The aetiopathogenesis of systemic sclerosis: thick skin—thin hypotheses

The Parkes Weber Lecture 1994

Carol M Black
MD FRCP

> Important advances in our knowledge of the disease of connective tissue are likely to come from biochemistry, immunology and biophysics, and in saying this I do not want to minimise the continuing importance of carefully controlled clinical observations [1].

The designation 'scleroderma' was first used by a Frenchman, Gintrac [2], in the 19th century to describe what was considered to be the hallmark (tight skin) of the disease, although Hippocrates, in the 5th century BC, is credited with the first description of the disorder. Scleroderma, which represents more than one disease (Table 1), with almost certainly more than one cause and pathogenic pathway, affects multiple organ systems resulting in widespread damage to blood vessels and the connective tissue [3]. When permanent vascular, dermal or internal organ changes begin to appear, several physical and serological differences separate scleroderma patients into distinct groups, each with a separate clinical presentation and a different disease course and progression [4].

Systemic sclerosis (SSc) is the most important and dangerous of the 'scleroderma spectrum' disorders; the extent of skin involvement and the associated pattern of internal organ damage form the basis for the current distinction between diffuse (dcSSc) and limited disease (lcSSc). Within the first year of observation, the practising physician is usually able to determine whether sclerosis of the skin of the upper arms, legs or trunk is occurring, which indicates dcSSc, or whether there is only cutaneous involvement restricted to the fingers, hands and face, indicating lcSSc [5] (Table 2). The subset lcSSc might be considered as 'vascular scleroderma' and will perhaps eventually be seen as a separate condition preceded by a lengthy prodrome of Raynaud's phenomenon and a 'pre-sclerotic' state in which patients have Raynaud's, abnormal capillaries and circulating antibodies. There are also rare cases in which the skin is never thickened but the internal organs are affected (scleroderma *sine* scleroderma). Other subdivisions within the spectrum include localised scleroderma, juvenile SSc and environmentally-induced scleroderma-like disease.

The pathological hallmarks of scleroderma are vascular and microvascular abnormalities characterised by capillary obliteration, endothelial injury/activation, intimal proliferation, medial thinning and a distinctive adventitial/interstitial cuff of collagen, consisting of perivascular and tissue infiltration of mononuclear inflammatory cells, 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 [6].

Pathogenesis

The pathogenesis of SSc is largely unknown, but contained within the present state of knowledge are the

Table 1. Spectrum of scleroderma and scleroderma-like syndromes.

Raynaud's phenomenon	Raynaud's disease (idiopathic)
	Raynaud's syndrome (secondary)
Scleroderma:	
• systemic	Limited cutaneous systemic sclerosis (lcSSc)
	Diffuse cutaneous systemic sclerosis (dcSSc)
	Scleroderma *sine* scleroderma
• localised	Morphea (plaque, guttate, generalised)
	Linear
	En coup de sabre
• juvenile	Localised forms (morphea, linear, *en coup de sabre*)
	Systemic forms (dcSSc, lcSSc)
• chemically induced	Environmental/occupational
	Drugs
Scleroderma-like diseases	Metabolic
	Immunological/inflammatory
	Localised systemic sclerosis and visceral diseases
	Eosinophilic fasciiitis
	Eosinomyalgic syndrome
	Mixed connective tissue disease (MCTD)
	Overlap syndromes

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technology, direction and ideas which could in the next 10 years answer some of the most perplexing questions. The triggers for the initial event are unknown, and nowhere are hypotheses so thin as when trying to define these aetiological factors. SSc is not considered to be primarily a genetic disorder, although there is evidence to support a genetic predisposition [7]. Environmental induction is also evident is some cases, and scleroderma is almost unique amongst the connective tissue diseases in being inducible by chemicals and drugs of several kinds; viruses, oncogenes and bacteria have also had their proponents, but to date such evidence is weak.

Genetic factors

The genetic background currently being pursued includes association with the major histocompatibility genes (MHC) and certain linked loci, chromosomal abnormalities, autoantibody/HLA associations and T cell polymorphism. All these factors are imprinted on gender, the strongest genetic marker in this disease; scleroderma is a female disease with, in some series, a female to male ratio of 15:1 in the reproductive years [7].

Table 2. Classification of systemic sclerosis (SSc) subsets.

1. *Pre-scleroderma:*
   Raynaud's plus nailfold capillary changes and circulating antinuclear antibodies (topoisomerase-1 anticentromere, nucleolar)
2. *Diffuse cutaneous SSc (dcSSc):*
   Onset of skin changes (puffy or hidebound) within one year of onset of Raynaud's
   Truncal and acral skin involvement
   Presence of tendon friction rubs
   Early and significant incidence of interstitial lung disease, oliguric renal failure, diffuse gastrointestinal disease, and myocardial involvement.
   Nailfold capillary dilatation and capillary drop out
   Anti-topoisomerase-1 (Scl-70) antibodies (30% of patients)
3. *Limited cutaneous SSc (lcSSc):*
   Raynaud's for years (occasionally decades)
   Skin involvement limited to hands, face, feet and forearms (acral) or absent
   A significant (10–15 years) late incidence of pulmonary hypertension, with or without interstitial lung disease, skin calcifications, telangiectasia and gastrointestinal involvement
   A high incidence of ACA (70–80%)
   Dilated nailfold capillary loops, usually without capillary dropout
4. *Scleroderma sine scleroderma:*
   Raynauds +/–
   No skin involvement
   Presentation with pulmonary fibrosis, scleroderma renal crisis, cardiac disease, gastrointestinal disease
   Antinuclear antibodies may be present (Scl 70, ACA, nucleolar)

