THESIS

THE ROLE OF THE EPIDERMIS IN PATHOGENESIS OF SYSTEMIC SCLEROSIS

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by

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DECLARATION CONCERNING THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF MEDICINE

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Solemnly and sincerely declare, in relation to the thesis entitled: THE ROLE OF THE EPIDERMIS IN PATHOGENESIS OF SYSTEMIC SCLEROSIS

(a) That work was done by me personally;

(b) The material has not previously been accepted in whole, or in part, for any other degree or diploma.

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ABSTRACT

Studies into the pathogenesis of systemic sclerosis (SSc) skin fibrosis to date have concentrated on dermal changes in the disease. Little attention has been paid to the epidermis in SSc. Epithelial-fibroblast interactions are believed to regulate wound healing and contribute to a number of fibrotic diseases. Recent proteomic data from our laboratory reveals altered keratinocyte (KC) specific proteins in SSc skin consistent with a wound healing phenotype of the disease epidermis. I therefore studied SSc KCs focusing on differentiation and KC-fibroblast interaction.

I found that KC maturation is altered in SSc with abnormal persistence of cytokeratins 1, 10 and 14 into suprabasal layers. Cytokeratins 6 and 16, induced in wound healing KCs, were shown to be expressed in SSc epidermis. In addition, IL-1, a pivotal cytokine involved in KC and fibroblast events post epidermal injury, and its downstream signalling phosphoproteins p38 and JNK were elevated in SSc epidermis.

I went on to study the effect of SSc epidermis on normal human fibroblasts. I found that SSc epidermis promoted fibroblast activation in an ET-1, TGF-β, and IL-1 dependent fashion. I suggest a double paracrine loop initiated by KC-derived IL-1 as a mechanism for epidermal-dermal co-activation in the disease, similar to that previously demonstrated for wound healing. There is a need for developing antifibrotic agents targeting epithelium-derived factors and their signalling pathways.

I went on to study normal epidermal wound healing. A paradox during epithelial repair is that KCs proliferate despite a TGF-β dominated environment, which is known to be anti-proliferative. Our laboratory previously showed that prostanoids

antagonise TGF-β-dependent events in human cells. The induction of prostanoids following injury could transiently free KCs from the anti-proliferative effects of TGF-β. I test this hypothesis by confirming transient induction of epidermal COX II and PGE_2 following injury. I also show that PGE_2 antagonises the anti-proliferative and pro-migratory effects of TGF-β on KCs. My work supports a model where induction of epidermal wound edge COX II leads to antagonism of TGF-β and allows KCs to proliferate prior to migration over the wound.

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AC	Adenylate cyclase
AEC	Alveolar Epithelial Cell
αSMA	α Smooth Muscle Actin
CBP	CREB-binding protein
COX	Cyclo-oxygenase
CTGF	Connective Tissue Growth Factor
CREB	cAMP response element binding protein
CRE	cAMP-responsive element
DGK	Diacyglycerol Kinase
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ET	Endothelin
FPCL	Fibroblast Populated Collagen Lattice
IL-1	Interleukin 1α
IL-1R	Interleukin 1 Receptor
IPF	Interstitial Pulmonary Fibrosis
KC	Keratinocyte
KGF	Keratinocyte Growth Factor
K-SFM	Keratinocyte Serum Free Medium
LAP	Latency associated Peptide
MAPK	Mitogen-activated Protein Kinase
MMP	Metalloproteinase
N	Normal
PDGF	Platelet Derived Growth Factor
PGE_2	Prostaglandin E2
PKA	Protein Kinase A
PKC	Protein Kinase C
pSMAD	Phosphorylated SMAD
SGF	Sarcoma Growth Factor
SSc	Scleroderma
STAT	Signal Transducers and Activators of Transcription
TGF-β	Transforming Growth Factor β
TβR	Transforming Growth Factor β Receptor
TH	Tyrosine Hydroxylase
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

Introductory Review of Scientific Literature

1.1.1 Scleroderma: definition and classification:

Scleroderma (SSc) is a clinically heterogeneous disorder of unknown aetiology which affects small arteries, microvessels and the diffuse connective tissue of the skin and internal organs, resulting in uncontrolled scarring and fibrosis. Hidebound skin is the clinical hallmark of SSc, and organ compromise is the prognostic keystone (E. C. LeRoy *et al.* 1988a).

Cases of skin disease compatible with scleroderma have been mentioned in the writings of Hippocrates (460-370 B.C.), Galen (131-201 A.D.), and Paulus Aegineta (625-690 A.D.). The first documented description, however, comes from Carlo Curzio from Naples in 1753. In 'An Account of an Extraordinary Disease of the Skin, and its Cure' he described a young woman with extensive tension, hardness, and woodiness of the skin. Dr Curzio claimed to have cured the young woman, which casts some doubt over the diagnosis. Following this, the disease was 're-discovered' in France in 1847 by Grisolle and Forget. The term scleroderma, however, was introduced by Gintrac, also in 1847, as the skin was the most obvious organ involved in the disease.

