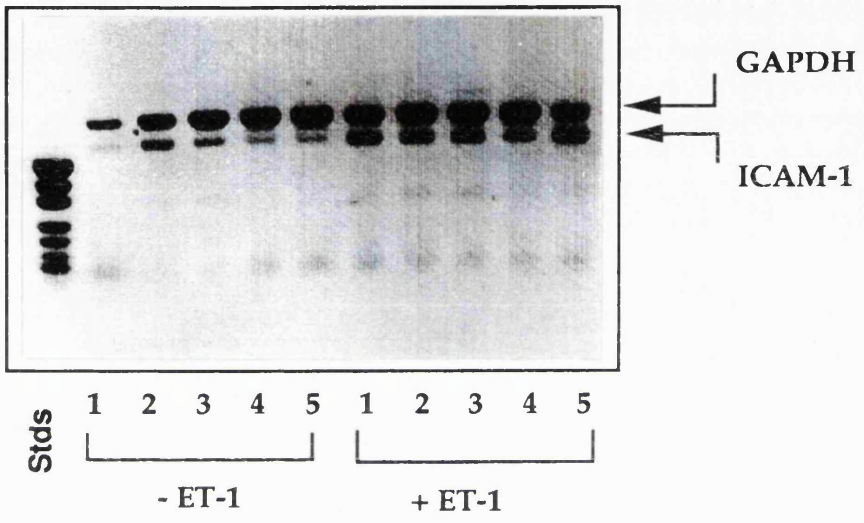


Figure 6.7. Modulation of ICAM-1 gene expression in normal fibroblasts by ET-1. Agarose gel showing products of PCR amplification of ICAM-1 (563 bp) cDNA and GAPDH (604bp) from 5 different fibroblast cultures. Lanes: pUC msp I, control normal fibroblasts (lines 1-5), ET-1 stimulated fibroblasts (lines 1-5).

6.3.6 Expression of ET-1 receptors by autoradiography

In vitro autoradiography has shown ET-1 binding sites on fibroblasts (Figure 6.8). I postulated that ET-1 could enhance leucocyte-fibroblast interaction through its induction of ICAM-1. This hypothesis was tested by stimulating U937 cells bind to fibroblasts with ET-1, in the absence or presence of mixed ET receptor antagonist. This indicates that the stimulatory effect of ET-1 on fibroblasts is mediated via ET receptors.

6.3.7 ET-1 treatment of fibroblasts enhances U937 cell adhesion

A cell adhesion assay was performed to determine whether upregulation of ICAM-1 induced by ET-1 resulted in increased leukocyte-fibroblast binding. Exposure of normal fibroblasts to ET-1 resulted in increased binding of radiolabelled U937 cells (Figure 6.9) (Figure 6.10). To assess the contribution of ICAM-1 to U937 cell adhesion after ET-1 stimulation of fibroblasts, I used MoAb to ICAM-1 and ET receptor antagonists for blocking experiments. U937 cell binding to normal fibroblasts could be markedly diminished by anti-ICAM-1 antibody and bosentan (Figure 6.9, 6.10).

6.4 DISCUSSION

The skin represents an important immunological compartment, and lymphocyte entry and migration through this organ occurs both normally and as a prominent feature of a number of inflammatory pathologies (Nickoloff, 1992). Cutaneous leukocyte trafficking is mediated by cell surface (Abraham *et al.*, 1990) and matrix adhesion receptors which regulate and direct cell movement (Spinger, 1990; Abraham *et al.*, 1991; Needleman, 1990) and changes in the cell specific expression and level of these antigens, as occurs during inflammatory skin diseases, result in major modifications in cell recruitment and lymphocyte infiltration as part of the pathogenic process (Buchsbaum *et al.*, 1993). Increasing interest has therefore been focused on the molecular events involved in mediating the cellular interactions between lymphocytes and dermal cells.

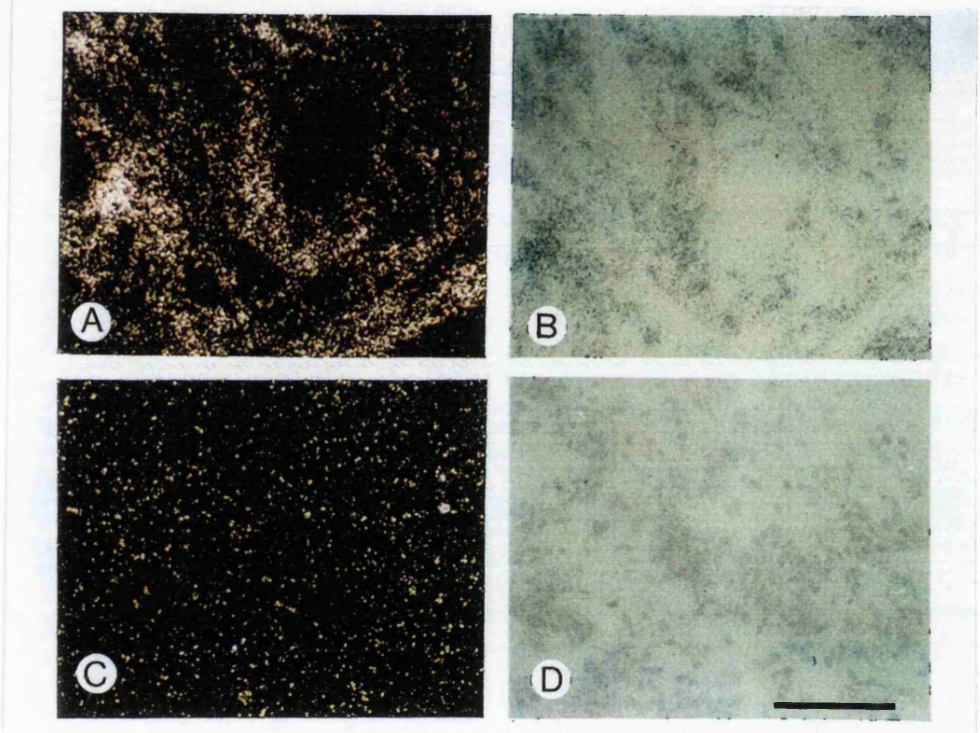


Figure 6.8. ET-1 receptors on cultured normal fibroblasts.
Dark-field (A and C) and bright-field (B and D) illumination autoradiographs of fibroblasts grown on slides incubated in the presence of 150 pM [^{125}I]ET-1 alone (total binding) (C and D) corresponding to A and B, but including 1.0 μM of unlabeled ET-1 (non-specific binding). Note accumulation of grains in A and B. Scale bar is 100 um.

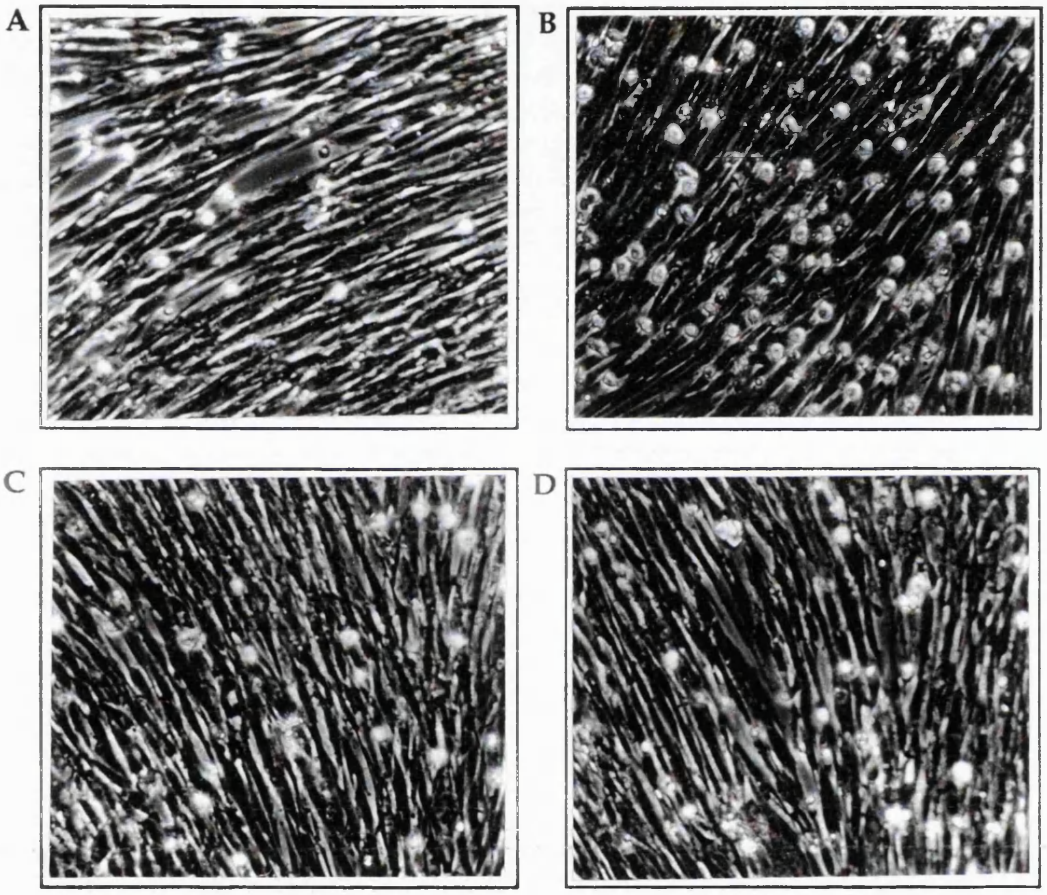


Figure 6.9. ET-1 upregulates the adhesiveness of normal fibroblasts for U937 cells. U937 cells were allowed to attach to unstimulated human normal fibroblasts (A) or fibroblasts after exposure to 100 nM ET-1 for 48 h ours (B) and then washed. Only adherent cells are observed. The adhesion of U937 cells to confluent dermal fibroblasts was inhibited by the ET-A and B receptor antagonist Ro 47-0203 (C) or the addition of a blocking antibody to ICAM-1 (D). (x100 Magnification)

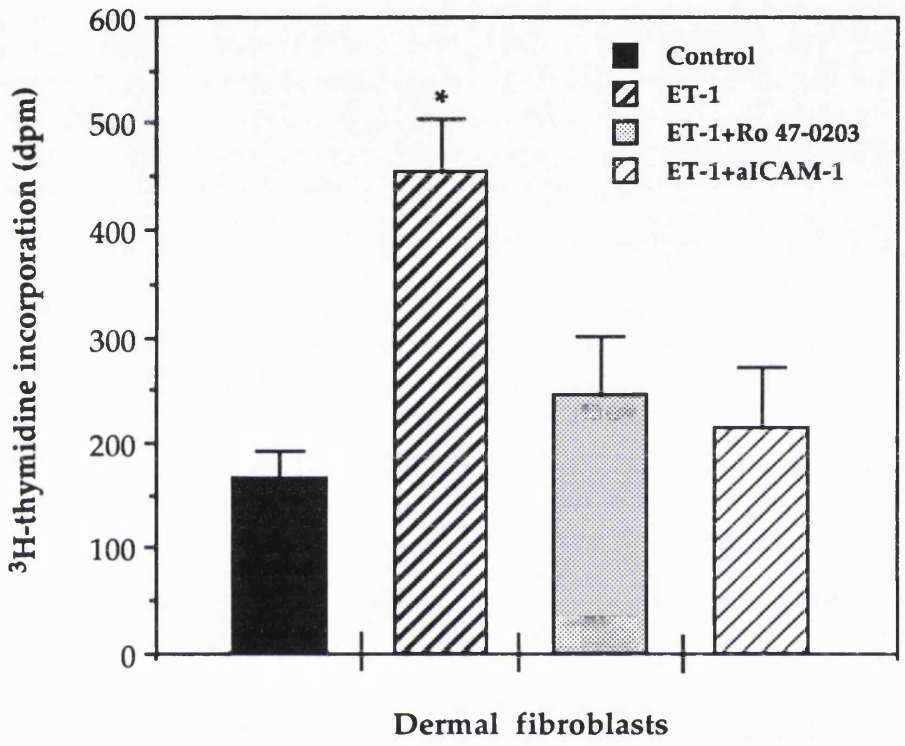


Figure 6.10. Inhibition of U937 cells adhesion to normal fibroblasts stimulated with ET-1. [^{3}H]thymidine-labelled U937 cells were added to unstimulated (control) normal fibroblasts or fibroblasts stimulated for 48 hours with ET-1(100nM) as shown. The inhibition of U937 cell binding is indicated materials and methods. Data represent the mean $\pm$ SEM of three experiments performed in triplicate. * $p<0.05$ significantly different from the incorporation in control fibroblasts.

In the present study, I have confirmed that fibroblasts from patients with dcSSc express significantly higher surface levels of the adhesion molecule ICAM-1 than fibroblasts from normal individuals and have extended this finding using an RNase protection analysis to show elevated mRNA encoding ICAM-1.

Increased expression of ICAM-1 has also been shown on other cells such as keratinocytes and endothelial cells and immunohistochemical studies have reported increased ICAM-1 expression in clinically involved and uninvolved scleroderma compared with normal skin (Sollberg *et al.*, 1992; Majewski *et al.*, 1992; Buchsbaum *et al.*, 1993). The present observations are thus consistent with previous reports of elevated surface expression of ICAM-1 on scleroderma fibroblasts (Needleman, 1990; Abraham *et al.*, 1991). This data also supports evidence that the cellular basis underlying scleroderma involves intrinsic abnormalities in fibroblast metabolism such as in the control of extracellular matrix synthesis and regulation of adhesion molecule expression (Whiteside *et al.*, 1988; Needleman, 1990).

It is notable that when cultured *in vitro*, although the main scleroderma fibroblast characteristic of increased collagen production reverts to normal, after a certain number of passages (LeRoy *et al.*, 1989), the results of my study show that elevated ICAM-1 expression persists for prolonged periods in culture. This is consistent with a previous study that suggested no direct relationship between the levels of high amounts of ICAM-1 on the fibroblast cell-surface and increased production of extracellular matrix components, primarily collagen (Needleman, 1990). However, the increased ICAM-1 mediated T cell adhesion to early passage scleroderma fibroblasts is lost with prolonged passage in culture (Abraham *et al.*, 1991). It is possible therefore that prolonged culture of fibroblasts *in vitro* results in the loss of other accessory adhesion or T cell activating factors which facilitate LFA-1/ICAM-1 mediated lymphocyte-fibroblast adhesion (Piela and Korn, 1988).

A number of studies have shown that elevated levels of these soluble adhesion molecules in the circulation are associated with a number of inflammatory

diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and scleroderma (Mason *et al.*, 1993; Sfikakis *et al.*, 1993; Gearing and Newman, 1993). The release or shedding of cell surface adhesion proteins has also been described *in vitro*, for example, L-selectin from neutrophils (Smith *et al.*, 1991) and lymphocytes (Jung and Dailey, 1990), ICAM-1 from melanoma cells, ICAM-1, VCAM-1 and E-selectin from the surface of endothelial cells following cytokine stimulation (Gearing and Newman, 1993). I have measured the levels of the 'soluble' ICAM-1 in culture supernatants of normal and scleroderma fibroblasts, and found that both types of cell release these molecules into their supernatants as soluble forms. I detected significantly higher levels of sICAM-1 in the media of scleroderma compared with normal fibroblasts.

The mechanism of ICAM-1 release into the culture media by fibroblasts is not fully elucidated. ICAM-1 has no alternative spliced form lacking the transmembrane domain and it is therefore likely that this ligand is proteolytically clipped at the cell surface. *In vitro* data suggests that, at least in endothelial cells, the soluble form of ICAM-1 is indeed generated by proteolytic cleavage at or near to the extracellular face of the membrane (Gearing and Newman, 1993). Fibroblasts, which express and release ICAM-1 as the soluble form, also express a plasma membrane-associated neutral endopeptidase (CD10, CALLA) (Letarte *et al.*, 1988). It is notable that half of the first 16 amino acids on the exposed extracellular carboxy-terminus of ICAM-1 are potential substrates for CD10 (Kerr and Kenny, 1974). Moreover, it is known that the rates at which certain adhesion molecules are removed from the cell surface differ (von Asmuth *et al.*, 1992). These variations in the half-life or residency time of particular molecules at the cell membrane suggests distinct mechanisms (internalization or shedding) by which cell-surface expression can be regulated, and may also partly explain the different levels of soluble adhesion molecules (Gearing and Newman, 1993).

ICAM-1 is a member of immunoglobulin gene superfamily and has five extracellular immunoglobulin-like domains, a single transmembrane region, and a short cytoplasmic domain. The organization of the regulatory elements required for both basal and cytokine-induced expression of ICAM-1 have been

defined (Stratowa and Audette, 1995). Expression of ICAM-1 can be induced or upregulated several fold by cytokines such as INFγ, IL-1β and TNFα (Shiwen *et al.*, 1994; Denton *et al.*, 1997). Although originally described as a potent smooth muscle constrictor, accumulating evidence indicates that ET-1 has a broader range of paracrine activities (Coker and Laurent, 1995). The experiments described above demonstrate that the ET-1 alters normal fibroblasts to express some of abnormal scleroderma disease phenotypes, including increased ICAM-1 expression.