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ACA = anticentromere autoantibody

HLA system

The first genetic area to be explored was the MHC. This was a natural choice because of the close association of the MHC with the rheumatic diseases. Increased frequencies of certain HLA class I, II and III antigens include HLA A9, A31, B8, B35, DR1, DR3, DR11, DRw6, DR52 and C4 null genes [7]. The antigens are associated with particular disease subsets or individual organ involvement, for example, DR52a and lung fibrosis [8], and C4A null alleles with an increased frequency of antinuclear antibodies (ANAs) in the relatives of scleroderma patients [9]. The interpretation of these associations has been confounded because of the variability of the association found in different centres. The results are probably influenced by ethnic variation—the degree of linkage disequilibrium differs between races: the Japanese, for example, lack the haplotype A1-B8-DR3 which is closely associated with scleroderma in North America and Europe. Other influences are variability in the classification of disease subsets throughout the world, and geographical and environmental factors which differ from country to country. Trans-racial gene mapping may be needed to answer some of the outstanding questions. The results are summarised in Table 3 of a recent large UK SSc family study (60 families) which employed a serological analysis of class I alleles and a combination of restriction fragment length polymorphism and oligonucleotide probing with polymerase chain reaction amplification for class II alleles. The C4A locus was the strongest of the

Table 3. Comparison of significance values for HLA associations in two studies [10].

HLA association	Cohort study (n = 115) p value	Family study (n = 63) p value
C4AQO	0.00064	0.000014
DQA2	0.0009	0.00008
DR3	0.011	0.003
DR11	0.015 (non-discriminatory to subset)	0.002 (lcSSc 0.00024)
DR52a	pulmonary fibrosis SSc 0.00012	–
DP	no association	no association

lcSSc = limited cutaneous systemic sclerosis
SSc = systemic sclerosis

potential markers of disease susceptibility analysed; C4AQ0, followed by DQA2, were found to be independent susceptibility factors for SSc [10].

Scleroderma is a heterogeneous disorder with a variety of autoantibodies associated with disease subsets (Table 4). Antibodies to centromeric peptides are usually found in patients with lcSSc. Anti-topoisomerase autoantibodies (anti-Scl-70), on the other hand, are correlated with widespread skin involvement and early onset systemic disease (dcSSc). Antibodies to RNA polymerases I, II and III (recently described in SSc) are found in 25% of patients, and may become important as markers for defining subsets of diffuse scleroderma patients with particular organ involvement and a poor prognosis. The antinucleolar autoantibody family includes a wide range of autoantigens, several of which are found in scleroderma. One, the anti-Pm-Scl antibody, has been closely linked to myositis in scleroderma patients [11–15]. Western Europeans and North American whites have a significantly higher frequency of anticentromere autoantibodies (ACA) and a lower frequency of anti-topoisomerase-1 than American blacks, Choctaw native Americans, Thais and Italians [16,17].

One of the primary roles of class II molecules is the presentation of processed antigen to the T cell receptor or helper T lymphocytes, resulting in an antigen-specific immune response, so autoantibody subsets in scleroderma might be expected to show correlations with class II MHC polymorphism—and indeed they do. ACA is associated with DR5 (DR11), DR4 (D13 subtypes), DR1 and DR8. These findings reflect linkage disequilibrium of DR5 (DR11) and many DR4 (D13 subtypes) with HLA DQ7 and of DR1 with DQ5. These HLA DR specificities share no unique amino acid sequences; this raised the possibility that another linked gene might be more highly correlated with this antibody response. Recently, Reveille *et al* [18] found DRB3, DQA1 and DQB1 alleles and the ACA response to be most closely associated with HLA DQB1 alleles in linkage disequilibrium with HLA DR1, DR4, DR5 (DR11) and DR8. These HLA DQB1 alleles had in common a polar tyrosine or a glycine amino acid at position 26 of the outermost domain of the HLA DQB molecule, as opposed to a hydrophobic leucine residue. In a British study in 1993, implication of the HLA DQB1 locus was inferred, as virtually all the ACA positive patients had either HLA DR1 or DR4 [10] (Fig 1). However, a recent paper by McHugh *et al* [19] has indicated that, although at least one HLA DQB1 allele not coding at position 26 of the first domain appears to be necessary, it may not be sufficient to

Table 4. Serum autoantibodies with clinical and laboratory correlates.

Antigen	ANA staining pattern	HLA associations	Frequency in all patients (%)	Clinical associations	Organ involvement
Scl-70 topoisomerase-1	Speckled	DR5 (DR11) DR3/DR52a DQ7 DQB1	15–20	Diffuse	Lung fibrosis
RNA I, II & III	Speckled/ nucleolar	?	20	Diffuse	Renal, skin
U_3 RNP	Nucleolar	?	< 5	Diffuse (overlap)	Pulmonary hypertension, muscle
PM-Scl	Nucleolar	DR3 DR52	3–5	Overlap, mixed	Muscle
U_1RNP	Speckled	?	10	Limited, blacks, mixed, overlap	Muscle
ACA centromere	Centromere (kinetochore)	DR1 (DQ5) DQB1 DR4 (D13 subtypes)	25–30	Limited	Pulmonary hypertension
Th (To)	Nucleolar	?	5	Limited	Pulmonary hypertension, small bowel

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ACA = anticentromere autoantibody
ANA = antinuclear antibody

generate ACA. Reveille and colleagues have also extended the known associations of anti-Scl-70 with DR5, DR2 and DR52a to include four HLA DQB1 alleles [20]. Japanese workers have found a similar allele association [21].