The clinical presentation of SSc is a spectrum from localised skin fibrosis without internal organ pathology (localised scleroderma), to a systemic disorder with cutaneous and internal organ involvement (systemic sclerosis - SSc). The latter has a much higher mortality (V. D. Steen, T. A. Medsger 2007), and it is the pathogenesis of this form of the disease that we are interested in for the purposes of this study.

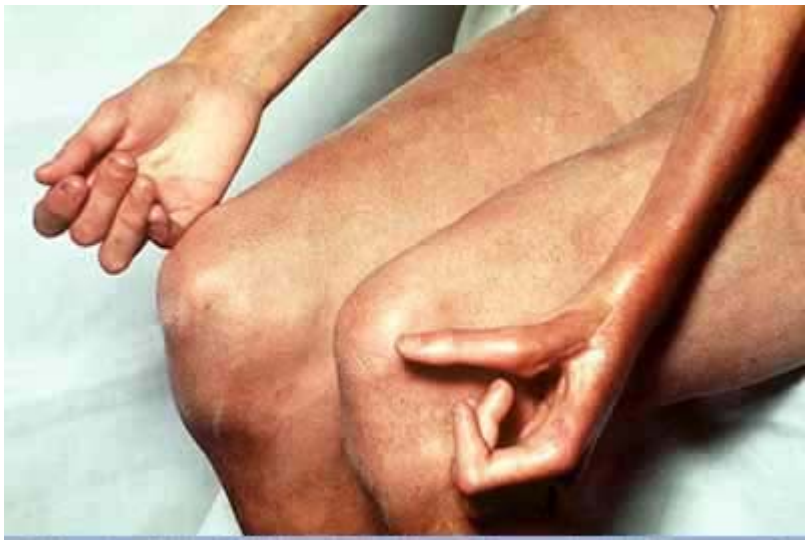


Figure 1.1: Diffuse skin sclerosis in SSc.

SSc can be limited or diffuse. The American College of Rheumatology (former American Rheumatism Association - ARA) has defined criteria, that are 97 % sensitive and 98 % specific for SSc as follows (American Rheumatism Association 1980b):

Major criterion:

- proximal diffuse (truncal) sclerosis (skin tightness, thickening, non-pitting induration)

Minor criteria:

- sclerodactyly (only fingers and/or toes)
- digital pitting scars or loss of substance of the digital finger pads (pulp loss)
- bibasilar pulmonary fibrosis

The patient should fulfil the major criterion or two of the three minor criteria. However, it is currently felt that the criteria need revision in view of the recent

advances in immunological and capillaroscopic studies (U. F. Haustein 2002). The classification of SSc used by most researchers and clinicians is one devised by LeRoy *et al.* in 1988 (E. C. LeRoy *et al.* 1988b):

Limited Cutaneous SSc:

- Raynaud's phenomenon for years at presentation
- Skin sclerosis limited to hands, feet, face, and forearms, or absent
- Significant late incidence of pulmonary hypertension, trigeminal neuralgia, calcinosis, and telangiectasia
- Dilated nail fold capillary loops, usually without capillary dropouts detected by wide field nail fold capillaroscopy.

Diffuse cutaneous SSc:

- Onset of Raynaud's phenomenon within 1 year of onset of skin changes
- 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
- Presence of anti-DNA topoisomerase I (anti-Scl-70) antibodies
- Absence of anticentromere antibodies
- Nail fold capillary dilatation and destruction detected by wide field nail fold capillaroscopy

1.1.2 Epidemiology:

The true incidence of SSc is probably underestimated since early features of the disease are often overlooked. However, it is estimated to be between 2.6 and 20 per million per year. The prevalence of SSc is reported to be 13 to 105 per million, although it can be as high as 290 in South California. There is a genetic, ethnic and environmental link in SSc expression (M. D. Mayes *et al.* 2003). The overall female/male ratio has been reported as 3:1. However, this ratio is larger in Great Britain (6:1), and in the USA (8:1).

Survival seems to be directly related to the severity of internal organ involvement. However, the skin score is a useful marker of both disease severity and prognosis. Raynaud's syndrome alone confers a 79-84% survival at 5 years, while truncal SSc at the onset of the disease reduces the survival rate to around 50% at 5 years (A. J. Barnett *et al.* 1988;E. V. Lally *et al.* 1988).

1.1.3 Scleroderma Pathogenesis:

SSc is a complex multifactorial disease. It is currently thought to be due to a combination of inflammatory, immunological and microvascular triggers, which result in massive fibroblast activation and extracellular matrix (ECM) deposition. This process ultimately leads to skin and internal organ fibrosis (D. J. Abraham, J. Varga 2005).