These results demonstrate that the level of expression of ICAM-1 could be significantly increased by incubation with ET-1. In chapter 5, I have shown that scleroderma fibroblasts downregulate ET_A surface receptors and gene level. In this experiment, ET-1 preferentially induced the expression of ICAM-1 on normal fibroblasts, where as scleroderma fibroblasts showed only a moderate upregulation of ICAM-1 expression. The results indirectly demonstrate that ET-1 induced expression of ICAM-1 on fibroblasts is mediated via the ET_A receptor. This is supported by a previous study on human fibroblast-like synovial cells (Schwarting *et al.*, 1996) and also by a study using rat neonatal cultured cardiac myocytes (Hayasaki *et al.*, 1996). This obervation also indicates a specific interaction between endothelial cells and dermal fibroblasts mediated via ET-1.

A considerable amount of evidence suggests that monocytes play an important role in the pathology of scleroderma (McColl and Buchanan, 1996; Kraling *et al.*, 1995). Treatment with ET-1 significantly increased U937 cell adhesion to fibroblasts and an anti-ICAM-1 antibody significantly inhibited the adhesion induced by ET-1. These results suggest that ET-1 increases U937 cell adhesion through induction of ICAM-1 expression at the cell surface. I could block the enhanced U937 cell binding after ET-1 stimulation by pre-incubating with bosentan, a mixed nonpetide receptor antagonist with high specificity and affinity for both $ET_{A/B}$ receptors, confirming that ET-1 is acting via one or both receptor subtypes (Clozel *et al.*, 1994). ET-1 may play a proinflammatory role through its ability to promote neutrophil migration (Elferink and de Koster, 1994). ET-1 also stimulates monocytes to produce IL-6, IL-8, IL-1, TNFα, TGFβ,

and PGE, all important in modulating immune responses (McMillen and Sumpio, 1995). Finally, ET-1-activated neutrophils, when placed into human umbilical cords, migrate into the vessel wall and cause tissue damage, illustrating that ET-1 may contribute to neutrophil-mediated injury (Halim *et al.*, 1995). So, ET-1 is likely to act as a key inflammatory mediator and mitogen through its ability to stimulate inflammatory cells, trigger cytokine and growth factor release, alter gene expression, and induce fibroblast cell growth.

In conclusion, these results show that ET-1 can upregulate expression of ICAM-1 by scleroderma fibroblasts thereby influencing leukocyte interactions *in vivo* and subsequently the pathogenesis of scleroderma. These results also suggest that the peptide ET-1 can modulate the function of fibroblasts in ways that may alter immune cell-fibroblast interactions. The ability of a non-peptide ET-1 antagonist to block this modulation may suggest additional ways in which such agents may be useful in treating inflammatory disease.

CHAPTER 7: SSc FIBROBLASTS RETAIN ABNORMAL COLLAGEN BIOSYNTHESIS AND ICAM-1 EXPRESSION FOLLOWING RETROVIRAL TRANSDUCTION

7.1 INTRODUCTION

Studies carried out on primary dermal fibroblasts obtained from scleroderma skin have shown that they have, at least at early passage, a coordinated increase in the synthesis of types I, III and VI collagen and fibronectin (Unemori *et al.*, 1991; Mauch *et al.*, 1993). *In situ* hybridisation studies of collagen mRNA and flow cytometric (FACS) analysis of isolated dermal fibroblasts have suggested that the expansion of a sub-population of abnormal fibroblasts *in vivo* might contribute disproportionately to these pathologic phenomena (Scharffetter *et al.*, 1988; Needleman *et al.*, 1990). This is supported by other observations that a subset of abnormal cells may also be responsible for the elevated expression of ICAM-1 and the down-regulation of certain proteolytic ectoenzymes (Abraham *et al.*, 1991; Needleman, 1990; Shiwen *et al.*, 1994; Bou-Gharious *et al.*, 1995). However, attempts to delineate regulatory mechanisms in disease-associated abnormal cells are often restricted by the limited numbers of cells and by their tendency to senescence or to revert to the normal phenotype on prolonged passage in tissue culture, presumably due either to the selection of a normal cell population or to modulation of fibroblast metabolism induced by altered growth conditions *in vitro* (Mauch *et al.*, 1993; Ivarsson *et al.*, 1993). Therefore, in order to further study the cellular pathology of scleroderma, experiments aimed at increasing the life span of primary fibroblasts under conditions which do not ablate their abnormal phenotypic and functional characteristics have been carried out.

Human cells which harbour DNA tumour viruses or their transforming genes (oncogenes) frequently show an extension of their life-span (Linder *et al.*, 1990; Stamps *et al.*, 1992). The SV40 large T antigen has been extensively used to extend the proliferative capacity of a number of types of human cell, including T lymphocytes (Ryan *et al.*, 1992), mammary epithelial cells (Bertek *et al.*, 1991; Stamps *et al.*, 1994), keratinocytes (Stacey *et al.*, 1990), fibroblasts (White *et al.*,

1992; Shay *et al.*, 1989), and myoblasts (Simon *et al.*, 1996). In this study I have used an amphotrophic retroviral vector which transfects a mutant temperature-sensitive, non-DNA-binding (tsA58-U19) construct of the SV40 large T antigen (Stamps *et al.*, 1994; Jat *et al.*, 1989) to establish long-term cultures of normal and scleroderma fibroblasts. The presence of this particular T antigen allows cell proliferation of the fibroblasts to be controlled by temperature-shift between a low (permissive) temperature, at which the cells continue to divide, and a non-permissive (high) temperature, at which growth arrest occurs. I report that some of the hallmark properties of the scleroderma fibroblasts were not affected by the presence of this tsT antigen and were maintained during prolonged culture *in vitro*. These cells may therefore facilitate detailed study of the regulatory mechanisms involved in the abnormal cellular pathology of scleroderma and may also provide important insights into disease mechanisms of other fibrotic conditions.

7.2 PATIENTS AND STATISTICS

7.2.1 Samples

Fibroblast cultures were grown from explants of punch biopsies taken from the involved skin of 4 patients with diffuse scleroderma and from 4 healthy normal volunteers. The group of 4 scleroderma patients all showed active, diffuse cutaneous disease (dcSSc) of 2-4 years' duration (average 3.1) at the time of biopsy. Their modified skin scores were between 18 and 48 (average 26) and all fulfilled the criteria of the American College of Rheumatology.

7.2.2 Statistical analysis

Statistical comparisons between the various parameters measured in normal and scleroderma fibroblasts were made using the Student's t-test. Data are expressed as the mean $\pm$ SEM. P values less than 0.05 were considered significant.

7.3 RESULTS

7.3.1 Retroviral infection and selection of normal and scleroderma fibroblasts

After three cycles of infection with the replication-defective retrovirus encoding the temperature-sensitive SV40 large T antigen, the fibroblasts were cultured at 35°C in growth medium contaning G418 for three weeks. During this period, the cells continued to proliferate rapidly and there was little sign of cell death. Indirect immunofluorescence staining was then carried out on cell cultures using antibodies against the SV40 large T antigen. Non-infected control primary parent fibroblast cultures (NF and SScF) were uniformly negative for the SV40 large T antigen (Figure 7.1A), whereas staining of the retrovirally infected cells after selection revealed a bright nuclear localisation of the large T antigen in almost all of the fibroblasts examined (Figure 7.1B) in both tsT-NF and tsT-SSc F cultures.

The G418-resistant cell cultures of both cell types have been repeatedly passaged over 50 times at a 1:5 split ratio, without any apparent crisis. The morphology of these cells, as monitored by phase-contrast microscopy, remained essentially the same as that of the parental fibroblasts.

7.3.2 Growth characteristics: temperature and anchorage-dependence

To determine whether the infected fibroblasts were under the proliferative control of the large T antigen, the effect of temperature on fibroblast cell growth was examined by culturing the cells at both permissive and non-permissive temperatures. Figures 7.2A and B show that, at 35°C, the parental fibroblasts (NF and SSc F) and the transduced fibroblasts (tsT-NF and tsT-SSc F) continued to divide, although the rate of proliferation was greater in the transduced cells, resulting in approximately twice the number of cells after 10 days of incubation. Moreover, although the non-transduced cells continued to proliferate when shifted to the non-permissive temperature of 39.5°C, the tsT-NF and tsT-SSc F cells ceased to divide after 48 hours, as observed with other tsT-expressing cells (Jat *et al.*, 1989; 1991).

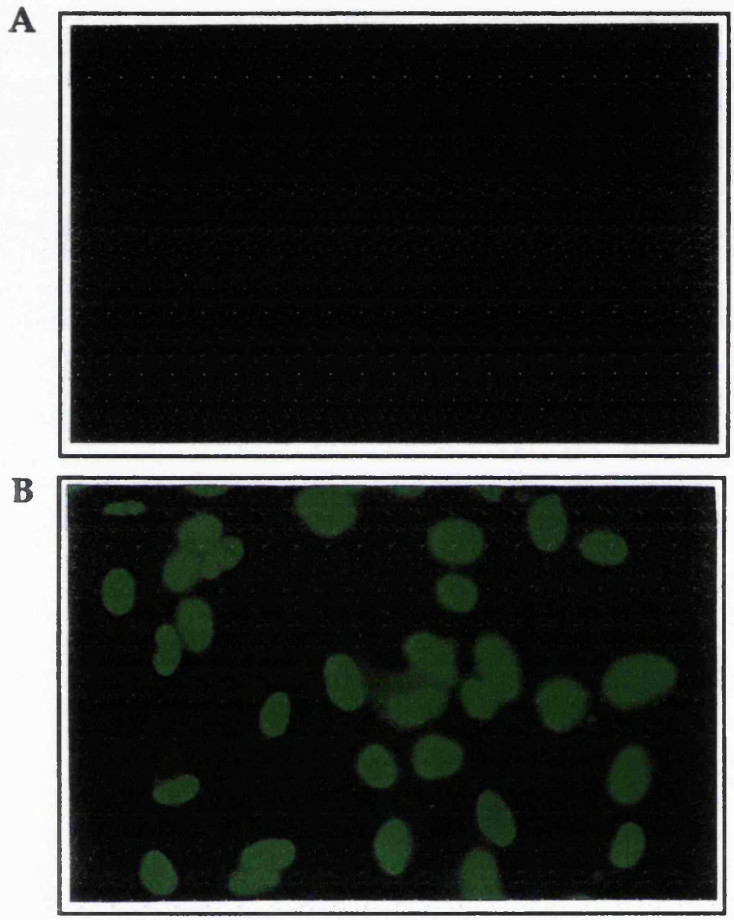


Figure 7.1. Indirect immunofluorescence staining of the SV40 large T antigen. Fibroblast cultures grown at 35°C were examined by indirect immunofluorescence. The transformed nature of these cell lines is demonstrated by positive staining of fixed cells using antibody specific for the SV40 large-T antigen. In (A), control normal fibroblasts (NF) showed no staining, whereas in (B), all the retrovirally-transduced G418-selected normal cells (tsT-NF) exhibited intense nuclear staining, demonstrating the presence of the SV40 large T antigen. Both types of cultures were examined at passage 10. Magnification, X400.

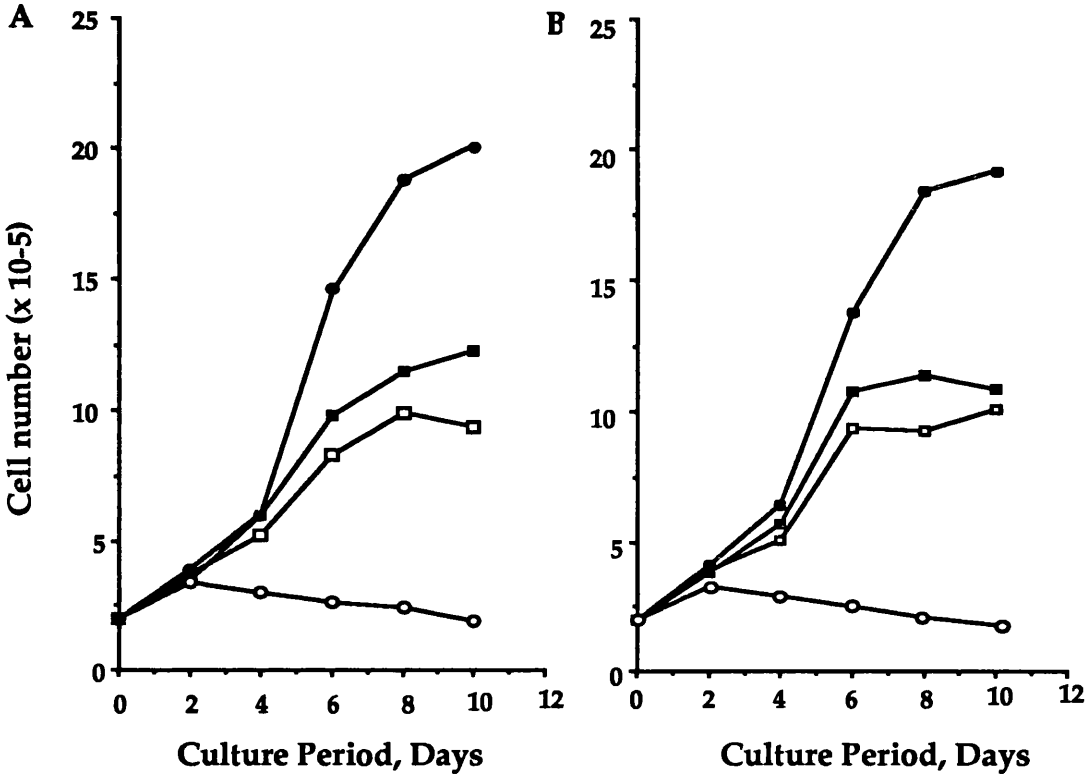


Figure 7.2. Temperature-dependence of fibroblast growth. Replicate fibroblast cultures between passages 6 and 10 were seeded at an initial density of $2x10^5$ cells per culture vessel and cultured on at either 35°C (solid symbols) or 39.5°C (open symbols) as shown. At the time points indicated, triplicate cultures were harvested by trypsinisation and the total cell number determined by direct counting using an haemocytometer after staining with trypan blue. (A) shows normal (squares) and tsT-transduced normal (circles) fibroblasts, and (B) shows SSc fibroblasts (squares) and tsT-transduced SSc fibroblasts (circles).

To investigate the anchorage dependence of the cells, they were grown in soft agar and their ability to form colonies at 35°C and 39.5°C was assessed as described in the Materials and Methods. After 3 weeks at the lower temperature, all of the cultures had only very few, very small colonies (<100μm; colony forming efficiencies of $<2 \times 10^{-4}$). None of the cell cultures examined formed colonies in soft agar when grown at 39.5°C. The results in Table 7.1 summarize some of the characteristic growth features of these cells.

7.3.3 Type I collagen secretion

Measurement of collagen type I secretion using an inhibition ELISA showed that the scleroderma cells released greater amounts of collagen type I than did the NF cells. The difference in collagen secretion was maintained at both 35 and 39.5°C (438 ± 102.3 and 414 ± 78.9 ng/ml/10^6 by the SSc F cells at the low and high temperatures, respectively, compared with 211 ± 56.4 and 214 ± 28.4 ng/ml/10^6 by the NF cells at the two temperatures respectively). Moreover, at 39.5°C the tsT-NF and tsT- SSc F cultures produced the same relative amounts of type I collagen (231 ± 27.7 and 422 ± 70.9 ng/ml/10^6) as did their non-transduced NF and SScF counterparts. Thus, the infected scleroderma cells retained the characteristic abnormal feature of increased collagen over-production typical of the original scleroderma phenotype.