Localisation of 'susceptible epitopes', however, has been less definitive. Suggestions include an American population with a tyrosine at position 30 or the TRAELDT sequence spanning positions 71–77 of the HLA DQB1 outermost domain [20], and there is a Japanese SSc population with a tyrosine at position 26 of the HLA DQB1 outermost domain [21]. In a British study, an HLA DPB1 association was suggested with the presence of an acidic amino acid residue at position 69 in the third hypervariable region of the outermost domain [10]. Autoantibodies to the anti-Pm-Scl antigen are nearly 100% correlated with the presence of the HLA DR3-DQw2 haplotype [22].

Autoantibodies

We are still waiting for direct evidence that these antibodies are pathogenic. It has been suggested that they arise secondary to cell damage or because of molecular mimicry. The latter theory is supported by work which shows homology between the target epitopes and retroviral proteins, suggesting a possible role for retroviruses in initiating autoantibody production [23,24]. Studies of epitope specificity of antibodies in scleroderma argue, however, for an antigen-driven process rather than fortuitous cross-reactivity, and sera from scleroderma patients usually recognise more than one epitope of topoisomerase-1. The fact that highly specific IgG antibodies closely related to HLA genes are produced in scleroderma is in favour of a T cell driven reaction connecting the two, although its specificity is unknown at present.

Skin and lung tissue from patients with SSc contains CD3+, CD4+, CD450+, interleukin(IL)-2-producing HLA DR+, lymphocyte-function associated antigen (LFA)-1+, α/β+ T cells. Skin, however, has a less florid cellular infiltrate and a paucity of eosinophils and neutrophils which are prominent features in lung disease, and skin does not contain the abundant secondary lymphoid follicles with the germinal centres which indicate that the follicles are actively producing antibody. Because of the presence of 'memory' T cells in the skin and lung interstitium, attempts have been made to see if these cells have been exposed to a common antigen, and evidence of clonality has been sought. To date, no specific α, β or γ T cell receptor gene has been implicated in the pathogenesis of the skin changes in SSc. Interest was aroused when a γ chain T cell receptor restriction fragment length polymorphism was found to occur more often in SSc patients [25]. This finding has not yet been confirmed. Investigation of the lung has been equally disappointing and the T cell response [26], as defined by Vα family expression, broad-based. Oncogenes have also been implicated both as stimuli to the immune response and as perpetuators of the increased matrix production seen in SSc [27]. However, to date, no study has implicated these genes as independent risk factors for the development of SSc.

Chromosomal instability

Chromosome instability was first reported in SSc by Housset *et al* in 1969 [28]. This observation has been confirmed and extended in several studies. A high chromosomal breakage rate, acentric fragments and

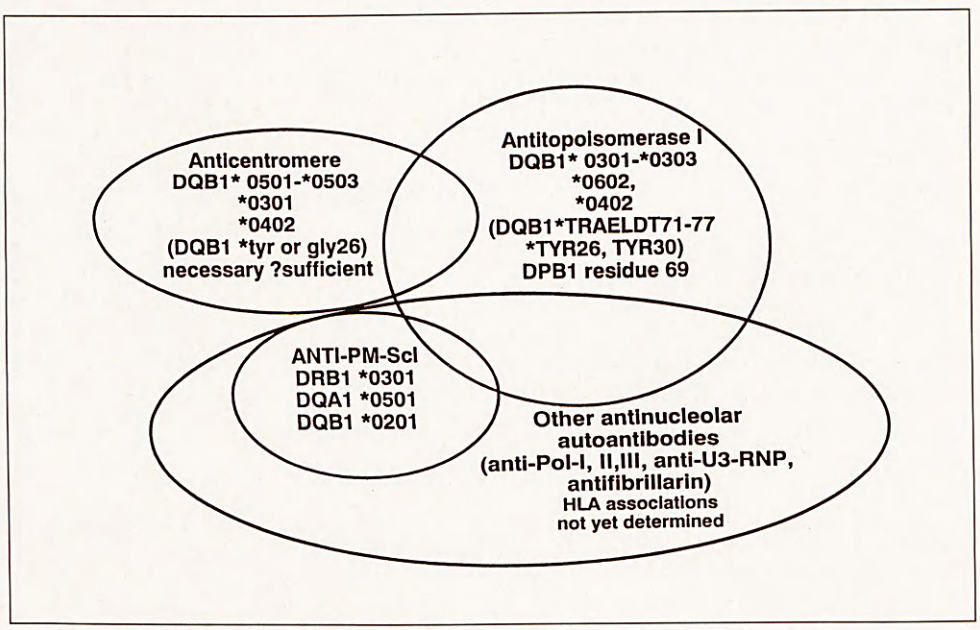

Fig 1. Autoantibody subsets of systemic sclerosis with HLA associations. Modified from Reveille JD. Molecular genetics of systemic sclerosis. *Current opinion in rheumatology* 1993;5:753–9.

deletions are found in patients' lymphocytes and fibroblasts. Similar abnormalities occur in many Raynaud's patients, but are rare in healthy individuals [29,30]. Clastogenic agents such as viruses, radiation, chemicals and drugs can cause chromosomal damage in SSc, but do the aberrations contribute to the aetiology? This area is controversial but a high rate of chromosomal instability is seen in patients with 'prescleroderma' (ie ANA positive, capillaroscopy-positive Raynaud's patients who subsequently go on to develop SSc [31]), and the clastogenic agent bleomycin has been shown to induce SSc [32].

Attempts have been made in the last few years to use more modern technologies to describe and define chromosomal abnormalities. Following on from work in tumours, we have used variable number tandem repeat analysis (VNTR) to identify chromosomal abnormalities in SSc. VNTRs are areas in the genome consisting of short repeat sequences that can exist over many kilobases [33]. These tandem repeats have been identified on every arm of each chromosome: they are areas of high recombinational activity, and are associated with deletions and insertions of the repeat unit, but do not necessarily involve chromosomal breaks. The telomere is the end section of the chromosome and its length can vary. Telomeres have specialised repeat units (TRUs) that confer stability to a chromosome, but TRUs are also located within the genome as a genetic fingerprint. We have shown that scleroderma patients and their family members have shortened telomeres and VNTR mutations. Using five highly polymorphic VNTRs, mutations were found in 36.7% of probands, 16.3% of siblings and 21.7% of offspring. In contrast, only 4.1% of the control group had VNTR abnormalities. Approximately 65% of all VNTR alleles that have altered in size have become larger. The most common VNTR site for mutation was pYNZ22 (17p1B4) [34]; it maps close to the RNA polymerase II gene *(rapII)*, and a small percentage of SSc patients (often those with a poor prognosis) have been reported to have antibodies against RNAPII. These antibodies may be generated if there is a disruption or alteration in the genetic code close to this site. They may, therefore, be markers of such an event and it is now possible to screen such patients. The reason for the size alteration remains obscure. A viral or chemical clastogen would be a possibility; it is not related to smoking, age, disease subset or therapy.

Drug metabolism

Allotypic variation in the ability to metabolise certain drugs may promote susceptibility to SSc, particularly drugs whose use is associated with SSc-like illnesses [35]. Poor metabolisers of both dapsone and mephenytoin hydroxylation have a tenfold greater risk of SSc [36]. This observation, yet to be confirmed by other workers, resembles the susceptibility to hydralazine-induced lupus which depends on an interaction with gender, MHC and acetylator phenotype [37]. Consideration of gender, MHC polymorphism and metabolic phenotypes could be informative in SSc.

Pathogenesis of connective tissue deposition

The three major hallmarks of SSc—vascular injury, a perivascular accumulation of mononuclear leukocytes and increased deposition of connective tissue matrix—are now known to be closely related and can be explained to a degree not previously possible. Vascular injury is associated with mononuclear leukocyte vascular adhesion and migration into the interstitium, resulting in clustering of these cells around blood vessels, with subsequent mediator release, development of a fibroblast-fibrogenic phenotype, matrix production and deposition, and leading to organ dysfunction.

Fibroblast activity

This theory places fibroblast activity and subsequent fibrosis secondary to activation and damage of other cell types. An alternative interpretation of fibroblast deregulation is to view it as an intrinsic defect which occurs separately or in addition to vascular damage. Numerous laboratories have performed fibroblast cultures from both the papillary and reticular dermis of SSc patients, and compared them with carefully matched normal skin fibroblasts. The most striking and consistent abnormality is the capacity of a significant number of the SSc fibroblasts in mass culture to secrete two- to fourfold higher levels of extracellular matrix (ECM) and ECM-precursor molecules, especially type I and III collagens, fibronectin and proteoglycans [38,39]. They continue to do this through many passages without stimulation.

The SSc fibroblast also appears to have an *in vitro* cell growth abnormality under conditions of sparse (weekly) and frequent (daily) replenishment of serum. When the collagen secreting ability of SSc and control fibroblasts was studied, the former continued at the same rate of growth and matrix synthesis whatever their feeding condition, but the control fibroblasts increased their collagen output briskly in response to frequent serum replenishment. This suggests that SSc fibroblasts have an endogenous autocrine capacity to direct their own proliferation *in vitro*. A formal study of this phenomenon by Trojanowska *et al* [40] has demonstrated a persistent proliferation of SSc fibroblasts in serum-free conditions, as measured by nuclear grain counts of tritiated thymidine uptake and by the continued expression of the proliferation-dependent proto-oncogene, *c-myc*. The precise molecular mechanism of this cell growth abnormality remains to be elucidated. This alternative and/or additional hypothesis must be borne in mind as the pathogenesis of SSc is as yet far from clear.

Endothelial damage

The hypothesis of vascular damage with subsequent fibrous deposition is well supported, and abundant evidence suggests that the earliest pathological changes occur in or near the microvascular endothelium [41]; however, it is not known whether the immune activation is triggered before or after endothelial changes have occurred. Two recent reports have thrown some light on this fundamental question. Prescott *et al* [42] studied, in a sequential fashion, the pathological changes in the perivascular spaces in the skin of 60 scleroderma patients and 25 controls. The first defect was functional and structural endothelial changes with subendothelial oedema, followed by platelet aggregation and lymphocyte migration of both CD4+ and CD8+ T cells; tissue fibrosis occurred at a later stage after the inflammation had subsided. Support for this finding comes from the work of Harrison *et al* [43] in which damage to endothelial/epithelial surfaces is shown to be the first ultrastructural change observed in lung biopsies from SSc patients. Lung sections which appeared to be normal under light microscopy, without evidence of infiltrating inflammatory cells, showed widespread endothelial/epithelial damage on electron microscopy. The nature of the initial vascular trigger to immune stimulation is critical to our understanding of the disease: environmental agents or an endothelium-seeking virus are possible candidates. In addition, intense vasospasm (Raynaud's phenomenon), an early event which occurs both in the extremities and internal organs of SSc patients, could lead by reperfusion injury to structural and functional changes in the endothelium and subsequent immune activation.