7.3.4 Expression of fibroblast antigens and adhesion molecules

To examine the phenotypic characteristics of the tsT-NF cells, they were evaluated for the expression of a number of surface-associated antigens compared with the normal, uninfected fibroblasts. The results in Table 7.2 show that more than 95% of both cell types expressed MHC Class I and only a small proportion (less than 5%) expressed MHC Class II antigens. A similar proportion of both cells also expressed fibronectin, intracellular procollagen type I and collagens types I and III (Table 7.2). FACS analysis of adhesion molecules which regulate interactions with the extracellular matrix and with other cells (Figure 7.3) revealed that the tsT-NF cells expressed the same levels of these antigens as did the non-transduced NF cells. Thus, CD29, CD44, CD49d and LFA-3 were all

Table 7.1. Summary of fibroblast cultures

Fibroblast Type	Passage	Expression of tsA58 T-antgen	G418 resistance (0.5 mg/ml)	Growth Characteristics	
				Temperature dependence *	Colonies in agar at 39.5°C
NF	2-6	-	-	-	-
tsT-NF	35 **	+	+	+	-
SScF	2-6	-	-	-	-
tsT-SScF	33**	+	+	+	-

* growth arrest at 39.5°C

** indicates number of passages post-infection

Table 7.2. Expression of human fibroblast antigens

Specificity	Antibody	% Postive cells*	
		tsT-NF	NF
MHC I	W6/32	98.9 (93.2 - 99.4)	99.1 (94.9 - 99.8)
MHC II	L234	3.5 (1.8 - 5.4)	2.9 (0.9 - 6.3)
Fibronectin	α-FN	93.6 (88.2 - 97.7)	94.1 (87.6 - 98.7)
Procollagen type I	M-38	89.2 (85.8 - 95.9)	87.3 (81.5 - 93.2)
Collagen type I	α-coll-I	95.9 (89.9 - 97.3)	94.8 (90.3 - 98.5)
Collagen type III	α-coll-III	21.1 (13.7 - 32.4)	22.9 (10.3 - 35.7)

* The proportion of antigen expressing cells were measured by FACS using a non-reactive antibody as negatve control, as described in the Materials and Methods. The values shown are averages of six experiments with ranges in parenthesis.

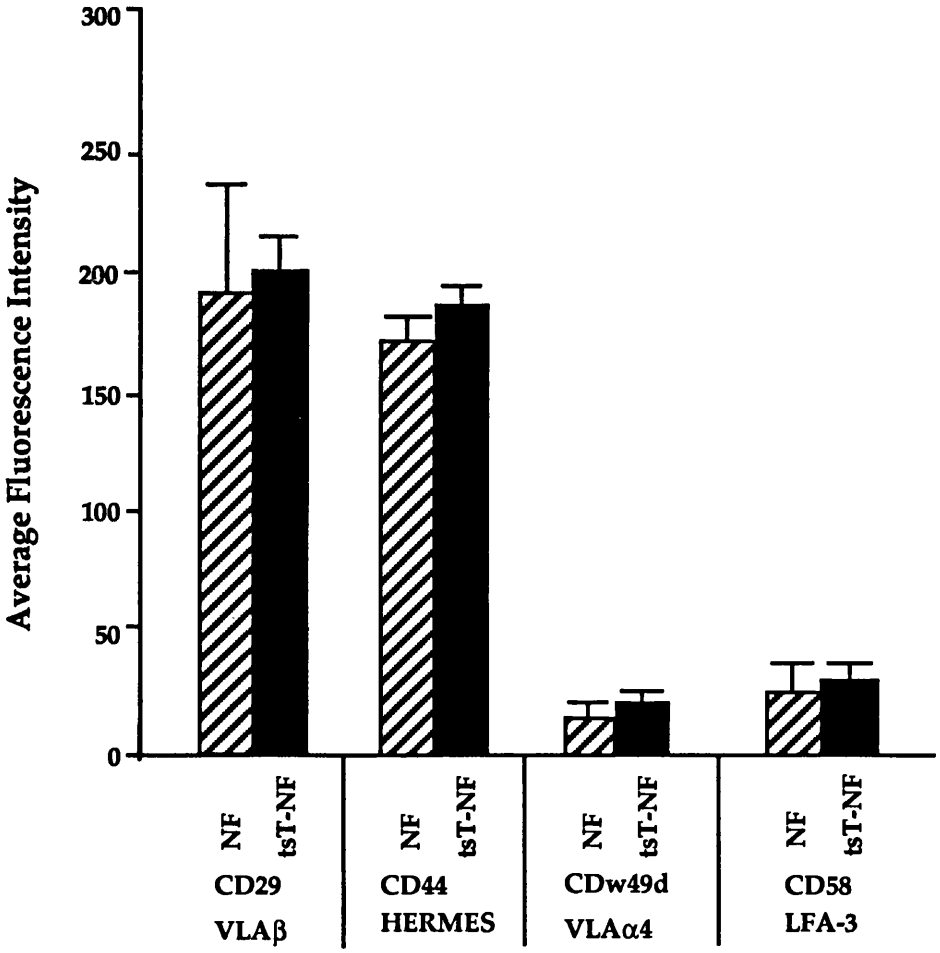


Figure 7.3. Expression of adhesion molecules on non-transduced (NF) and transduced (tsT-NF) normal fibroblasts. The effect of the temperature-sensitive SV40 large T antigen on the relative expression of a number of adhesion molecules was examined by FACS. Following staining with the respective mAb, the adhesion molecule was detected using an FITC-conjugated secondary antibody and the level of expression measured using a FACScan. The hatched plots represent normal fibroblasts (NF) and the solid plots the transduced normal fibroblasts (tsT-NF). The results are expressed as the average fluorescence intensities of three individual experiments ± SEM (error bars).

found to be expressed equally by both cell types, although the levels of the latter two adhesion molecules were much lower than CD29 and CD44.

7.3.5 Expression and cytokine-induction of ICAM-1

The elevated expression of ICAM-1 is a common feature of inflammatory reactions and has been shown to be associated with scleroderma (Abraham *et al.*, 1991; Needleman *et al.*, 1990; Springer, 1990). Moreover, ICAM-1 expression by both normal and scleroderma fibroblasts *in vitro* is up-regulated by cytokines such as IL-1, TNF-α and IFN-γ (Abraham *et al.*, 1991). In the present experiments, ICAM-1 expression was again found to be higher in the scleroderma fibroblasts compared with normal controls (AFIs of 77.3 and 42.1, respectively), as shown in Figure 7.4. The elevated level of this adhesion molecule was also apparent in the tsT-SScF cells, which had an AFI of 75.9 (Figure 7.4). This was substantially higher than ICAM-1 expression in the tsT-NF cells, which remained at the same, low level as in the non-infected NF cells. The results in figure 7.4 also show that both the tsT-NF and tsT-SScF cells retained their responsiveness to cytokine induction, since ICAM-1 expression was enhanced by 225 and 165% ($p<0.05$), respectively, when the cells were cultured in the presence of IFN-γ. In addition, both cell types were also similarly sensitive to the presence of TNF-α, which elicited equally elevated expression of ICAM-1.

7.3.6 Expression of cell surface proteases

To further examine the characteristic features of tsA58-U19-transduced human fibroblast cultures, we used FACS to measure the expression of the cell surface proteases, neutral endopeptidase (CD10), aminopeptidase N (CD13) and dipeptidyl peptidase IV (DPPIV; CD26). These enzymes have previously been shown to be present in both normal and scleroderma fibroblasts, although CD26 was expressed at a lower level in scleroderma cells (Bou-Gharios *et al.*, 1995). In the present study, the level of expression of CD10 was found to be slightly decreased in both non-transduced and tsT-transduced scleroderma cells, compared with the normal cells (Figure 7.5). CD13 was found to be expressed at very similar levels on the surface of all the types of fibroblasts examined.

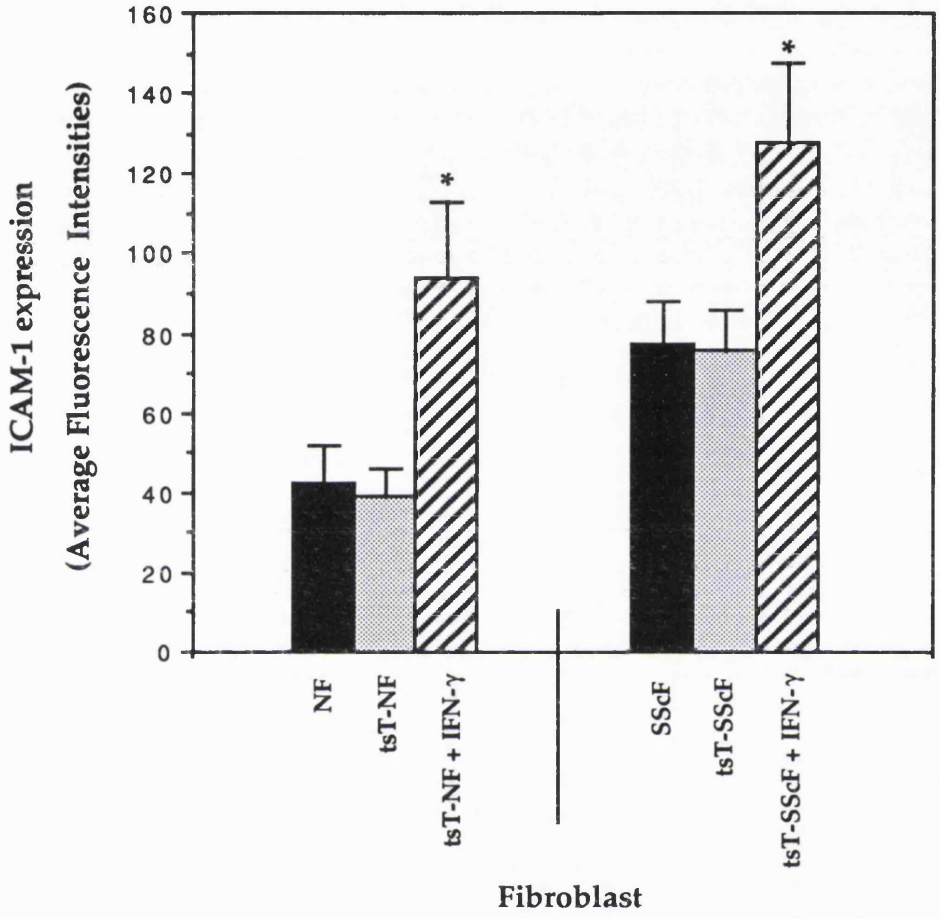


Figure 7.4. Expression and cytokine-induction of ICAM-1.
The endogenous and IFN-γ-inducible expression of ICAM-1 was measured by FACS, as described in the Materials and Methods. Normal, non-transduced fibroblasts (NF) are shown by solid bars and tsT-expressing normal cells (tsT-NF) by dotted and hatched bars (in the absence and presence of the optimal concentration IFN-γ respectively). The data represent values from three individual experiments ± SEM. * $p<0.05$.

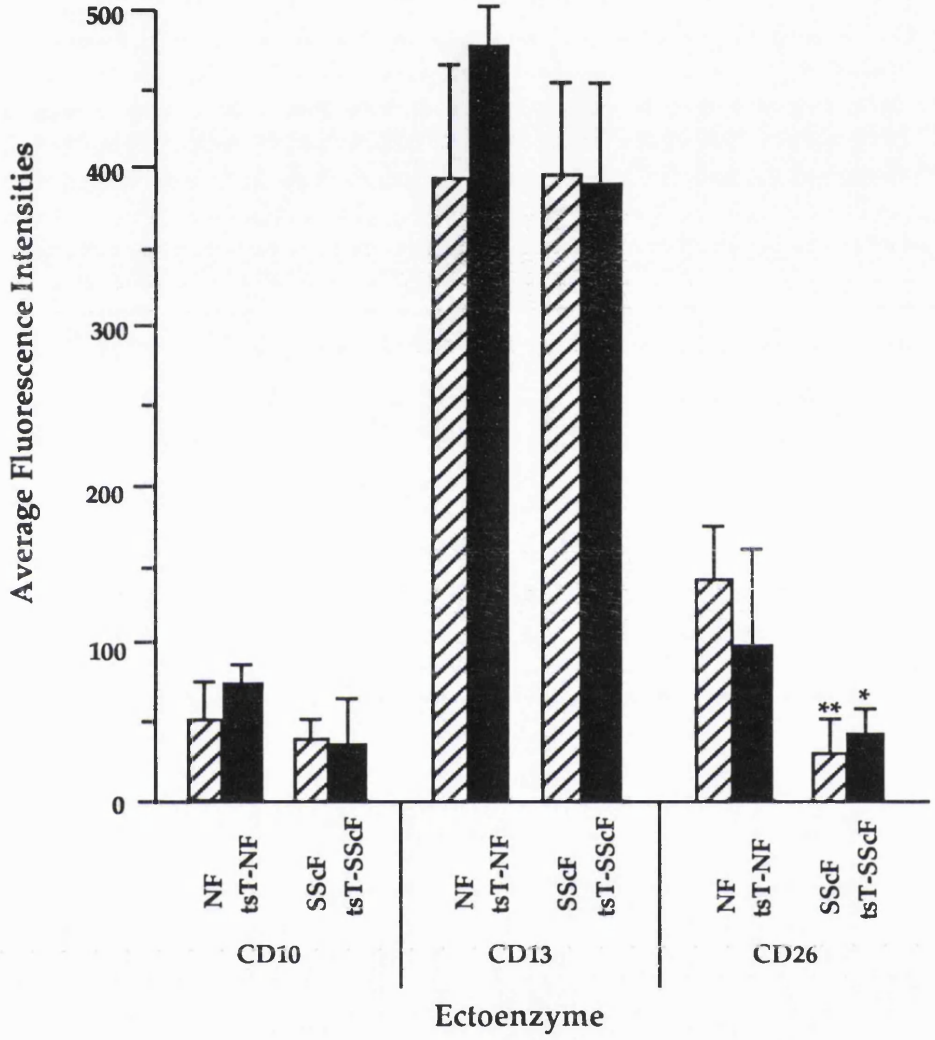


Figure 7.5. Expression of cell surface proteases (ectoenzymes). The level of expression of the ectoenzymes, CD10, CD13 and CD26 were measured in non-transduced (hatched bars) and retrovirally transduced (solid bars) cell cultures. The level of each surface protease is shown for normal fibroblasts (NF) and their tsT-transduced counterparts (tsT-NF) in comparison with non-transduced and transduced scleroderma fibroblasts, SScF and tsT-SScF cells respecively. Surface expression is given as the AFI of four individual experiments $\pm$ SEM. * $p<0.05$, ** $p<0.01$.

However, the results in Figure 7.5 show that there was no significant difference in the expression of dipeptidyl peptidase IV when the non-transduced normal human fibroblasts were compared with their transduced counter-parts. Similarly, the levels of CD26 expressed by SScF cells was not markedly affected by transduction with the tsT antigen. In contrast, CD26 was significantly lower in scleroderma fibroblasts (SScF) (AFI of 29.9 ± 13.9) compared with both the non-transduced and transduced normal cells (AFIs of 139.8 ± 23.1, P=0.004 and 99.9 ± 47.9, P= 0.01, respectively). Notably, the significant reduction in the expression of CD26 was found to be retained by the scleroderma cells (AFI of 43.3 ± 9.8 ; P<0.05), after transduction with the SV40 large T antigen.

7.4 DISCUSSION

In this study, an amphotropic retroviral vector system has been used to generate temperature-dependent normal and scleroderma fibroblast cultures containing the temperature-sensitive SV40 large T antigen. Both types of culture appear morphologically normal and are not transformed in that they have not acquired the capacity of anchorage-independent growth. However, the transduced scleroderma-derived cells retained, for extended periods of time *in vitro*, a phenotype which corresponds with their behaviour *in vivo* and in short-term primary culture. Scleroderma fibroblasts, with extended growth potential but nevertheless retaining the disease phenotype even after extended passage, are of considerable potential value. They enable large numbers of cells to be generated, permit prolonged investigations *in vitro* and they provide a model system with which to study the role of the fibroblast component of the scleroderma disease process. Previous results have shown these types of tsT-expressing cells can be reproducibly generated with a retroviral vector system which enables a high efficiency of gene transduction and a stable pattern of genomic integration (Stamps *et al.*, 1994).

In contrast to the present studies with retrovirally engineered cells, long-term cultures of non-modified scleroderma fibroblasts have often been observed to revert from a typical disease phenotype with increased collagen synthesis

(Jimenez *et al.*, 1986; Uitto *et al.*, 1979; Scharffetter *et al.*, 1988), elevated surface ICAM-1 (Abraham *et al.*, 1991; Needleman, 1990) and depressed activity of the ectoenzyme dipeptidyl peptidase IV (DPPIV, CD26) (Bou-Gharios *et al.*, 1995) to the patterns typical of normal fibroblasts. One possible reason for this change is the gradual take-over of such cultures by normal fibroblasts. This hypothesis is supported by our observation in the present study that the introduction of the growth-promoting tsT-gene has enabled cells with the disease phenotype to persist for at least 50 passages.

The SV40 large T antigen is capable of greatly extending the life-span of different types of human cells *in vitro*. This oncogenic protein provides a mitogenic stimulus whilst at the same time eliminating the function of cell cycle regulating tumor-suppressor proteins, most notably p53 and Rb, by binding to them (Stamps *et al.*, 1992; DeCaprio *et al.*, 1988). However, the presence of the T antigen does not itself immortalise human cells (Stamps *et al.*, 1992), since most cells expressing this gene eventually reach a 'crisis' at which they cease to grow normally, accumulate many chromosomal aberrations and usually die. Immortal cell lines do emerge at a reproducible, but generally very low, frequency. Although our results clearly show that the scleroderma cultures exhibit stringent temperature-dependant growth, it still remains uncertain whether they are 'immortal' cell lines at the present stage. After 50 passages the cells have undergone at least 120 population doublings *ex vivo*. This is far more than the 20 to 40 cycles we have observed with non-transduced scleroderma fibroblasts and in excess of the normal life-span of most adult human fibroblasts *in vitro*. (Stamps *et al.*, 1992). While no overt crisis was observed, this does not preclude the generation of truly immortal cell lines since establishment without crisis has sometimes been observed using similar vector systems (Bartek *et al.*, 1991). Nonetheless, the retention of the disease phenotype for extended periods *in vitro* provides a useful and important model for studying the cellular and molecular pathology of scleroderma.

In the results reported here the abnormal phenotype of increased ICAM-1 expression is retained even after prolonged periods in culture. Needleman *et al*

(1990) showed two subpopulations of fibroblasts which were delineated based on their surface expression of ICAM-1 into high and low expressor populations, although no correlation between ICAM-1 expression and collagen secretion was found. Notably, the tsT- SScF populations did not express any heterogeneity with regard to ICAM-1 expression although there was an increased expression of collagen type I. The expression of other fibroblast adhesion molecules examined in this study notably LFA-3, CD29, CD44, CD49d, were similar to that observed in the non-transduced scleroderma cells.

Dermal fibroblasts from scleroderma lesions have been shown to synthesise increased type I and III collagen and other matrix components (Jimenez *et al.*, 1986; Uitto *et al.*, 1979). Moreover, recent observations using three dimensional collagen gels show an impaired regulation of collagen pro-α1 (I) mRNA turnover and an abnormal interaction with the extracellular matrix, possibly resulting from an aberrant expression of the collagen binding α1β1 integrins, in particular, reduced levels of the α1 sub-unit (Ivarsson *et al.*, 1993). I have shown that there is no difference in the expression of CD29 (β1 integrin) between normal and scleroderma fibroblasts either before or after the retroviral transduction of the tsA58-U19 construct. Based on the above observation and the retention of increased collagen synthesis, it is therefore likely that the aberrant regulation is due to an abnormal expression of α1 and not β1 integrin. Furthermore, the tsT-SScF cells retained this ability even after prolonged periods in culture, suggesting that extrinsic environmental factors are not responsible for the reversion to normal levels of collagen synthesis by scleroderma fibroblasts in long-term culture *in vitro*.

Surface proteases belong to a group of ectoenzymes which are integral membrane proteins, whose active site is located at the cell surface, allowing substrate hydrolysis in the extracellular environment. These plasma membrane antigens are thought to have a number of functional roles including cell activation, regulation of interactions with and turnover of matrix components and the metabolism of peptide hormones (Kenny *et al.*, 1989). Phenotypically distinct fibroblast sub-populations have been previously described based on the

differential expression of these surface antigens (Bou-Gharios *et al.*, 1995; Atherton *et al.*, 1994). In scleroderma, the expression of the serine protease CD26 (DPPIV) has been reported to be substantially lower in scleroderma fibroblasts compared with normal cells (Bou-Gharios *et al.*, 1995). In previous studies using cultures derived directly from primary explants, without the use of viral oncogenes to enhance cell growth, this phenotype was observed at low passage in scleroderma cultures, while at later passages CD26 levels were similar to those found in normal fibroblasts (Bou-Gharios *et al.*, 1995). In this context it is interesting to note that, in cultures of normal inter and intra-lobular human mammary fibroblasts, differences that were initially present in the levels of CD26 also disappeared with time (Atherton *et al.*, 1994), while differences in another ectoenzyme, CD10, emerged and were retained *in vitro*. In the present study no significant difference in the levels of expression of either CD10 or CD13 were found in normal compared with scleroderma-derived cells. However, the level of CD26 was significantly lower in the primary scleroderma cells and this difference was maintained after retroviral infection of the scleroderma fibroblasts and transduction of the tsT antigen and continued passage, again indicating that intrinsic abnormalities rather than extrinsic factors are responsible for the difference.

In this study I have shown that expression of a temperature-sensitive SV40 large T antigen does not alter the expression of a number of fibroblast membrane antigens and synthetic functions when the gene is introduced into cells via an amphotropic retroviral vector system. The most important observation is the conservation of expression and regulation of the abnormal scleroderma disease phenotype with respect of collagen synthesis and the expression of certain adhesion molecules and ectoenzymes. These cultures may therefore play an important role in investigations of an abnormal cell type in the pathogenesis of scleroderma and possibly other fibrotic conditions.

CHAPTER 8: FINAL DISCUSSION

The research work covered in this thesis highlights a number of aspects of fibroblast biology which are likely to be highly relevant to the development and progression of scleroderma (SSc). In this chapter I will consider the areas of research that I have addressed, summarise what I think are the pertinent findings that can be drawn from the studies outlined in the preceding chapters and try to place into perspective my results within the overall knowledge of field and discuss their relevance to the human disease.

The pathological consequences of scleroderma involve, as outlined in chapter 1 abnormal immune cell activation and vascular damage accompanied by specific alterations in certain fibroblast properties and ensuing connective tissue fibrosis. The focus of the study presented in this thesis is centred on the fibroblasts, the cell type that is critical to the fibrotic nature of scleroderma.

One of the cardinal features of scleroderma that is universally recognised by all researchers is the increased synthesis and deposition of extracellular matrix components notably type I collagen by connective tissue fibroblasts. This notion is strongly supported by the extensive literature examining the *in vivo* characteristics of dermal fibroblasts from scleroderma patients. Here I have studied in some detail the properties of pulmonary fibroblasts from scleroderma patients with lung fibrosis and I have been able to directly compare the phenotype of fibroblasts from two different scleroderma lesional tissue fibrosis. This was achieved experimental by simultaneously studying the synthesis of collagen in both dermal and lung fibroblast within monolayer and 3-D collagen gel. In addition I was able to directly examine both normal and scleroderma fibroblasts from both organs (skin and lung) with respect to their collagen biosynthesis and the transcriptional regulation of the collagen type I gene (COL1A2). In concert with increase collagen production, other important aspects of the fibrotic phenotype appears to be aberrant cytokine/growth factor production within scleroderma and an altered responsiveness by fibroblasts to a number of these factors. It is therefore likely that both autocrine and paracrine

regulatory pathways have an influence on the disease manifestations. To address the potential pathways that may exert major influences in modulating fibroblast phenotype and function I studied two specific cytokines/growth factors, connective tissue growth factor (CTGF) and endothelin-1 (ET-1). Of particular interest was the regulation of collagen biosynthesis by CTGF, a factor thought to be a potential down-stream effector cytokine induced following TGF-β activation. I also examined the fibroblast response to endothelin-1 peptide produced by activated endothelial cells. In addition to its role in vascular tone endothelin-1 has been recently shown to be a fibroblast mitogen and have a potential role in the modulation of fibroblast matrix production. This peptide is a particularly attractive candidate as it represents a link in the disease pathology between vascular activation/damage with the accompanying fibrotic process. The role of ET-1 in scleroderma became more apparent when examining its influence on the expression of ICAM-1. ICAM-1 has been reported to be elevated on scleroderma fibroblasts, where it mediates lymphocyte-fibroblast interaction, important in the recruitment of activated T cells into extravascular tissues. In view of the enhanced extravasation of leukocytes into the skin in the acute early phase of scleroderma, the potential influence of endothelin-1 which may function as a potent pro-inflammatory mediator in regulating ICAM-1 expression was also studied. Finally, the most persuasive evidence for a central fibroblast defect in scleroderma would be a persistence in the disease specific phenotype *in vitro*. In order to extend the life-span of scleroderma fibroblasts, I generated cell lines that were transduced with a temperature-sensitive SV40 large T antigen and studied the persistence of the altered phenotype (elevated collagen type I and ICAM-1 expression) upon prolonged passage.

The results of the first study (Chapter 3) showed that *in vitro* both dermal and lung scleroderma fibroblasts exhibit markedly elevated collagen (I) protein production and increased mRNA levels. In addition despite normal gel contraction both scleroderma skin and lung fibroblasts fail to down-regulate collagen type I mRNA. Although skin fibroblasts showed higher levels of collagen production than lung fibroblasts, both demonstrated increased activation of the COL1A2 gene in scleroderma strains compared with controls,

suggesting that the elevated collagen (I) mRNA levels are due, at least partly, to transcriptional activation. This agrees with the previous reports by several groups studying scleroderma skin fibroblasts (Ivarsson *et al.*, 1993; Kikuchi *et al.*, 1992). In chapter 3, I demonstrated that there are substantial similarities between the abnormal *in vitro* properties of scleroderma lung and dermal fibroblasts (Shiwen *et al.*, 1997).

In view of the similarly elevated collagen (I) levels exhibited by both dermal and lung fibroblast, dermal fibroblasts were use for the majority of the further experimental work. Growth factors and cytokines are important regulators of collagen and other matrix synthesis by fibroblasts. It is interesting that different paracrine and autocrine factors, such as connective tissue growth factor (CTGF) (Chapter 4) and ET-1 (Chapter 5), modulate fibroblast properties independently.

The overall research goal in chapter 4 was to increase the understanding of the role of CTGF in connective tissue fibrosis. CTGF was first described and cloned in 1991 and shown to be a potent mitogen secreted by human endothelial cells, with the ability to induce the growth and matrix production of fibroblasts *in vitro* (Bradham *et al.*, 1991, Igarashi *et al.*, 1993). A potential role in a number of fibrosing diseases has been suggested by strong expression of the peptide in scleroderma skin lesions as well as skin from other conditions such as keloid scars. These observations point to CTGF as a possible effector gene that significantly contributes to the disease process (Igarashi *et al.*, 1996; Oemar *et al.*, 1997). I feel that this protein represents a good fibrogenic candidate in view of the significant correlation between CTGF expression and skin sclerosis not only in scleroderma but also in other fibrotic disorders. In this thesis, I have demonstrated that only scleroderma fibroblasts exhibit basal expression of intracellular CTGF and are able to secrete the protein. Normal fibroblasts were clearly shown not to constitutively produce CTGF. However, both normal and scleroderma fibroblasts can be induced to produce high levels of CTGF protein after activation with TGFβ. It therefore appears that CTGF may be a significant autocrine growth factor for fibroblasts once activated (Frazier *et al.*, 1996), with the potential to exert an significant influence on the development of fibrotic

disorders such as scleroderma. Indeed, in further experiments I was able to demonstrate that recombinant CTGF (rCTGF) protein stimulated fibroblast proliferation and pro-α1(I) collagen synthesis in a manner similar to TGFβ. Up-regulation of collagen biosynthesis by rCTGF was markedly greater for scleroderma fibroblasts than normal strains. There was a correlation between fibroblast proliferation, collagen synthesis and CTGF expression. Based on these observation, it is likely that CTGF exerts its activity through binding to a cell surface receptor which may be up-regulated in fibrotic conditions. Identification of the CTGF receptor and quantitation of binding sites, as well as understanding how CTGF signalling results in extracelluler matrix production, will remain important goals of future research. This data will provide evidence to test our hypothesis that CTGF represents a major autocrine growth factor involved in fibrotic conditions.

The involvement of vascular injury in the pathology of scleroderma has been suggested by the presence of early vascular changes associated with endothelial cell damage preceding the onset of tissue fibrosis (Campbell and LeRoy, 1975), and the relationship between disease outcome and the severity of vascular lesions (Kahaleh, 1990). The data presented in chapter 5 confirm that ET-1, a peptide produced by activated or damaged endothelium is able to regulate both cell proliferation and the turnover of matrix components such as type I and III collagen, fibronectin and collagenase (MMP-1). Interestingly, when compared with normal cells scleroderma fibroblasts appear to exhibit a reduced response to ET-1. It is well documented that ET-1 mediates varied biological effects through different ET receptors, therefore it is likely that an altered expression of ET receptors may be associated with different functional responses. Using ET receptor selective ligands, I examined the expression of different receptor subtypes in scleroderma and normal fibroblasts. In scleroderma fibroblasts there was a decrease in the levels of ET_A receptors. There is no doubt that in addition to a change in receptor expression there is a substantial change in the specific cell responding ET-1 occurring during the disease process which provides evidence for the involvement of ET-1 and its receptors in the pathophysiology of scleroderma.

In addition to matrix over-production, another characteristic property of scleroderma fibroblasts is intercellular adhesion molecule-1 (ICAM-1) expression. Previous studies showed increased ICAM-1 expression on scleroderma fibroblasts (Needleman, 1990; Abraham *et al.*, 1991). Circulating ICAM-1 levels are increased in plasma of scleroderma patients (Denton *et al.*, 1995) and bronchoalveoler lavage fluid (BALF) (Du Bois *et al.*, 1992). It is possible that fibroblasts may be a source of circulating ICAM-1 based on the shedding of soluble ICAM-1 (sICAM-1) which is increased in culture media from scleroderma fibroblasts grown *in vitro* (Shiwen *et al.*, 1994). Interestingly, ET-1, as sICAM-1, is also increased in plasma, BALF from scleroderma patients (Cambery *et al.*, 1994; Vancheeswaran *et al.*, 1994), as well as culture medium from scleroderma fibroblasts (Kawaguchi *et al.*, 1994). In chapter 6 data is presented that confirm that ET-1 is able to up-regulate fibroblast ICAM-1 expression. These observations indicate that ET-1 may function as a potent proinflammatory mediator by regulating interactions between immune cells and fibroblasts in scleroderma. The modulating effect of ET-1 can be inhibited by the pretreatment of fibroblasts with ET-receptor antagonists.

From the above studies, scleroderma fibroblasts cultured *in vitro* are shown to be an important resource with which to study and increase our understanding of the pathogenesis of scleroderma, and in particular the fibrotic component of the disease. However, understanding the precise cellular and molecular pathology of scleroderma has been limited by the difficulty in obtaining sufficient numbers of cells for investigation and the observation that the scleroderma phenotype reverts with passage *in vitro*. Establishment of fibroblast cell lines from early passage primary cells of scleroderma patients using a temperature-sensitive SV40 mutant (tsA58-U19) could overcome these difficulties. In this study I have shown that expression of a temperature-sensitive SV40 large T antigen following retroviral transduction does not alter the expression of a number of fibroblast membrane antigens and synthetic functions. The most important observation is the conservation of the abnormal scleroderma disease phenotype with respect to collagen synthesis and the expression of ICAM-1 (Shiwen *et al.*, 1995). These

cultures may therefore be useful in investigations of an abnormal cell type in the pathogenesis of scleroderma and possibly other fibrotic conditions.

In summary, this thesis provides further clues about the pathogenesis of scleroderma. The abnormal regulation of fibroblast phenotype in scleroderma supports the hypothesis that extrinsic modulation *in vivo* leads to sustained abnormalities of scleroderma fibroblast properties. The potential mechanisms explored are also likely to be highly relevant to other, more common, fibrotic disorders (e.g. liver cirrhosis, atherosclerosis). The results discussed above also suggest possible targets for the further development of therapeutic strategies and intervention. These might include neutralising antibodies or pharmacological antagonists directed against ET-1 and CTGF. The demonstration of persistent gene activation in scleroderma fibroblasts also suggests that molecular strategies for reducing the activity of such genes may also be effective. It is especially important to consider that some of the pathways of altered fibroblast regulation are likely to operate in the established fibrotic lesion such as scleroderma which has hitherto been impossible to treat effectively.

REFERENCES

Abraham D, Bokth S, Bou-Gharios G, Beauchamp J, Olsen I. Interactions between lymphocytes and dermal fibroblasts: an *in vitro* model of cutaneous lymphocyte trafficking. Exp Cell Res 190. (1990): 118-126.

Abraham D, Lupoli S, McWhirter A, Plater-Zyberk C, Piela TH, Korn JH, Olsen I, Black C. Expression and function of surface antigens on scleroderma fibroblasts. Arthritis Rheum 34. (1991): 1164-1172.

Abraham D, Vancheeswaran R, Dashwood MR, Rajkumar V, Pantelide P, Shiwen X, du Bois RM and Black CM. Increased levels of endothelin-1 and differential endothelin type$_{A/B}$ receptor expression in scleroderma-associated fibrotic lung disease. Am J Pathol 151. (1997): 831-841.

Adamson IY and Bowden DH. The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. Am J Pathol 77. (1974): 185-197.

Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC and Lawley TJ. HMEC-1: Establishment of an immortalised human microvascular endothelial cell line. J Invest Dermatol 99. (1992): 683-690.

Altmann RD, Medsger TA, Bloch DA and Michel BA. Predictors of survival in systemic sclerosis (scleroderma). Arthritis Rheum 34. (1990): 403-413.