Vasoconstriction

Numerous publications in the last five years have enlarged our understanding of the mediators of vascular tone in large and small blood vessels [44]; in addition to hypoxia, physical stress, neural, humoral and inflammatory mediators, there are secreted products of the endothelial cells such as endothelium-derived relaxing factor (EDRF, nitric oxide (NO)) and endothelium-derived constricting factors (EDCF, endothelins), which alter vascular tone and may be important in the creation of the scleroderma lesion.

Endothelins are the most potent vasoconstrictors known. Endothelin(ET)-1 also has a fibrogenic potential. It is raised in a number of disorders, including trauma, inflammatory arthritides, bowel disease, pulmonary hypertension and hypertensive renal failure [45–47]. Increased levels of this peptide have also been reported in the circulation of patients with primary Raynaud's phenomenon and SSc [48,49]. Work by Kahaleh suggests that endothelin contributes not only to vascular change but also to the fibrotic lesion in SSc [50]. Our recent studies showed significantly raised circulating levels of ET-1 not only in the minimally fibrotic subset of SSc patients with primarily vascular disease and associated pulmonary hypertension but also in the diffuse subset in whom widespread fibrosis is the major hallmark [51]. We and others used immunohistochemical and autoradiographic techniques to localise ET-1 and its binding sites in skin biopsies from SSc patients. The presence of ET-1 in association with superficial vessels was demonstrated in both clinically involved and uninvolved skin and ET-1 was bound to its putative receptor in the yet-to-be involved skin. This binding decreases with increasing tissue fibrosis. These results suggest that ET-1 may play a role in the earliest events in SSc when fibrosis is being initiated [52,53].

In health, the vasoconstrictive action of ET-1 would be balanced by the opposing action of NO (EDRF). Data presented recently at the American College of Rheumatology's annual meeting [54] showed that the total NO-producing compounds measured by chemiluminescence in plasma of SSc patients were decreased, and that the increase in plasma NO seen on exposing normal subjects to a cold challenge was absent in SSc patients. NO may therefore not only be a marker of endothelial function but, by causing local reductions in blood flow and increasing platelet sensitivity, impairment of its synthesis may also be directly relevant to the disturbance of SSc.

Endothelial cytotoxic factors

Superoxide anions may be additional vascular factors. These anions are released from the endothelium, and can not only neutralise NO but also oxidise circulating low-density lipoproteins (LDL). Several years ago Blake and co-workers suggested that LDL could be the source of the cytotoxicity of stored SSc serum on cultured endothelial cells [55], a finding originally reported by Kahaleh in 1979 [56]. Our own recent studies have shown that LDLs in SSc patients, both with limited and with diffuse disease, are much more susceptible to oxidation, in contrast to patients with primary Raynaud's and other rheumatic diseases, indicating either an inherent difference in the lipoproteins or, more likely, that lipoproteins are subject to free radical attack in the circulation [57]. Thus, the evolution of the vascular lesion in SSc is highly complex.

The initial reports of a circulating endothelial cell cytotoxic factor naturally led to a search for an immune-mediated damaging agent; both cellular and humoral reactivity has been extensively considered. Some patients with scleroderma have circulating anti-endothelial cell antibodies, but this is not thought to be a critical event; some sera are blocked by monoclonal antibodies to tumour necrosis factor (TNF) α or β, while other sera are blocked by prior incubation with protease inhibitors. The cell source of the proteases and their nature are as yet uncertain, but

retroviral proteases and the granzyme family of proteases produced by activated T cells are under current investigation [58].

Adhesion molecules

Following damage to and inflammation of the vascular endothelium, it is believed that adhesion molecules such as endothelial leukocyte adhesion molecule-1 (ELAM-1), vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) are upregulated in response to cytokines and other factors. These endothelial adhesion molecules (ligands) bind to specific integrins on T and B lymphocytes, platelets, neutrophils, monocytes and natural killer (NK) cells [59,60]. This results in adhesion of cells to the vascular endothelium and subsequent migration and chemotaxis of these cells through what have now become 'leaky vessels' into the extracellular matrix (ECM). Evidence to support this hypothesis is growing.

The adhesion molecules can be identified in tissue samples and measured as soluble forms in the circulation. Recent work has shown positive immunostaining with monoclonal antibodies for E-selectin and ICAM-1 on endothelial cells in scleroderma skin, but not controls [61–64]; in other studies in small groups of patients, increased circulating levels of E-selectin and ICAM-1 have been found [65,66]. In an attempt to study both the circulating levels and tissue expression in a large well-defined group of scleroderma patients, we have shown not only differential expression of circulating levels of E-selectin, ICAM-1 and VCAM-1 in dcSSc and lcSSc, Raynaud's phenomenon and morphoea, but also a significant increase in the expression of E-selectin with progression of the disease from clinically uninvolved to lesional skin (Fig 2). This was associated with raised levels of the circulating form of E-selectin [67].