Anonymous. Subcommittee for scleroderma criteria of the american rheumatism association diagnostic and therapeutic criteria committee: preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 23. (1980): 581-590.

Artlett CM, Black CM, Briggs DC, Stevens CO, Welsh KI. Telomere reduction in scleroderma patients: a possible cause for chromosomal instability. Br J Rheum 35. (1996): 723-737.

Assoian RK, Fleurdelys BE, Stevenson HC, Miller PJ, Madtes DK, Raines EW, Ross R, Sporn MB. Expression and secretion of type β transforming growth factor by activated human macrophages. Proc Natl Acad Sci USA 84. (1987): 6020-6024.

Atherton AJ, O'Hare MJ, Buluwaela L, Tetley J, Monaghan P, Paterson HF, Warburton MJ, and Gusterson BA. Ectoenzyme regulation by phenotypically distinct fibroblast sub-populations isolated from the human mammary gland. J Cell Sci 107. (1994): 2931-2939.

Avila JJ, Lympany PA, Pantelidis P, Welsh KI, Black CM, du Bois RM. Fibronectin gene polymorphisms associated with fibrosing alveolitis in systemic sclerosis. (1997) Am J Resp Crit Care Med In press.

Bailey A and Black CM. The role of connective tissue in the pathogenesis of scleroderma. In Black CM, Myers AR, eds. systemic sclerosis (scleroderma). New York: Gower, 1985. pp 75-105.

Barnett AJ. History of scleroderma. 1996. systemic sclerosis Ed. Clements PJ and Furst DE. Williams & Wilkins. p3-22.

Barnet RL, Borisy GG, Rothfield NF, Earnshaw WC. Injection of anticentromeric antibodies in interphase disrupts events required for chomosome movement in mitosis. J Cell Biol 111. (1990):1519-1533.

Bartek J, Bartkova j, Kyprianou N, Lalani E, Staskova Z, Shearer M, Chang S, Taylor-Papadimitritriou J. Efficient immortalization of luminal epithelial cells from human mammary gland by introduction of simian virus 40 large tumor antigen with a recombinant retrovirus. Proc Natl Acad Sci USA 88. (1991): 3520-3524.

Battegary EJ, Raines EW, Seifert RA, Bowen-Pope DF, Ross R. Transforming growth factor beta induces bimodal proliferation of connective tissue cells via complx control of an autocrine platelet-derived growth factor loop. Cell 63. (1990): 515-524.

Bell E, Ivarsson B, Merrill C. Production of tissue like structure by human fibroblasts of different proliferative potential. Proc Natl Acad Sci USA 76. (1979): 1274-1278.

Bennett VD and Adams SL. Identification of a cartilage-specific promoter within intron 2 of the chick alpha 2(I) collagen gene. J Biol Chem 265. (1990): 2223-2230.

Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA. Matrix metalloproteinases: a review. Crit Rev Oral Biol Med 42. (1993): 197-250.

Black CM. The aetiopathogenesis of systemic sclerosis: thick skin-thin hypotheses. J Royal Coll Physi London 29. (1995): 119-130.

Black CM and Denton CP. Scleroderma and related disorders in adults and childern. In Oxford Textbook of Rheumatology ed. Oxford University Press. (1997): In press

Black CM, Welish KI, Walker AE, Bernstein RM, Catoggio LJ, McGregor AR, Lloyd JK. Genetic susceptibility to scleroderma-like syndrome induced by vinyl chloride. Lancet i. (1983): 53-55.

Blake DR, Winyard P, Scott DGI, Brailsford S, Blann A, Lunec J. Endothelial cell cytotoxicity in inflammatory vascular disease-the possible role of oxidised lipoproteins. Ann Rheum Dis 44. (1985): 176-184.

Boast S, Ming-Wan S, Ramirez F, Sanchez M, Avvedimento EV: Functional analysis of cis-acting DNA sequences controlling transcription of the human type I collagen genes. J Bio Chem 265. (1990): 13351-13356.

Bocchieri MH, Christner PJ, Henriksen PD, Jimenez SA. Immunological characterisation of (tight skin/NZB) F1 hybrid mice with connective tissue and autoimmune features resembline human systemic sclerosis. J Autoimmun 6. (1993): 337-351.

Bolster MB, Ludwicka A, Sutherland SE, Strange C, Silver RM. Cytokine concentrations in bronchoalveolar lavage fluid of patients with systemic sclerosis. Arthritis Rheum 40. (1997): 743-451.

Border WA and Noble NA. Transforming growth factor beta in tissue fibrosis. NEJM 331. (1994): 1286-1292.

Bou-Gharios G, Osman J, Atherton A, Monoghan P, Vancheeswaran R, Black CM, Olsen I. Expression of ectopeptidases in scleroderma. Ann Rheum Dis 54 (1995): 111-115.

Bradham DM, Igarashi A, Potter RL, Grotendorst GR. Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. J Cell Biol 114. (1991): 1285-1294

Briggs D, Black C, Welsh K. Genetic factors in Scleroderma. Rheum Dis Clin N Am 16. (1990): 31-51.

Briggs DC, Stephens C, Vaughan R, Welsh K, Black C. A molecular and serological analysis of the major histocompatibility complex and complement component C4 in systemic sclerosis. Arthritis Rheum 366. (1993): 943-954.

Briggs DC, Vaughan RW, Welsh KI, Myers A, duBois RM, Black CM. Immunogenetic prediction of pulmonary fibrosis in systemic sclerosis. Lancet 338. (1991): 661-662.

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 38. (1995): 1060-1067.

Buchsbaum ME, Kupper TS, Murphy GF. Differential induction of intercellular adhesion molecule-1 in human skin by recombinant cytokines. J Cutan Pathol 20. (1993): 21-27.

Cambrey AD, Harrison NK, Dawes KE, Southcott AM, Black CM, du Bois RM, Laurent GJ, McAnulty RJ. Increased levels of endothelin-1 in bronchoalveolar

lavage fluid from patients with systemic sclerosis contribute to fibroblast mitogenic activity *in vitro*. Am J Resp Cell Mol Biol 11. (1994): 439-445.

Campbell PM, LeRoy EC. Pathogenesis of systemic sclerosis: a vascular hypothesis. Semin Arthritis Rheum 4. (1975): 351-368.

Carpentier H, Maricq HR. Microvasculature in systemic sclerosis. Clin Exp Rheum 16. (1990): 75-92.

Carson CW, Beall LD, Hunder GG, Johnson CM, Newman W. Serum ELAM-1 is increased in vasculitis, scleroderma, and systemic lupus erythematosus. J Rheum 20. (1993): 809-814.

Carvalho D, Savage CO, Black CM. and Pearson JD. IgG antiendothelial cell autoantibodies from scleroderma patients induce leukocyte adhesion to human vascular endothelial cells in vitro. Induction of adhesion molecule expression and involvement of endothelium-derived cytokines. J Clin Invest 97. (1996): 111-119.

Casciola-Rosen L, Wigley F, Rosen A. Scleroderma autoantigens are uniquely fragmented by metal-catalyzed oxidation reactions: implications for pathogenesis. J Exp Med 185. (1997): 71-79.

Cepko CL, Roberts BE, Mulligan RC. Construction and applications of a highly transmissible murine retrovirus shuttle vector. Cell 37. (1984): 1053-1062.

Chanez P, Lacoste JY, Guillot B, Giron J, Michel FB, Bousquent J. Mast cells' contribution to the fibrosing alveolitis of the scleroderma lung. Am Rev Respir Dis 147. (1993): 1479-1502.

Chomczynski P and Sacchi N. Single step method method of RNA isolation by acid-guanidinium-phenol-chloroform extraction. Anal Biochem 162. (1987): 156-159.

Christner PJ, Peters J, Hawkins D, Siracusa LD, Jimenez SA. The tight skin 2 mouse: An animal model of scleroderma displaying cutaneous fibrosis and mononuclear cell infiltration Arthritis Rheum 38. (1995): 1791-1798.

Chu M-L, de Wet W, Bernard M, Ding JF, Marabito M, Myer J, Williams C, Ramirez F. Human pro-α(I) collagen gene structure reveals evolutionary conservation of a pattern of introns and exons. Nature 310. (1984): 337-340.

Claman, H.N. On scleroderma - mast cells, endothelial cells and fibroblasts. JAMA 262. (1989): 1206-1209.

Claman HN, Giorno RC, Seibold JR. Endothelial and fibroblastic activation in scleroderma. Arthritis Rheum 34. (1991): 1495-1501.

Clozel M, Breu V, Gray GA, Kalina B, Loffler B-M, Burri K, Cassal J-M, Hirth G, Muller M, Neidhart W, Ramuz H. Pharmacological characterisation of bosentan, a new potent orally active non-peptide endothelin receptor antagonist. J Pharmacol Exp Ther 270. (1994): 228-235.

Coker KC and Laurent GJ. Pathogenesis of pulmonary fibrosis: Implications for pharmacological intervention. In Walters EH, du Bois RM, eds. Immunology and Management of Interstitial lung diseases London: Chapman & Hall, 1995. pp 19-36.

Colige A, Sokolov BP, Nugent P, Baserga R, Prockop DJ. Use of an antisense oligonucleotide to inhibit expression of a mutated human procollagen gene (COL1A1) in transfected nouse 3T3 cells. Biochem 32. (1993): 7-11.

Dang H, Dauphinee MJ, Talal N. Serum antibody to retroviral gag proteins in systemic sclerosis. Arthritis Rheum 34. (1991): 1336-1337.

Dashwood MR, Sykes RM, Muddle JR. Autoradiographic localisation of [^{125}I] endothelin binding sites in human blood vessels and coronary tissue: functional correlates. J Cardiovasc Pharmacol 17. (1991): S458-S462.

Davis GE. Affinity of integrin for damaged extracellular matrix αvβ3 binds to denatured collagen type I through RGD sites. BBRC 182. (1992): 1025-1031.

DeCaprio J, Ludlow J, Figge J. Shew JY, Huang CM, Lee WH, Marsilio E, Paucha E, Livingston DM. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell 54. (1988): 275-283.

de Crombrugghe B, Vuorio T, Karsenty G. Control of type I collagen genes in scleroderma and normal fibroblasts. Rheum Dis Clin N Am 16. (1990): 109-123.

Degiannis D, Seibold J, Czarnecki M, Raskova J, Raska K. Soluble and cellular markers of immune activation in patients with systemic sclerosis. Clin Immunol Immunopathol 56. (1990): 259-270.

Deguchi Y, Negoro S, Kishimoto. Mutant fibronectin gene in skin fibroblasts of sclertic lesions from patients with progressive systemic sclerosis. Arthritis Rheum 32. (1989): 247-250.

Del Papa N, Guidali L, Sironi M, Shoenfeld Y, Mantovani A, Tincani A, Balestrieri G, Antonella R, Sinico RA and Meroni PL. Anti-endothelial cell IgG antibodies from patients with Wegener's granulomatosis bind to human endothelial cells in vitro and induce adhesion molecule expression and cytokine secretion. Arthritis Rheum 39. (1996): 758-766.

Denton CP, Bickerstaff MCM, Shi-Wen X, Carulli MT, Haskard DO, Du bois RM, Black CM. Serial circulating adhesion molecule levels reflect disease severity in systemic sclerosis. Br J Rheum 34. (1995): 1048-1054.

Denton CP, Shi-Wen X, Welsh KI, Pearson JD, Black CM Scleroderma fibroblasts phenotype is modulated by endothelial cell co-culture. J Rheum 23. (1996): 633-638.

Denton CP, Shi-Wen X, Pearson JD, Black CM: Scleroderma fibroblast show increased responsiveness to endothelial cell derived Interleukin 1 and basic fibroblast growth factor. J Invest Dermatol 108. (1997): 269-274.

Du Bois RM, P.G. H, Hemingway I, Gearing AJ. Soluble cell adhesion molecules ICAM-1, ELAM-1 and VCAM-1 are present in epithelial lining fluid in patients with interstitial lung disease. Am Rev Resp Dis 145. (1992): A190.

Dzamba BJ, Wu H, Jaenisch R, Peters DM. Fibronectin binding site in type I collagen regulates fibronectin fibril formation. J Cell Biol 121. (1993): 1165-1172.

Diamond MS, Staunton DE, de Fougerolles AR, Stacker SA, Springer TA. ICAM-1 (CD54): a counter-receptor for MAC-1. J Cell Biol 111. (1990): 3129

Eckes B, Mauch C, Huppe G, Kreig T. Downregulation of collagen synthesis in fibroblasts within three dimensional collagen lattices involves transcriptional and post-transcriptional mechanisms. FEBS Lett 318. (1993): 129-133.

Eckes B, Mauch C, Huppe G, Kreig T: Differential regulation of transcription and transcript stability of pro-α1(I) collagen and fibronection in activated fibroblasts derived from patients with systemic scleroderma. Biochem J 315. (1996): 549-554.

Edwin WA, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, Lawley TJ. HMEC-1: Establishment of an immortalized human microvascular endothelial cell line. J Invest Dermatol 99. (1992): 683-690.

Ehrenreich H, Anderson RW, Fox CH. Endothelins, peptides with potent vasoactive properties are produced by human macrophages. J Exp Med 172. (1990): 1741-1748.

Elferink JGR and de Koster BM. Endothelian-induced activation of neutrophil migration. Biochem Pharmacol 48. (1994): 865-871.

Fagundus DM and LeRoy EC. Cytokines in systemic sclerosis Clin in Dermatol 12. (1995): 407-418.

Fanning GC, Bunce M, Black CM, Wilsh KI. Polymerase chain reaction haplotyping using 3′ mismatches in the forward and reverse primers: application to the biallelic polymorphisms of tumor necrosis factor and lymphotoxin a. Tissue Antigens 50. (1997): 23-31.

Feghali CA, Boulware DW, Ferriss JA and Levy LS. Expression of c-myc, c-myb, and c-sis in fibroblasts from affected and unaffected skin of patients with systemic sclerosis. Autoimmun 16. (1993): 167-171.

Feldmen SR, Trojanowska M, Smith EA, LeRoy EC. Differential responses of human papillary and reticular fibroblasts to growth factors. Am J Med Sci 305. (1994): 203-207.

Fickling SA, Tooze JA and Whitely G. Characterisation of human umbilical vein endothelial cell lines produced by transfection with the early region of SV40. Exp Cell Res 201. (1992): 517-521.

Fingerman E and Helmer ME. Regulation of proteins in the VLA cell substrate adhesion family: Influence of cell growth conditions on VLA-1, VLA-2 and VLA-3 expression. Exp Cell Res 177. (1988): 132-142.

Flanders KC, Thompson NL, Cissel DS, Van Obberghen E, Baker CC, Kass ME, Ellingsworth LR, Roberts AB, Spron MB. Transforming growth factor beta-1: histochemical localization with antibodies to different epitopes. J Cell Biol 108. (1989): 653-660.

Flaumenhaft R, Kojima S, Abe M, Rifkin DB. Activation of latent transforming growth factor beta. Adv Pharmacol 24. (1993): 51-76.

Fleishmajer R, Perlish JS. Capillary alterations in scleroderma. J Am Acad Dermatol 113. (1980): 1661-1666.

Fleishmajer R, Perlish JS, Reeves JRT. Cellular infiltrates in scleroderma skin. Arthritis Rheum 20. (1977): 975-984.

Frazier K and Grotendorst GR. Expression of connective tissue growth factor mRNA in the fibrous stroma of mammary tumors. Int J Biochem Cell Biol 29. (1997): 153-161.

Frazier K, Williams S, Kothapalli D, Klapper H, Grotendorst GR. Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. J Invest Dermol 107. (1996): 404-411

Fries KM, Blieden T, Looney RJ, Sempowsk GD, Slivera MR, Willis RA, Phipps RP. Evidence of fibroblast heterogeneity and the role of fibroblast subpopulations in fibrosis. Clin Immunol Immunopathol 72. (1994): 283-292.

Fritzler MJ, Kinsella TD, Garbutt E. The CREST syndrome: A distinct serologic entity with anti-centromere antibodies. Am J Med 69. (1980): 520-526.

Gay S, Jones RE and Huang G, Gay RE. Immunohistologic demonstration of platelet-derived growth factor and *sis*-oncogene expression in scleroderma. J Invest Dermatol 92. (1989): 301-303.

Gay S, Trabandt A, Moreland LW and Gay RE. Growth factors, extracellular matrix, and oncogenes in scleroderma. Arthritis Rheum 35. (1992): 304-310.

Gearing JH and Newman W. Circulating adhesion molecules in disease [Review]. Immunol Tod 14. (1993): 506-512.

Gershwin ME, Abplanalp HA, Castles JJ, Ikeda RM, Van der Water J, Eklund J, Haynes D. Characterisation of a spontaenous disease of white Leghorn chickens resembling progressive systemic sclerosis (scleroderma). J Exp Med 153. (1981): 1640-1659.

Gillery P, Maquart F-X, Le Corre Y, Kalis B, Borel J-P. Variability in the retraction of collagen lattices by scleroderma fibroblasts - relationship to protein synthesis and clinical data. Clin Exp Derm 16. (1991): 324-330.

Giltay EJ, Moens HJB, Riley AH, Tan RG. Silicone breast prostheses and rheumatic symptoms: a retrospective follow up study. Ann Rheum Dis 53. (1994): 194-196.

Goetz RH. Pathology of progressive systemic sclerosis (generalised scleroderma) with special reference to changes in the viscera. Clin Proc 4. (1945): 337-360.

Gonzalez-Amaro R, Alarcon-Segovia D, Alcocer-Varela J, Rosenstein Y. Mononuclear cell-fibroblast interactions in scleroderma. Clin Immunol Immunopathol 46. (1988): 412-420.

Gorman CM, Moffat LF, Howard B. Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. Mol Cell Biol 2. (1982): 1044-1051.

Green MC, Sweet HO, Bunker LE. Tight-skin, a new mutation of the mouse causing excessive growth of connective tissue and skeleton. Am J Pathol 82. (1976): 493-512.

Grotendorst GR, Okochi H, Hayashi N. A novel transforming growth factor β response element controls the expression of the connective tissue growth factor gene. Cell Growth Differ 7. (1996): 469-480

Gruschwitz M, Driesch P, Kellner I, Hornstein OP, Sterry W. Expression of adhesion proteins involved in cell-cell and cell-matrix interactions in the skin of patients with progressive systemic sclerosis. J Am Acad Dermatol 27. (1992): 169-177.

Gruschwitz MS, Hornstein OP, von den Driesch P. Correlation of soluble adhesion molecules in the peripheral blood of scleroderma patients with their in situ expression and with disease activity. Arthritis Rheum 38. (1995): 184-189.

Guarda E, Myers PR, Brilla CG, Tyagi SC, Weber KT. Endothelial cell induced modulation of cardiac fibroblast collagen metabolism. Cardiovasc Res 27. (1993): 1004-1008.

Guarda E, Katwa LC, Myers PR, Tyagi SC, Weber KT. Effects of endothelins on collagen turnover in cardiac fibroblasts. Cardiovasc Res 27. (1993): 2130-2134.

Gullberg D, Gehlsen KR, Turner DC, Ahlen K, Zijenah LS, Barnes MJ, Rubin K. Analysis of alpha 1 beta 1, alpha 2 beta 1 and alpha 3 beta 1 intergrins in cell-collagen interactions: identification of conformation dependent alpha 1 beta 1 binding sites in collagen type I. EMBO J 11. (1992): 3865-3873.

Halim A, Kanayama E, Maradny E, Maehara K, Terao T. Activated neutrophil by endothelin-1 caused tissue damage in human umbilical cord. Thromb Res 77. (1995): 321-327.

Harris P and Ralph P. Human leukaemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. J Leucocyte Biol 37. (1985): 407-422.

Harrison NK, Myers AR, Corrin B, Soosay G, Dewar A, Black CM, duBois RM, Turner-Warwick M. Structural features of interstitial lung disease in systemic sclerosis. Am Rev Resp Dis 144. (1991): 706-713.

Harrison NK, Cambrey AD, Myers A, Southcott AM, Black CM, Du Bois RM, Laurent GJ, McAnulty RJ. Insulin-like growth factor-1 is partially responsible for fibroblast proliferation induced by bronchoalveolar lavage fluid from patients with systemic sclerosis. Clin Sci 86. (1994): 141-148.

Hautala T, Byers MG, Eddy RL, Shows TB, Kivirikko KI, Myllyla R. Cloning of human lysine hydroxylase: complete cDNA-derived amino acid sequence and assignment of the gene (PLOD) to chromosome 1p36.3-p36.2. Genomics 13. (1992): 62-69.

Hautala T, Heikkinen J, Kivirikko KI, Myllyla R. Minoxidil specifically decreases the expression of lysine hydroxylase in cultured human skin fibroblasts. Biochem J 283. (1992): 51-54.

Hawkins RA, Clamon HN, Clark RAF, Steigerwald JC. Increased dermal mast cell population in progressive systemic sclerosis: A link to fibrosis? Ann Intern Med 102. (1985): 182-186.

Hayasaki Y, Nakajima M, Kitano Y, Iwasaki T, Shimamura T, Iwaki K. ICAM-1 expression on cardiac myocytes and aortic endothelial cells via their specific endothelin receptor subtype. BBRC 229. (1996): 817-824.

Haynes WG and Webb DJ. The endothelin family of peptides: local hormones with diverse roles in health and disease? Clin Sci 84. (1993): 485-500.

Helaakoski T, Vuori K, Myllyla R, Kivirikko KI, Pihlajaniemi T. Molecular cloning of the alpha-subunit of human prolyl 4-hydroxylase: the complete cDNA-derived amino acid sequence and evidence for alternative splicing of RNA transcripts. Proc Natl Acad Sci USA 86. (1989): 4392-4396.

Heldin CH and Westermark B. Platelet-derived growth factor: mechanism of action and possible in vivo function. Cell Regul 1. (1990): 555-66.

Hernandez-Rodriguez NA, Cambrey AD, Harrison NK, Chambers RC, Gray AJ, Southcott AM, du Bois RM, Black CM, Scully MF, McNulty RJ, Laurent GJ. Role of thrombin in pulmonary fibrosis. Lancet 346. (1995): 1071-1072.

Herrick AL, Rieley F, Schofield D, Hollis S, Braganza JM, Jayson MIV. Micronutrient antioxidant status in patients with primary raynaud's phenomenon and systemic sclerosis. J Rheum 21. (1994): 1477-1483.

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 37. (1994): 278-288.

Hitraya EG, Jimenez SA. Transcriptional activation of the α1(I) procollagen gene in systemic sclerosis dermal fibroblasts. Arthritis Rheum 39. (1996): 1347-1354.

Hochberg UF, White B, Steen V, Medsger TA, Weisman M, Wigley FM. The association of augmentation mammoplasty with systemic sclerosis: preliminary results from a case-control study. Arthritis Rheum 36(Suppl). (1993): S71.

Holt CM, Lindsey N, Moult J, Malia RG, Greaves M, Hume A, Rowell NR. Antibody-dependent cellular cytotoxicity of vascular endothelium. Clin Exp Immunol 78. (1989): 359-365.

Housset E, Emerit I, Daulon A. Anomalies chromosomiques dans la sclerodermie generalisee. Etudes de 10 malades. Cr Hebd Seanc Acad Sci (Paris) 1969; 269-274.

Hulmes DJ. The collagen superfamily-diverse structures and assemblies (Review). Essays Biol 27. (1992): 49-67.

Hutchinson J. Congenital defects and inherited proclivitis. Arch Surg 4. (1893): 305-309.

Hynes RO. Integrins: versatility, modulation and signalling in cell adhesion. Cell 69. (1992): 11-25.

Ichihashi H, Shinkai H, Takei M, Sano S. Analysis of the mechanism of bleomycin-induced cutaneous fibrosis in mice. J Antibiot (Tokyo) 26. (1973): 238.

Igarashi A, Nashiro K, Kikuchi K, Sato S, Ihn H, Grotendorst GR, Takehara K. Significant correlation between connective tissue growth factor gene expression

and skin sclerosis in tissue sections from patients with systemic sclerosis. J Invest Dermatol 105. (1995): 280-284

Igarashi A, Nashiro K, Kikuchi K, Sato S, Ihn H, Fujimoto M, Grotendorst GR, Takehara K. Connective tissue growth factor gene expression in tissue sections from localized scleroderma, Keloid, and other fibrotic skin disorders. J Invest Dermatol 106. (1996): 729-733

Igarashi A, Okochi K, Bradham DM, Grotendorst GR. Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. Mol Biol Cell 4. (1993): 637-645.

Inagaki Y, Truter S, Ramirez F. Transforming growth factor β stimulates α2(I) collagen gene expression through a cis-acting element that contains an Sp1-binding site. J Biol Chem 269. (1994): 14828-14832.

Ivarsson M, McWhirter A, Black CM, Rubin K. Impaired regulation of collagen pro-a1(I) mRNA and change in pattern of collagen-binding integrins on scleroderma fibroblasts. J Invest Dermatol 101. (1993): 216-221.

Jaffe EA. Cell biology of endothelial cells. Hum Pathol 18. (1987): 234-239.

Jaffee BD and Claman HN. Chronic gaft-host disease as a model for scleroderma. Cell Immunol 77. (1983): 1-12.

Jat PS, Cepko CL, Mulligan RC, and Sharp PA. Recombinant retroviruses encoding simian virus 40 large T antigen and polyomavirus large and middle T antigens. Mol Cell Biol 6. (1986): 1204-1217.

Jat PS and Sharp PA. Cell lines established by a temperature-sensitive simian virus 40 large-T-antigen gene are growth restricted at the nonpermissive temperature. Mol Cell Biol 9. (1989): 1672-1681.

Jimenez SA, Diaz A, Khalili K. Retrovirses and the pathogenesis of systemic sclerosis. Inter Rev Immunol 12. (1995): 159-176.

Jimenez SA, Feldman C, Bashey RI. Co-ordinate increase in the expression of type I and III collagen genes in progressive systemic sclerosis fibroblasts. Biochem J 237. (1986): 837-843.

Jimenez SA, Millan A, Bashey RI. Scleroderma-like alterations in collagen metabolism occurring in the TSK (tight skin) mouse. Arthritis Rheum 27. (1984): 180-185.

Jimenez SA, Varga J, Olson A. Functional analysis of human α1(I) procollagen gene promoter. J Biol Chem 269. (1994): 12684

Jonhnson RL and Ziff M. Lymphokine stimulation of collagen accumulation. J Clin Invest 78. (1976): 240-246.

Jung TM and Dailey MO. Rapid modulation of homing receptors (p90 MEL-14) induced by activators of protein kinase C; receptor shedding due to accelerated proteolytic cleavage at the cell surface. J Immunol 144. (1990): 3130-3136.

Kadler KE, Hojima Y, Prockop DJ. Assembly of collagen fibrils de novo by cleavage of the type I pC-collagen with procollagen C-proteinase. J Biol Chem 262. (1987): 15696-16701.

Kahaleh MB. Endothelin, an endothelial-dependant vasoconstrictor in scleroderma. Arthritis Rheum 34. (1991): 978-983.

Kahaleh MB: Vascular disease in scleroderma. Rheum Dis Clin N Am 16. (1990): 53-75.

Kahaleh MB and Fan PS. Cytotoxic T cell involvement in scleroderma: Detection of granzyme-a gene expression in systemic sclerosis skin. Arthritis Rheum 35. (1992): S22.

Kahaleh MB, Sultany GL, Smith EA, Huffstutter JE, Loadholt CB, LeRoy EC. A modified scleroderma skin scoring method. Clin Exp Rheuma 4. (1986): 367-369.

Kahaleh MB, Yin T. The molecular mechanism of endothelial cell (EC) injury in scleroderma (SSc). Identification of granzyme 1 (a product of cytolytic T cells in SSc sera (Abstract). Arthritis Rheum 33 (Suppl). (1990): 67.

Kahari VM, Sondberg M, Kalimo H, Vuorio T, Vuorio E. Identification of fibroblasts responsible for increased collagen production in localised scleroderma by in situ hybridisation. J Invest Dermatol 90. (1988): 664-670.

Kallenberg CGM. Early detection of connective tissue disease in patients with Raynaud's phenomenon. Rheum Dis Clin N Amer 16. (1990): 11-31.

Katayama K, Armendariz-Borunda J, Raghow R, Kang AH, Seyer JM. A pentapeptide from type I procollagen promoters extracellular matrix production. J Biol Chem 268. (1993): 9941-9944.

Katwa LC, Guarda E, Weber KT. Endothelin receptors in cultured adult rat cardiac fibroblasts. Cardiovasc Res 27. (1993): 2125-2129.

Kawaguchi Y, Suzuki K, Hara M, Hidaka T, Ishizuka T, Kawagoe M, Nakamura H. Increased endothelin-1 production in fibroblasts derived from patients with systemic sclerosis. Ann Rheum Dis 53. (1994): 506-510.

Kehrl JH, Wakefield LM, Roberts AB, Jakowelew S, Alvarez-Mon M, Derynck R, Sporn MB, Fauci AS. Production of transforming growth factor-β by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med 163. (1986): 1037-1050.

Kenny AJ, O'Hare MJ, and Gusterson BA. Cell-surface peptidases as modulators of growth and differentiation. Lancet 2. (1989): 785-787.

Kerr LD, Miller DB, Matrisian LM. TGFβ1 inhibition of transin/stromelysin gene expression is mediated through a Fos binding sequence. Cell 61. (1990): 267-278.

Kerr MA and Kenny AJ. The purification and specificity of a neutral endopeptidase from rabbit kidney brush border. Biochem J 137. (1974): 477-488.

Khillan JS, Li SW, Prockop DJ. Partial rescue of a lethal phenotype of fragile bones in transgeneic mice with a chimeric antisense gene directed against a mutated collagen gene. Proc Natl Acad Sci USA 91. (1994): 6298-6302.

Kikuchi K, Hartl CW, Smith EA, LeRoy EC, Trojanowska M: Direct demonstration of transcriptional activation of collagen gene expression in systemic sclerosis fibroblasts: insensitivity to TGF-β1 stimulation. BBRC 187. (1992): 45-50.

Kikuchi K, Kadono T, Ihn H, Sato S, Igarashi A, Nakagawa H, Tamaki K, Takehara K. Growth regulation in scleroderma fibroblasts: Increased response to transforming growth factor-β1. J Invest Dermatol 105. (1995): 280-284.

Kikuchi K, Kubo M, Sato S, Fujimoto M and Tamaki K. Serum tissue inhibitor of metalloproteinases in patients with systemic sclerosis. J Am Acad Dermatol 33. (1995): 973-978.

Kikuchi K, Kadono T, Sato S, Tamaki K, and Takehara K. Imparied growth response to endothelin-1 in scleroderma fibroblasts. BBRC 207. (1995): 829-838.

Kireeva ML, Latinkic BV, Kolesnikova TV, Chen CC, Yang GP, Abler AS, Lau LF. Cry61 and Fisp12 are both ECM-associated signaling molecules: activities, metabolism, and localization during development. Exp Cell Res 233. (1997): 63-77.

Kirk TZ, Mark ME, Chua CC, Chua BH and Mayes MD. Myofibroblasts from scleroderma skin synthesize elevated levels of collagen and tissue inhibitor of metalloproteinase (TIMP-1) with two forms of TIMP-1. J Biol Chem 270. (1995): 3423-3428.

Kivirikko KI. Collagens and their abnormalities in a wide spectrum of diseases (Review). Ann Med 25. (1993): 113-126.

Kivirikko KI, Helaakoski T, Tasanen K, Vuori K, Myllyla R, Pihlajaniemi T. Molecular biology of prolyl 4-hydroxylase. Ann New York Acad Sci 580. (1990): 132-142.

Kivirikko KI, Myllyla R, Pihlajaniemi T. Protein hydroxylation: prolyl 4-hydroxylase, an enzyme with four cosubstrates and a multifunctional subunit. FASEB J 3. (1989): 1609-1617.

Klareskog L, Gustafsson R, Scheynius A, Hällgren R. Increased expression of platelet-derived growth factor type B receptors in the skin of patients with systemic sclerosis. Arthritis Rheum 33. (1990): 1534-1541.

Knock GA, Terenghi G, Bunker CB, Bull HA, Dowd PM, Polak JM. Characterisation of endothelin-binding sites in human skin and their regulation in primary Raynaud's phenomenon and systemic sclerosis. J Invest Dermatol 101. (1993): 73-78.

Korn JH, Thrall RS, Wilbur DC, Kream BE, Piela-Smith TH. Fibroblast heterogeneity: clonal selection of fibroblasts as a model for fibrotic disease. In Fibroblast lung heterogeneity ed. R. Phipps. Boca Raton: CRC Press, 1992. pp119-133.

Kothapalli D, Frazier KS, Welply A, Segarini PR, Grotendorst GR. Transforming growth factor-β induces anchorage-independent growth of NRK fibroblasts via a connective tissue growth factor-dependent signaling pathway. Cell Growth Differ 8. (1997): 61-68.

Kourembana S, Marsden PA, McQuillan LP, Faller DV. Hypoxia induced endothelin gene expression and secretion in cultured human endothelium. J Clin Invest 88. (1991): 1054-1057.

Kraling BM, Maul GG, Jimenez SA. Mononuclear cellular infiltrates in clinically involved skin from patients with systemic sclerosis of recent onset predominantly consist of monocytes/macrophages. Pathobiology 63. (1995): 48-56.

Krieg TH, Hein R, Hatamochi A, Auxmailley M. Molecular and clinical aspects of connective tissue (Review). Eur J Clin Invest 18. (1988): 105-123.