These findings, which suggest continuing endothelial activation throughout the course of SSc, underscore the importance of further studies on vascular activation and the interaction and adhesion of the lymphocyte with the endothelial cell. That such adhesion occurs in scleroderma has been implied in studies by Gruschwitz *et al* [63] who found that in early SSc the expression of ELAM-1 on endothelial cells correlated with the amount of mononuclear cell infiltration. In addition, a recent study by Rudnicka *et al* [68] showed a reduction in scleroderma peripheral blood mononuclear cell (PBMC) adhesion to endothelial cells but enhanced adhesion of the patient's active rosette-forming cells (ARFCs), activated cytotoxic inducer T lymphocytes, NK cells and some T helper leukocytes to the endothelium. Decreased adhesion of PBMCs to endothelial cells was found to correlate with a diminished percentage of ARFC in the peripheral blood. This enhanced adhesion with subsequent migration might be responsible for the diminished circulating numbers of ARFCs. The migrating cells are almost certainly moving through 'leaky vessels'. Although the exact cellular or molecular basis for deranged permeability in SSc is as yet unknown, possible candidates are histamine, kinins, complement, antibodies, free radicals, thromboxanes, oxidised LDLs and cytolytic T cells. A permeable endothelium undoubtedly facilitates the next stage in the pathogenic process. The role of the mast cells and eosinophils in the evolution

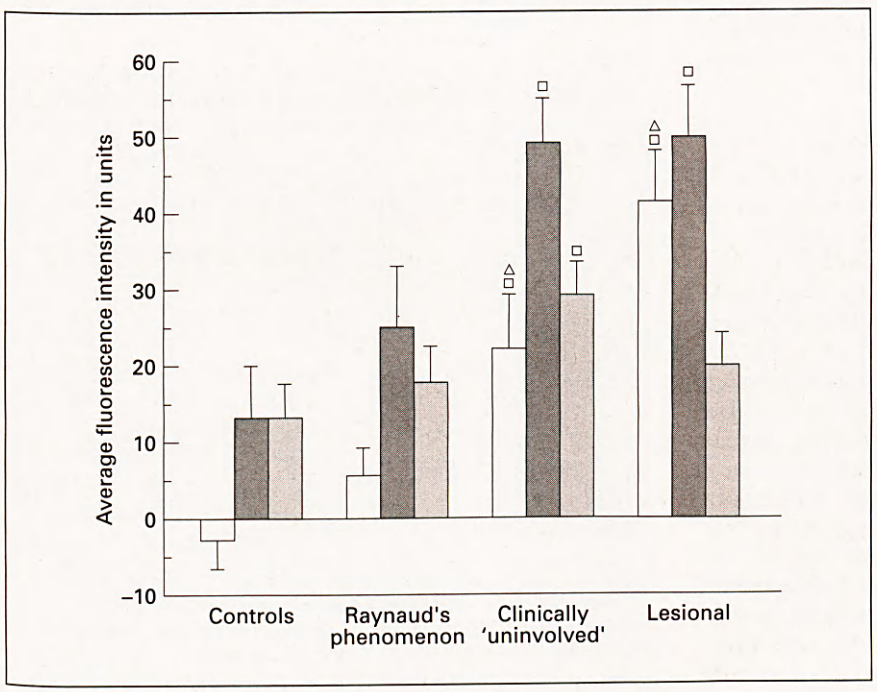

Fig 2. Dermal vascular expression of E-selectin ICAM-1 and VCAM-1 in RP SSc patients and normal controls.

of the scleroderma lesion, although potentially important, has received far less attention and is incompletely studied [69].

The mononuclear cells which settle in the perivascular spaces have subsets of integrin molecules on their surface, including those of the β^1 and β^2 class, that facilitate binding to other cells such as fibroblasts, and to tissue components, including type I and IV collagens, fibronectin and laminin [70–73]. The cell-cell and cell-matrix interactions which are now facilitated may be expected to stimulate synthesis of various growth factors and cytokines able to mediate the proliferation and activation of vascular and connective tissue cells—particularly fibroblasts—resulting in increased matrix deposition.

Cytokines

Large numbers of cytokines have been examined as potential effectors of the fibrogenic process; within the context of scleroderma, these include transforming growth factor (TGF) β, platelet-derived growth factor (PDGF), TNFα, insulin-like growth factors (IGFs), basic fibroblast growth factor (bFGF), IL-1, IL-4, IL-6, IL-8, and interferon (IF)γ. It is highly unlikely that any one individual mediator can account for the complex pathology of SSc. The importance of a single cytokine may be limited to the disease subset and the specific stage of the disease. These factors may set the conditions that permit the effector cells to respond to the next sequential signal. Cytokines can affect each other and the effect is usually mediated through the cell, either by internal mechanisms or through changed receptor levels. The functions of lymphocytes, monocytes and fibroblasts are modulated in part through these receptor molecules. As the full effects of the many known cytokines are only now being unravelled, a comprehensive understanding of the *in vivo* steps culminating in fibrosis is still in evolution.

TGFβ is a cytokine of major interest in scleroderma because of its capacity to stimulate synthesis of ECM proteins, including collagen and fibronectin. It is produced by megakaryocytes, macrophages and T cells, and nearly all cell types have TGFβ receptors [74, 75]. Despite its theoretical potential, studies of skin biopsies, bronchoalveolar lavage (BAL) and blood samples from SSc patients for TGFβ mRNA and protein have yielded inconsistent and conflicting results [76–79] — perhaps because these studies have used both different subsets of SSc patients with variable rates of progression at different times of their disease and also lesional, non-lesional and normal control skin. These variables make exact comparison of results difficult. It is still not known whether the growth factor may act at an early stage in the disease and lose its effect with chronicity (although the literature would support this concept), whether its effects vary in extent from organ to organ, and whether it acts alone or, as is most likely (as shown below), in concert with other cytokines. In addition, and most importantly, its role in the acquisition and maintenance of the SSc phenotype has not been worked out.