Kupper TS and Ferguson T. A potential role for α2β1 integrin in human eye disease involving vitroretinal traction. FASBE J 7. (1993): 1402-1405.

Laiho M, Saksela O, Andreasen PA, Keski-Oja J. Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-β. J Cell Biol 103. (1986): 2403-2410.

Langholz O, Rockel D, Mauch C, Kozlowska E, Bank I, Kreig T, Eckes B. Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by a1b1 and a2b1 integrins. J Cell Biol 131. (1995): 1903-1915.

Laptev AV, Lu Z, Colige A, Prockop DJ. Specific inhibition of expression of a human procollagen gene (COL1A1) with modified antisense oligonucleotides. Biochem 33. (1994): 11033-11039.

Laurent GJ, McAnulty RJ, Corrin B, Cockerill P. Biochemical and histological changes in pulmonary fibrosis induced in rabbits with intratracheal bleomycin. Eur J Clin Invest 11. (1981): 441-418.

Lee ME, Block KD, Clifford JD, Quetermous T. Functional analysis of the endothelin-1 promoter: evidence for endothelial cell-specific cis-acting sequence. J Biol Chem 265. (1990): 10446-10450.

LeRoy EC. Increased collagen synthesis by scleroderma skin fibroblasts *in vitro*. J Clin Invest 54. (1974): 880-889.

LeRoy EC. Systemic sclerosis a vascular perspective. Rheum Dis Clin N Amer 22. (1996): 675-694.

LeRoy EC, Black CM, Fleishmajer R, Jablonska S, Krieg T, Medsger T, Rowell N, Wollheim F. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheum 15. (1988): 202-205.

LeRoy EC, Smith EA, Kahaleh MB, Trojanowska M, Silver RM. A strategy for determining the pathogenesis of systemic sclerosis. Is transforming growth factor beta the answer? Arthritis Rheum 32. (1989): 817-825.

Letarte M, Vera S, Tran R, Addis JBL, Onizuka RJ, Quackenbush EJ, Jongeneel CV, McInnes RR. Common acute lymphocytic leukemia antigen is identical to neutral endopeptidase. J Exp Med 168. (1988): 1247-1253.

Levin ER. Endothelins [Review] NEJM 333. (1995): 356-363.

Linder, S. and Marshall, H. Immortalization of primary cells by DNA tumor virus. Exp Cell Res 191, (1990): 1-7.

Livingstone JZ, Scott TE, Wigley FM, Anhalt GJ, Bias WB, McLean RH, Hochberg MC. System sclerosis (scleroderma): Clinical, genetic and serological subsets. J Rheumatol 14. (1987): 512-518.

Lovell CR, Nicholls AC, Duance VC, Bailey AJ. Characterisation of dermal collagen in systemic sclerosis. Br J Dermatol 100. (1979): 359-369.

Ludwicka A, Trojanowska M, Smith EA, Baumann M, Strange C, Korn JH, Smith T, LeRoy EC, Silver RM. Growth and characteristics of fibroblasts obtained from bronchoalveolar lavage of patients with scleroderma. J Rheum 19. (1992): 1716-1723.

Lund LR, Riccio A, Andreasen PA, Nielsen LS, Kristensen P, Laiho M, Saksela O, Blasi F, Dano K. Transforming growth factor-β is a strong and fast acting positive regulator of the level of type-1 plasminogen activator-inhibitor mRNA in WI-38 human lung fibroblasts. EMBO J 6. (1987): 1281-1286.

McCabe RB, Kersey JH, Vendersteen PJ, Vosika GJ. Reverse transcriptase in a patient with scleroderma. Biochem Med 21. (1979): 47-54.

McCarron RM, Wang L, Stanimirovic DB, and Spatz M. Endothelin induction of adhesion moecule expression on human brain microvascular endothelial cells. Neuroscience Lett 156. (1993): 31-34.

McColl G. and Buchanan RRC. Unilateral scleroderma following ischaemic hand injury. J Rheum 21. (1996): 380-381.

McMillen MA, and Sumpio BE. Endothelians: polyfunctional cytokines. J Am Coll Surg 180. (1995): 621-637.

Maddison PJ, Stephens C, Briggs D, Welsh KI, Harvey G, Whyte J, McHugh N, Black CM, Group tUSS. Connective tissue disease and autoantibodies in the kindred of 63 patients with systemic sclerosis. Medicine 72. (1993): 103-112.

Martin ER, Brenner BM, Ballermann BJ. Heterogeneity of cell surface endothelin receptors. J Biol Chem 265. (1990): 14044-14049.

Martinerie C, Viegas-Pequignot E, Guenard I, Dutrillaux B, Nguyen VC, Bernheim A, Perbal B. Physical mapping of human loci homologous to the chicken *nov* proto-oncogene. Oncogene 7. (1992): 2529-2534.

Mason JC, Kapahi P, Haskard DO. Detection of increased levels of circulating intracellular adhesion molecule 1 in some patients with rheumatoid arthritis but not in patients with systemic lupus erthematosus: Lack of correlation with levels of circulating vascular cell adhesion molecule 1. Arthritis Rheum 36. (1993): 519-527.

Matsui S. Ober die Pathologie und Pathogenese von Sklerodermia Universalis. Mitteilungen Medizinischen Fakultat Universitat Tokyo 31. (1924): 55-116.

Mauch C, Eckes B, Hunzelmann N, Oono T, Kozlowska E, Krieg T. Control of fibrosis in systemic scleroderma. J Invest Dermatol 100. (1993): 92S-96S.

Mauch C, Kozlowska E, Eckes B, Krieg T. Altered regulation of collagen metabolism in scleroderma fibroblasts grown within three dimensional collagen gels. Exp Dermatol 1. (1992): 185-190.

Medsger TA. Epidemiology of systemic sclerosis. Clinics in Dermatol 12. (1994): 207-216.

Miller AD and Buttimore C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol Cell Biol 6. (1986): 2895-2902.

Mojock CF and Shevach EM. Adhesion molecules: a rheumatologic perspective. Arthritis Rheum 40. (1997): 991-1004.

Mountz JD, Downs Minor MB, Turner R, Thomas MB, Richards F, Pisko E. Bleomycin induced cutaneous toxicity in the rat: analysis of histopathology and ultrastructure compared with progressive systemic sclerosis (scleroderma). Br J Dermatol 108. (1983): 679-683.

Murad S, Tennant MC, Pinnell SR. Structure-activity relationship of minoxidil analogs as inhibitors of lysyl hydroxylase in cultured fibroblasts. Arch Biochem Biophys 292. (1992); 234-238.

Murphy G and Reynolds J. Extracellular matrix degradation. Connective Tissue and Its Heritable Disorders Wiley-Liss, Inc. 1993 p287-316.

Murrel DF. A radical proposal for the pathogenesis of scleroderma. J Am Acad Dermatol 28. (1993): 78-85.

Myers CL, Wertheimer SJ, Schembri-King J, Parks T, Wallace RW. Induction of ICAM-1 by TNF-α, IL-1β and LPS in human endothelial cells after downregulation of PKC. Am J Physiol 263. (1992): C767.

Nakai A, Satoh M, Hirayashi K, Nagata K. Involvement of the stress protein HSP47 in procollagen processing in the endoplasmic reticulum. J Cell Biol 117. (1992): 903-914.

Narayanan AS, Whithey J, Souza A, Raghu G. Effect of gamma-interferon on collagen synthesis by normal and fibrotic human lung fibroblasts. Chest 101. (1992): 1326-1331.

Needleman B. Increased expression of intercellular adhesion molecule 1 on the fibroblasts of scleroderma patients. Arthritis Rheum 33. (1990): 1847-1851.

Needleman B, Orodonez J, Taramelli D, Alms W, Gayer K, Choi J. *In vitro* identification of a subpopulation of fibroblasts that produces high levels of collagen in scleroderma patients. Arthritis Rheum 33. (1990): 842-852.

Needleman BW, Wigley FM, Stair RW. Interleukin-1, Interleukin-2, Interleukin-4, Interleukin-6, Tumor Necrosis Factor-a and Interferon gamma levels in sera of patients with systemic sclerosis. Arthritis Rheum 35. (1992): 67-72.

Neilson E, Phillips S, Jimenez S. Lymphokine modulation of fibroblast proliferation. J Immunol 128. (1982): 1484-1492

Nietert p, Sutherland S, Silver R, Pandey J, Dosemeci M. Occupational solvent exposure: a risk factor for systemic sclerosis. Arthritis Rheum 39. (1996): S318.

Nusgens B, Merrill C, Lapiere C, Bell E. Collagen biosynthesis by cells in a tissue equivalent matrix *in vitro*. Coll Rel Res 4. (1984): 351-364.

Oemar BS and Luscher TF. Connective tissue growth factor: friend or for? Arterioscler Thromb Vasc Biol 17. (1997): 1483-1489.

Oemar BS, Wener A, Garnier JM, Godoy N, Nauck M, Marz W, Rupp J, Pech M, Luscher FL. Human connective tissue growth factor is expressed in advanced atherosclerotic lesions. Circulation 95. (1997): 831-839.

Ong ACM, Jowett TP, Firth JD, Burton S, Karet FE, and Fine LG. An endothelin-1 mediated autocrine growth loop involved in human renal regeneration. Kidney International 48. (1995): 390-401.

O'Reilly G, Charnock-Jones DS, Davenport AP, Cameron IT, SmithSK. Presence of messenger ribonucleic acid for endothelin-1, endothelin-2 and endothelin-3 in human endometrium and a change in the ratio of ET-A and ET-B receptor subtype across the menstrual cycle. J Clin Endocrin Metab 75. (1992): 1545-1549.

Pablos JL, Everett ET, Harley R, LeRoy EC and Norris JS. Transforming growth factor-β1and collagen gene expression during postnatal skin development and fibrosis in the tight-skin mouse. Lab Invest 72. (1995): 670-678.

Pearson JD. The endothelium: its role in scleroderma. Ann Rheum Dis 50. (1991): 866-871.

Pearson JD. Markers of endothelial perturbation and damage. Br J Rheum 32. (1993): 651-652.

Peltonen L, Halila R, Ryhanen L. Enzymes converting procollagens to collagens. J Cell Biochem 28. (1985): 15-21.

Perez MI and Kohn SR. Systemic sclerosis. J Am Acad Dermatol 28. (1993): 525-547.

Peters I and Ball ST. Tight skin-2 (TSK-2). Mouse News Lett 74. (1986): 91-97.

Piccinini G, Luchetti MM, Caniglia LM, Carossino AM, Montroni M, Introna M, Gabrielli A. c-myb proto-oncogene is expressed by quiecent scleroderma fibroblasts and, unlike b-myb gene, does not correlate with proliferation. J Invest Dermatol 106. (1996): 1281-1286.

Picker LJ, Kishimoto TK, Smith CW, Warnock RA, Butcher EC. ELAM-1 is an adhesion molecule for skin-homing T cells. Nature 349. (1991): 796-799.

Piela TH, Korn JH. ICAM-1-dependent fibroblast-lymphocyte adhesion: discordance between surface expression and function of ICAM-1. Cell Immunol 129. (1990): 125-137.

Piela TH, Korn JH. Lymphocyte-fibroblast adhesion induced by inteferon-γ. Cell Immunol 114. (1988): 149-160.

Pober JS, Bevilacqua MP, Mendrick DL, Lapiere LA, Fiers W and Gimbrone MA. Two distinct monokines IL-1 and TNF each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. J Immunol 136. (1986): 1680-1687.

Pober JS, Gimbrone MA, Cotran RS, Reiss CS, Burakoff SJ, Fiers W, Ault KA. Ia expression by vascular endothelium is inducible by activated T cells and by human gamma interferon. J Exp Med 157. (1983): 1339-1353.

Pollock DM, Keith TL, Highsmith RF. Endothelin receptors and calcium signaling [review]. FASEB J 9. (1995): 1196-1204.

Postlethwaite AE, Seyer JM, Kang AH. Chemotactic attraction of human fibroblasts to type I and III collagen and collagen-derived peptides. Proc Natl Acad Sci USA 75. (1978): 871-874.

Postlethwaite A, Synderman R, Kang A. The chemotactic attraction of human fibroblasts to a lymphocyte-derived factor. J Exp Med 144. (1976): 1188-1203.

Prescott RJ, Freemont AJ, Jones CJP, Hoyland J, Fielding P. Sequential dermal microvascular and perivascular changes in the development of scleroderma. J Pathol 166. (1992): 255-263.

Prockop DJ and Hulmes DJS. (1994) In Extracellular matrix assembly and structure, eds. Yurchenco PD, Birk DE, Mecham RP. San Diego, Academic, pp47-90.

Prockop DJ and Kivirikko KI. Collagens: Moleculer biology, diseases, and potentials for therapy. Annu Rev Biochem 64. (1995): 403-434.

Prockop DJ and Kivirikko KI. Heritable diseases of collagen (Review). NEJM 311. (1984): 376-386.

Query CC and Keene JD. A human autoimmune protein associated with U1 RNA contains a region of homology that is crossreactive with retroviral p30gag antigen. Cell 51. (1988): 211-214.

Raghu G, Masta S, Meyers D, Narayanan S. Collagen synthesis by normal and fibrotic human lung fibroblasts and the effect of TGFβ 1-3. Am Rev Resp Dis 140. (1989): 95-100.

Rannard SI, Hunninghake GW, Bitterman PB, Crystal RG. Production of fibronectin by the human alverolar macrophage: mechanism for the recruitment of fibroblasts to sites of tissue injury in interstitial lung diseases. Proc Natl Acad Sci USA 78. (1981): 7147-7151.

Reichlin M, Maddison PJ, Targoff IN, Bunch T, Arnett F, Sharp G, Treadwell E, Tan EM. Antibodies to a nuclear/nucleolar antigen in patients with polymyositis overlap syndromes. J Clin Immunol 4. (1984): 40-44.

Reimer G, Steen VD, Penning CA, Medsger TA, Tan EM. Correlates between autoantibodies to nuclear antigens and clinical features in patients with systemic sclerosis. Arthritis Rheum 31. (1988): 525-532.

Rennard SI, Berg R, Martin GR, Foidart JM, Gehron Robey P. Enzyme linked immunoassays for connective tissue components. Anal Biochem 104. (1980): 205-214.

Reynolds EE, Hwang O, Flynn MA, Welch KM, Cody WL, Steinbaugh B, He JX, Chung FZ, Doherty AM. Pharmacological differences between rat and human endothelin B receptors. BBRC 209. (1995): 506-512.

Reynolds EE, Kerser JA, Haleen SJ, Walker DM, Olszewski B, Schroeder RL, Taylor DG, Hwang O, Welch KM, Flynn MA, Thompson DM, Edmunds JJ, Berryman KA, Plummer M, Cheng X, Patt WC, Doherty AM. Pharmacological characterization of PD 156707, an orally active ET_A receptor antagonist. J Pharmacol Exp Ther 273. (1995): 1410-1417.

Romanic AM, Adachi E, Hojima Y, Engel J, Prockop DJ. Polymerization of pNcollagen I and copolymerization of pNcollagen I with collagen I. A kinetic, thermodynamic, and morphologic study. J Biol Chem 267. (1992): 22265-22271.

Rosenbaum J, Pottinger BE, Woo P, Black CM, Loizou S, Byron MA.and Pearson JD. Measurement and characterisation of circulating anti-endothelial cell IgG in connective tissue diseases. Clin Exp Immunol 72. (1988): 450-456.

Rossi GA, Bitterman PB, Rennard SI. Evidence for chronic inflammation as a component of the intertestitial lung disease associated with progressive systemic sclerosis. Am Rev Resp Dis 131. (1985): 612-617.

Rossi P and de Crombrugghe B. Identification of a cell-specific transcriptional enhancer in the first intron of the mouse α2 (type I) gene. Proc Natl Acad Sci USA 84. (1987): 5590-5594.

Rossouw CMS, Vergeer WP, duPlooy SJ. DNA sequences in the first intron of the human pro-α(I) collagen gene enhancer transcription. J Biol Chem 262. (1987): 15151-15157.

Rothlein R, Dustin ML, Marlin SD, Springer TA. A human intercellular adhesion moleccule distinct from LFA-1. J Immunol 137. (1986): 1270-1276.

Rubanyi GM and Polokoff MA. Endothelins: Molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol Rev 46. (1994): 325-415.

Rudnicka L, Majewski S, Blasczczyk M, Skiendzielewska A, Makiela B, Skopinska M. Adhesion of peripheral blood mononuclear cells to vascular endothelium in patients with systemic sclerosis. Arthritis Rheum 35. (1992): 771-775.

Ryan, QC, Goonewardene IM, and Murasko DM. Extension of lifespan of human T lymphocytes by transfection with SV40 large T antigen. Exp Cell Res 199. (1992): 387-391.

Ryseck RP, Macdonald BH, Mattei MG, Bravo R. Structure, mapping, and expression of fisp-12, a growth factor-inducible gene encoding a secreted cysteine-rich protein. Cell Growth Differ 2. (1991): 225-233.

Sacca R and Cochran BH. Identification of a PDGF-responsive element in the murine c-myc gene. Oncogene 5. (1990): 1499-505.

Saito, H. and Moses, R. E. Immortalization of Werner Syndrome and Progeria fibroblasts. Exp Cell Res 192. (1991): 373-379.

Salmon-Ehr V, Serpier H, Nawrocki B, Gillery P, Clavel C, Kalis B, Birembaut P and Maquart FX. Expression of interleukin-4 in scleroderma skin specimens and

scleroderma fibroblast cultures. Potential role in fibrosis. Arch Dermatol 132. (1996): 802-806.

Sandmeyer S, Gallis B, Bornstein P. Coordinate transcriptional regulation of type I procollagen genes by Rous sarcoma virus. J Biol Chem 256. (1981): 5022-5028.

Sappino A, Masouye I, Saurat J, Gabbian G. Smooth muscle differentiation in scleroderma fibroblastic cells. Am J Pathol 137. (1990): 585-591.

Sauk J, Smith T, Norris K, Ferreira L. Hsp47 and the translation-translocation machinery cooperate in the production of α1(I) chains of type I procollagen. J Biol Chem 369. (1994): 3941-3946.

Saunders WS, Chue C, Goebl M, Craig C, Clark. Molecular cloning of a human homologue of Drosophilia heterochromatin protein HPI using anti-centromere autoantibodies with anti-chromo specificity. J Cell Sci 104. (1993): 573-582.

Scharfetter K, Buttgeret BL, Krieg T. Localisation of collagen mRNA in normal and scleroderma skin by in situ hybridization. Eur J Clin Invest 18. (1988): 9-17.

Schwarting A, Schlaak J, Lotz J, Buschenfelde KH, Mayet WJ. Endothelin-1 modulates the expression of adhesion molecules on fibroblast-like synovial cells. Scand J Rheum 25. (1996): 246-256.

Sessa WC, Kaw S, Hecker M, Vane JR. The biosynthesis of endothelin-1 by human polymorphonuclear leucocytes. BBRC 174. (1991): 613-618.

Sfikakis PP, Tesar J, Baraf H, Lipnick R, Klipple G, Tsokos GC. Circulating intercellular adhesion molecule-1 in patients with systemic sclerosis. Clin Immunol Immunopathol 68. (1993): 88-92.

Shay JW, Woodring EW. Quantitation of the frequency of immortalization of normal human diploid fibroblasts by SV40 large T-antigen. Exp Cell Res 184. (1989): 109-118.

Shi-Wen X, Denton CP, McWhirter A, Bou-Gharious G, Abraham DJ, duBois RM and Black CM: Scleroderma lung fibroblasts exhibit elevated and dysregulated collagen type I biosynthesis. Arthritis Rheum 40. (1997): 1237-1244.

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 37. (1994): 1689-1697.

Shi-Wen X, Vancheeswaran R, Bou-Gharios G, O'Hare M, Olsen I, Abraham D, Black CM. Scleroderma-derived human fibroblasts retain abnormal phenotypic and functional characteristics following retroviral transduction with the SV40 tsT antigen. Exp Cell Res 220. (1995): 407-414.

Silman AJ. Epidemiology of scleroderma. Ann Rheum Dis 50. (1991): 846-853.

Silman AJ. Scleroderma. Baillieres Clin Rheum 9. (1995): 471-482.

Silman AJ and Hochberg. Occupational and environmental influences on scleroderma. Rheum Dis Clin N Am 22. (1996): 737-749.

Silver RM, Miller SK, Kinsella MB, Smith EA, Schabel SI. Evaluation and management of scleroderma lung disease using bronchoalveolar lavage. Am Med J 88. (1990): 470-475.

Silver RM, Metcalf JF, LeRoy EC. Interstitial lung disease in scleroderma. Immune complexes in sera and bronchoalveolar lavage fluid. Arthritis Rheum 29. (1986): 525-531.

Simmons D, Makgoba MW, Seed B. ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. Nature 331. (1988): 624-627.

Simon LV, Beauchamp JR, O'Hare M, Olsen I. Establishment of long-term myogenic cultures from patients with Duchenne muscular dystrophy by retroviral transduction of a temperature-sensitive SV40 large T antigen. Exp Cell Res 224. (1996): 264-271.

Smith CW, Kishimoto TK, Abbassi O, Hughes B, Rothlein R, McIntire LV, Butcher E, Anderson DC. Chemotactic factors regulate lectin adhesion molecule-1 (LECAM-1)-dependent neutrophil adhesion to cytokine stimulated endothelial cells *in vitro*. J Clin Invest 87. (1991): 609-618.

Smith CW, Marlin SD, Rothlein R, Toman C, Anderson DC. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils *in vitro*. J Clin Invest 83. (1989): 2008-2017.

Smith EA and LeRoy EC. A possible role for transforming growth factor beta in systemic sclerosis. J Invest Dermatol 95. (1990): 125-127.

Snowden N, Coupes B, Herrick A, Illingworth K, Jayson MI, Brenchley PE. Plasma TGF beta in systemic sclerosis: a cross-sectional study. Ann Rheum Dis 53. (1994): 763-767.

Sokolovsky M, Ambar I, Galron R. A novel subtype of endothelin receptors. J Biol Chem 267. (1992): 20551-20554.

Sollberg S, Peltonen J, Uitto J, Jimenez SA. Elevated expression of β1 and β2 integrins, intercellular adhesion molecule-1, and endothelial leukocyte adhesion molecule-1 in the skin of patients with systemic sclerosis of recent onset. Arthritis Rheum 35. (1992): 290-298.

Soma Y and Grotendorst GR. Transforming growth factor beta stimulates primary human skin fibroblast DNA synthesis via an autocrine production of PDGF-related peptides. J Cell Physiol 140. (1989): 246-253.

Southcott AM, Jones KP, Li D, Majumder S, Cambery AD, Pantelides P, Black CM, Jeffrey PK, Davies BH, Du Bois RM. Interleukin-8 - differential expression in lung fibrosing alveolitis and systemic sclerosis. Am J Resp Crit Care Med 151. (1995): 1604-1612.

Sporn MB, Roberts A, Wakefield LM, de Crombrugghe B. Some recent advances in the chemistry and biology of transforming growth factor-β. J Cell Biol 105. (1987): 1039-1045.

Springer TA. Adhesion receptors of the immune system. Nature 346. (1990): 425-434.

Springer TA, Dustin ML, Kisimoto TK. The lymphocyte function-associated LFA-1 CD2 and LFA-3 molelcules cell adhesion receptors of the immune system. Ann Rev Immunol 5. (1987): 223-252.

Stacey M, Gallimore P, McConville C, Taylor A. Rearrangement of the same chromosome regions in different SV40 transformed human skin keratinocyte lines is associated with tumourigenicity. Oncogene 5. (1990): 727-739.

Stamps AC, Gusterson BA, O'Hare M. Are tumours immortal?. Eur J Cancer 28A. (1992): 1495-1500.

Stamps AC, Davies SC, Burman J, O'Hare MJ. Analysis of proviral integration in human mammary epithelial cell lines immortalized by retroviral infection with a temperature-sensitive SV40 T-antigen construct. Inter J Cancer 57. (1994): 865-874.

Statsny P, Stembridge VA, Ziff M. Homologous disease in the adult rat, a model for autoimmune disease. J Exp Med 118. (1963): 635-642.

Stein CM, Tanner SB, Awad JA, Roberts LJ, Morrow JD. Evidence of free radical-mediated injury (isoprostane overproductin) in scleroderma. Arthritis Rheum 39. (1996): 1146-1150.

Stratowa C and Audette M. Transcriptional regulation of the human intercellular adhesion molecule-1 gene. Immunobiol 193. (1995): 293-304.

Stupi AM, Steen VD, Owens GR, Barnes EL, Rodnan GP and Medsger TA. Pulmonary hypertension in the CREST syndrome varient of systemic sclerosis. Arthritis Rheuma 29. (1986): 515-524.

Takayanagi R, Kitazumi K, Takasaki C, Ohnaka K, Aimoto S, Takasa K, Ohashi M, Nawata H. Presence of non-secletive type of endothelin receptor on vascular endothelium and its linkage to vasodilation. FABS Lett 282. (1991): 103-106.

Takeda K, Hatamochi A, Ueki H, Nakata M, Oishi Y. Decreased collagense expression in cultured systemic sclerosis fibroblasts. J Invest Dermatol 103. (1994): 359-363.

Tan EM. Antinuclear antibodies: Diagnostic markers for autoimmune diseases and probes for cell biology. Adv Immunol 44. (1989): 93-151.

Tarai M, Kohno Y, Namba M. Class II major histocompatibility antigen expression of coronary arterial endothelium in a patient with Kawasaki disease. Hum Pathol 21. (1990): 231-234.

Taylor JP, Cupp C, Diaz A, Chowdhury M, Khalili K, Jimenez SA, Amini S. Activation of expression of genes coding for extracellular matrix proteins in Tat-producing glioblastoma cells. Proc Natl Acad Sci USA 89. (1992): 9617-9621.

Trojanowska M, Wu L, LeRoy EC. Elevated expression of c-myc proto-oncogene in scleroderma fibroblasts. Oncogene 3. (1988): 477-481.

Trojanowska M, Wu L and LeRoy EC. Elevated expression of c-myc proto-oncogene in scleroderma fibroblasts. Oncogene 3. (1996): 477-481.

Tsutamoto T, Hisanaga T, Fukai D, Wada A, Maeda Y, Maeda K, Kinoshita M. Prognostic value of plasma soluble intercellular adhesion molecule-1 and endothelin-1 concentration in patients with chronic congestive heart failure Am J Cardiol 76. (1995): 803-808.

Tuveri MA, Passiu G, Mathieu A, Aloe L. Nerve growth factor and mast cell distribution in the skin of patients with systemic sclerosis. Clin Exp Rheum 20. (1993): 319-322.

Uitto J, Bauer EA, Eisen AZ. Scleroderma. Increased biosynthesis of triple-helical type I and type III procollagens associated with unaltered expression of collagenase by skin fibroblasts in culture. J Clin Invest 64. (1979): 921-930.

Unemori EN, Amento EP. Connective tissue metabolism including cytokines in scleroderma. Curr Opin Rheum 3. (1991): 953-959.

van der Rest M and Garrone R. Collagen family of proteins. FASEB J 5. (1991): 2814-2823.

van de Water J and Gershwin ME. Avian scleroderma: An inherited fibrotic disease of white leghorn chickens resembling progressive systemic sclerosis. Am J Pathol 120. (1985): 478-482.

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

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 21. (1994): 1838-1844.

Varga J, Rosenbloom J, Jimenez SA. Transforming growth factor β causes a persistent increase in steady-state amounts for type I and type III collagen and fibronectin mRNAs in normal human dermal fibroblasts. Biochem J 297. (1987): 597-604.

von Asmuth EJU, Smeets EF, Ginsel LA, Onderwater JJM, Leeuwenberg JFM, Buurman WA. Evidence for endocytosis of E-selectin in human endothelial cells. Eur J Immunol 22. (1992): 2519-2526.

von Notthafft A. Neure arbeiten und anshihten uber sklerodermia. Zentralbl Allg Pathol Anat 9. (1898): 108-114.

Voraberger G, Schafer R, Stratowa C. Cloning of the human gene for intercellular adhesion molecule-1 and analysis of its 5'-regulatory region. J Immunol 147. (1991): 2777-2786.

Vuorio E and de Crombrugghe B. The family of collagen genes. Ann Rev Biochem 59. (1990): 837-872.

Walsh SM, Wong H, McCartney-Francis N. Role of growth factors in inflammation and repair. J Cell Biochem 40. (1989): 193-199.

Welsh KI, Black CM. Environmental and genetic factors in scleroderma. In Systemic sclerosis: scleroderma 1988. Ed. MIV Jayson & CM Black. London: John Wiley and Sons. p33-47.

White B. Pathogenesis: Immune aspects. Systemic sclerosis 1996. Ed. Clements PJ and Furst DE. Williams & Wilkins. p229-250.

White JA, Carter SG, Ozer HL, Boyd AL. Cooperativity of SV40 T antigen and ras in progressive stages of transformation of human fibroblasts. Exp Cell Res 203. (1992): 157-163.

Whiteside TL, Ferrarini M, Hebda P, Buckingham R. Heterogeneous synthetic phenotype of cloned scleroderma fibroblasts may be due to aberrant regulation in the synthesis of connective tissues. Arthritis Rheum 31. (1988): 1221-1229.

Yamane K, Miyauchi T, Suzuki N, Yuhara T, Akama T, Suzuki H, Kashiwagi H. Significance of plasma endothelin-1 levels in patients with SSc. J Rheum 19. (1992): 1566-1571.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, and Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332. (1988): 411-415.

Yurovsky VV, Sutton PA, Schulze DH, Wigley FM, Wise RA, Howard RF, White B. Expansion of selected Vd1+ gd T cells in systemic sclerosis patients. J Immunol 153. (1994): 881-891.

APPENDIX I

SOURCES FOR REAGENTS

REAGENTS

1. Tissue culture reagents

DMEM	Dulbecco's modified Eagles medium - Gibco BRL
MCDB	Serum-free complete medium - Gibco BRL
RPMI 1640	RPMI 1640 medium - Gibco BRL
Trypsin-EDTA	Gibco BRL
Penicillin	Sigma Chemical Co, St Louis, MO, USA 100 μg/ml
Gentamycin	Sigma Chemical Co, St Louis, MO, USA 50 μg/ml
Amphotericin B	Sigma Chemical Co, St Louis, MO, USA 2.5 μg/ml
Vitrogen-100	Celltrix, Santa Clara, CA, USA 3 mg/ml

2. Cytokines and growth factors

CTGF	FibroGen Inc, South San Francisco, USA
ET-1	Sigma Chemical Co, St Louis, MO, USA
TGFβ	R&D Systems Inc. Minneapolis, MN, USA

3. Antibodies

Anti-ICAM-1	R&D Systems Inc. Minneapolis, MN, USA
Anti-collagen type I&III	Southern Biotechnology Associates Inc., Birmingham, AL,USA
Anti-human fibronectin	Southern Biotechnology Associates Inc., Birmingham, AL,USA
Anti-Collagenase (MMP-1)	Amersham Life Sciences, UK

3. Antibodies

CD10	Serotec, Oxford, UK
CD13	Serotec, Oxford, UK
CD26	Serotec, Oxford, UK
CD29	Serotec, Oxford, UK
CD49d	Serotec, Oxford, UK
VLA-4 α-chain	Serotec, Oxford, UK
M-38	Developmental Studies Hybridoma Bank, Baltimore, MD. USA
MHC I	American Type Culture Collection, Rockville, USA
MHC II	American Type Culture Collection, Rockville, USA
CD58 (LFA-3)	American Type Culture Collection, Rockville, USA

4. Radioisotopes

[^{32}P]-dCTP	[α-^{32}P]-doxycytidine triphosphate 1 mCi/ml, 800 Ci/mmol Amersham Life Sciences, UK
[^{3}H]-TdR	methyl-^{3}H thymidine 1 mCi in 1ml, specific activity 5Ci/mmol Amersham Life Sciences, UK
[^{125}I]-labelled ET-1	Amersham Life Sciences, UK
[^{125}I]-labelled PD151242	Amersham Life Sciences, UK
[^{125}I]-labelled BQ3020	Amersham Life Sciences, UK

5. Transfection

pGL3 plasmid	Promega, Visconsin, USA
Lipofectamine	Gibco BRL, Paisley, UK
Dual-Luciferase Reporter Assay System	Promega, Maddison, USA

Appendix II

Commonly used buffers and solutions

20 x SSC

3.0 M	sodium chloride	
0.3 M	sodium citrate	pH7

10 x TBE

0.89 M	boric acid	
0.2 M	Tris	
0.025 M	Ethylenediaminetetraacetic acid (EDTA)	pH8

10 x MOPS

0.2 M	3-[N-morpholino]-propane-sulphonic acid (MOPS)	
0.05 M	sodium acetate	
0.01 M	EDTA	pH8.3

TE

0.01 M	Tris HCI	
0.001 M	EDTA	pH8.0, pH7.6, pH7.4

Denature solution (used in RNA preparation)

4 M	guanidium thiocyanate	
0.025 M	sodium citrate	pH7.0
0.5%	N-lauryl sarcosin (sodium salt)	
0.1 M	2-mercaptoethanol (to be added just before use)	

TENS

0.01 M	Tris	pH8.0
0.001 M	EDTA	
0.1 M	sodium hydroxide	
0.5%	Sodium dodecyl sulphate (SDS)	