We have tried to answer this last question. Our studies demonstrate that SSc fibroblasts are not characterised by elevated TGFβ synthesis, and there was no evidence of coordinate regulation of TGFβ and collagen over passage number as the cells aged in culture, suggesting that collagen is not under autocrine control by TGFβ in SSc fibroblasts. Furthermore, repeated pulses of TGFβ did not significantly induce sustained procollagen-α1(I) mRNA synthesis in normal fibroblasts, nor did this treatment significantly alter collagen regulation by normal fibroblasts in a collagen gel. Collagen and TGFβ-type II receptor mRNA were inducible by TGFβ in both SSc and control cells, indicating that the failure to sustain increased collagen synthesis is not due to lack of responsiveness by the fibroblasts but is rather a reflection of the transient nature of TGFβ-induced fibrogenesis (maximum at 12 hours). The ability of SSc fibroblasts to activate exogenous latent forms of TGFβ was found to be impaired. Therefore, our data give no support to the hypothesis that TGFβ maintains the SSc phenotype *in vitro* or that it can induce this phenotype [80].

TGFβ does, however, influence other cytokines and is an indirect mitogen for fibroblasts acting via PDGFα receptor interactions. Yamakage *et al* demonstrated that in response to $TGF\beta_1$ [81] fibroblasts from SSc patients, in contrast to normal adult and newborn foreskin fibroblasts, express greater numbers of PDGFα receptors on their surface, and that there is a corresponding increase in PDGFα receptor protein and messenger RNA. TGFβ increased the mitogenic responses to PDGF-AA which binds only to the α receptor, in contrast to PDGFβ which binds to both

Table 5. Mediators, cytokines and collagen metabolism.

Fibrotic lesion		Mediator
Fibroblast chemotaxis	↑	(TGFβ, IL-4, TNFα and β, PDGF, IFγ)
Fibroblast proliferation	↑	(TGFβ, IL-1α and β, PDGF, IFγ TNFα and β)
Collagen synthesis	↑	(TGFβ, IL-1α andβ, PDGF, IL-4, IGF)
Collagen synthesis	↓	(IFγ, TNFα and β, EGF, relaxin, leukoregulin)
Collagenase synthesis	↑	(IL=1α and β, TNFα and β)
Collagenase synthesis	↓	(TGFβ)
TIMP synthesis	↑	(IL-1α and β, TGFβ)
TIMP synthesis	↓	(IL-6)

EGF = epidermal growth factor; IF = interferon; IGF = insulin-like growth factor; IL = interleukin; PDGF = platelet-derived growth factor; TGF = transforming growth factor; TIMP = tissue inhibitor of metalloproteinases; TNF = tumour necrosis factor

the α and β receptors of scleroderma cells. PDGF-AA was found immunohistochemically in SSc dermis near blood vessels and hair follicles, but not in normal skin [82].

These results suggest that the increased expression of fibroblast populations in SSc may be due in part to activation of the PDGF-AA ligand/α receptor pathway, induced by TGFβ. PDGF can also increase the rate of transcription of bFGF in normal fibroblasts. In scleroderma both PDGF and bFGF are deposited around blood vessels in the lower dermis of early lesions, suggesting that these growth factors may be related to endothelial injury in scleroderma [27]. PDGF regulates the expression of genes that are important in the mitogenic response, and Gay *et al* found increased *ras* oncogene product in early lesional skin in association with vascular endothelium and mononuclear cells [83]. Other studies have established that fibroblasts expressing the *ras* oncogene produce more bFGF, suggesting that bFGF may play an autocrine role leading to growth stimulation [83].

The multifactorial cytokine IL-6 is an additional potential factor in the complex pathway that leads to fibrosis. IL-6 is not only a potent stimulant of T and B lymphocyte function and a fibrogenic cytokine, but also an inhibitor of fibroblast-derived tissue inhibitor of metalloproteinases (TIMP). Increased expression of IL-6 in the basal and squamous layers of scleroderma epidermis was reported by Romero and Pincus [84], and *in vitro* work by Feghali *et al* [85] showed a 6–30 fold increase in IL-6 production in fibroblasts from involved skin compared with matched cultures from uninvolved skin or fibroblasts from healthy skin. This suggests that IL-6 could be produced locally by fibroblasts, and that the effects of IL-6 could not only contribute to T and B lymphocyte abnormalities and collagen synthesis but also reduce degradation of newly synthesised matrix by inhibiting collagenase and other metalloproteinases. Raised levels of circulating IL-2, IL-4, IL6–IL1, IL-8, TNFα and IFγ have been found in scleroderma [86] and anticytokine antibodies to IL-6 and IL-8 have also been reported [87]. The biological importance of antibodies is unknown, but their presence suggests involvement in the disease. Antibodies to cytokines may not only inhibit the biological function of the molecule but may, paradoxically, enhance cytokine function, prolong its half-life and deliver the cytokine to its target cell.

IL-8, a potent neutrophil chemoattractant, may play an important role in the pathogenesis of pulmonary fibrosis in scleroderma. BAL fluid from scleroderma patients with lung fibrosis and patients with fibrosing alveolitis contains more IL-8 than healthy controls and patients with sarcoidosis; the amount is proportional to neutrophil influx into the lower respiratory tract. *In situ* hybridisation demonstrated the predominance of the IL-8-positive signals from cells within the alveolar spaces [88]. Serum levels of IF8γ were depressed in patients with scleroderma-related fibrosing alveolitis but elevated in patients with sarcoidosis [89]. IFγ levels in the lavage fluid were not notable; there was no spontaneous production of IFγ by mononuclear cells and a poor response to mitogen-stimulated production.

The results of this study suggest a defect in T-cell reproduction of IFγ in patients with fibrosing alveolitis. Because IFγ can suppress synthesis of collagen by fibroblasts *in vitro,* decreased production may enhance tissue fibrosis and contribute to the development of pulmonary fibrosis in patients with scleroderma. Impairment of IFγ production in scleroderma has been reported by all investigators who studied this mediator, yet there is well documented expression of HLA class II on skin fibroblasts in scleroderma. As IFγ is the most potent mediator of HLA class II induction, either a different peptide is responsible for *in vivo* HLA DR expression or there is a difference between the *in vitro* and *in vivo* environments. These interactions indicate the highly complex nature of the cytokine pathways in the development of the fibrotic process in scleroderma.

Stimuli to collagen synthesis

There are numerous possible stimuli to collagen synthesis. Not only do the integrins integrate the intracellular cytoskeleton with the extracellular environment but, for example, the increased expression of ICAM-1, a member of the Ig superfamily, on scleroderma fibroblasts is responsible for increased binding of T cells to those fibroblasts through ICAM-1/LFA-1 interactions [90–92]. This interaction may provide important signals to the fibroblast. In addition, the integrins are known to participate in signal transduction events and could be of relevance to the scleroderma lesion [93]. An alteration in integrin expression could well influence cell behaviour which would result in aberrant regulation of collagen synthesis. The ability of normal fibroblasts to contract collagen gels (which are a simulated ECM) is directly proportional to their ability to downregulate mRNA encoding procollagen-α1(I) [93,94]. Recent research both from our own and other laboratories has shown that SSc fibroblasts do not exhibit a similar relationship and cannot downregulate their collagen synthesis to a similar extent, thus suggesting a loss of feedback control (Fig 3)—which may be related to a loss from the cell surface of collagen-binding integrin receptors. We have shown that the expression of the α_1 chain of collagen-binding integrin α_1, β_1, an important early messenger in signal transduction, is lower in some scleroderma fibroblasts than in matched normal cells. There is supportive evidence for this from Krieg's laboratory in Germany [95].

Fibroblasts form intimate contacts with the ECM at sites called focal adhesions, where the integrins accumulate. Part of the intracellular signalling process following fibroblast attachment to ECM is mediated by

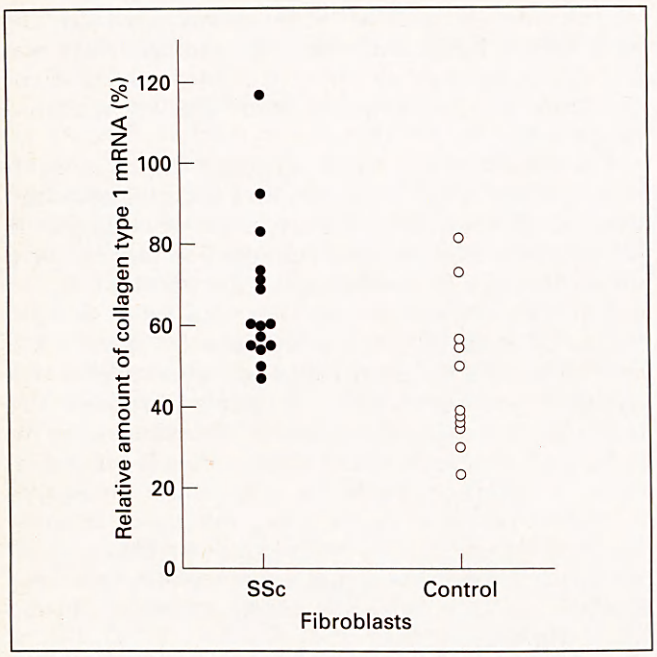

Fig 3. Proα(I) mRNA down-regulation by normal (n = 11) and SSc (n = 15) fibroblasts.

phosphorylation of several proteins including focal adhesion kinase (p125^{FAK}). This enzyme binds β1 integrin, talin and α-actinin. Phosphorylated p125^{FAK} [96,97] localises in the focal adhesion following integrin engagement and forms a crucial component of the regulatory pathways leading to control of transcription of the collagen gene in response to the attachment of the fibroblast to ECM. Our finding that a significant proportion of SSc fibroblast cell line extracts expresses low levels of α1 integrin chains raises the possibility that the impaired collagen regulation of SSc fibroblasts grown in a collagen gel may be due to their inability to form a functional association between the collagen-binding integrins and p125^{FAK}. The mechanism for transcriptional regulation of collagen synthesis is extremely complex; however, information about this process is essential to further our understanding of the pathogenesis of systemic sclerosis.

At best, our present therapies are inadequately targeted, often given at too late a stage and used in uncertain combinations. The future management of systemic sclerosis depends on an understanding of these complex pathogenic processes, which can then aid directed immunosuppression, endothelial protection and healing, prevention of excessive deposition of ECM and enhancement of ECM turnover.

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Address for correspondence: Professor Carol M Black, Head of Rheumatology, Royal Free Hospital, Pond Street, London NW3 2QG