Initiation triggers for the disease are not known but certain risk factors are identified. For example, polyvinyl chloride exposure in the US and Germany in 1930s resulted in a SSc-like illness in factory workers (P. J. Nietert, R. M. Silver 2000a). Exposure to silica dust in miners, stone masons, and others, exposure to aromatic and aliphatic

organic solvents, and exposure to epoxy resins and formaldehyde have all been linked to the occurrence of SSc (A. J. Silman, S. Jones 1992).

Some genetic links have also been identified, although small family numbers have prevented linkage analysis. Genetic susceptibility to SSc is thought to be multifactorial. Monzygotic twin studies show a 4.7% concordance for clinical disease expression, while the concordance for having antinuclear antibodies is 90%, suggesting that genetic susceptibility alone is not enough to lead to the disease (C. Feghali-Bostwick *et al.* 2003). Examples of polymorphisms found to be associated with SSc are those in fibrillin-1 gene, IL-1α, CTLA4, IL-10 and SPARC (Y. Allanore *et al.* 2007).

1.1.4 Scleroderma Histopathology:

Fibrosis is defined as the formation or development of excess fibrous connective tissue in an organ or tissue as a reparative or reactive process, as opposed to formation of fibrous tissue as a normal constituent of an organ or tissue. Cutaneous scarring is defined as the macroscopic disturbance of the normal structure and function of the skin, arising as a consequence of wound healing, and owing to the changes in epidermal, dermal and subcutaneous tissue at the time of wounding (M. W. Ferguson *et al.* 1996).

In its mild form fibrosis may result in hypertrophic scar or keloid formation, but in the most severe form fibrotic disease can occur with organ destruction and ultimately death. SSc is one such disease.

Sclerosis of the skin is a predominant and defining feature of SSc. It usually starts in the distal limbs and gradually progresses to involve other areas of the skin. Skin sclerosis starts of as active inflammation and oedema, progresses to the fibrotic

stage, and ends in atrophy. The skin score has been shown to be a useful predictor of SSc outcome (P. J. Clements *et al.* 2000b).

Histopathological findings in SSc depend on the stage of the disease. At the early stage one observes mild inflammatory infiltrates consisting of lymphocytes (mostly T helper cells), monocytes, histiocytes and plasma cells around the blood vessels and ducts of the eccrine sweat glands. The collagen fibres appear oedematous. In the later stage these infiltrates are reduced or disappear completely. The vessel walls are thickened and hyalinised, their lumen is narrowed leading to devascularisation (M. B. Kahaleh *et al.* 1979). Abundant accumulation of connective tissue and matrix proteins is first seen in the vicinity of blood vessels, in the reticular dermis, and at the border of the subcutaneous tissue. The combination of cellular infiltrates and connective tissue consisting of collagen, fibrillins and proteoglycans makes up extracellular matrix (ECM). Increased production and decreased breakdown of this fibrotic substance leads to progressive replacement of normal tissues and organs resulting in their functional impairment (J. Varga, D. Abraham 2007).

Tissue fibrosis is felt to be due to the failure of normal wound healing processes to terminate appropriately (A. Desmouliere *et al.* 2005c). The cell responsible for fibrosis is the fibroblast. In its resident, migrating and activated forms fibroblasts play an important role in wound healing. This means that in order to understand fibrosis, we must understand normal wound healing as well as its dysregulation.

1.1.5 Wound Healing:

1.1.5.1 Phases of normal wound healing:

The process of normal wound healing is highly regulated (S. Werner, R. Grose 2003g). It is thought of in three phases that occur sequentially and simultaneously.

Inflammatory phase. Immediately after injury -5 days.

In the inflammatory or reactive phase cell injury causes cytokines, growth factors, and other low molecular weight compounds to be released from serum of injured blood vessels and degranulating platelets. There is an influx of inflammatory cells which in turn release their own inflammatory mediators. Mechanical injury to the epidermis results in the release of IL-1 stored in the KC cytoplasm. Transforming growth factor β (TGF-β), resident in its latent form and bound to ECM, becomes activated. Some of the mediators (such as IL-1, TGF-β, PDGF, and EGF) contribute to the next phase of wound healing (B. J. Faler *et al.* 2006a).

Proliferative phase. 2 days -3 weeks.

The repair process restores tissue continuity through deposition of repair tissue. The proliferative phase starts with the migration and proliferation of KCs at the wound edge. This is followed by two fundamental processes: fibroplasia and angiogenesis. Massive angiogenesis leads to new blood vessel formation from capillaries growing towards the repair zone. There is migration and proliferation of dermal fibroblasts, which start laying down collagen and ECM. They are activated to form myofibroblasts and begin the wound contraction process. New nerve endings sprout around the wound edge. The resulting wound connective tissue is known as granulation tissue due to the granular appearance of the numerous capillaries it contains.

Remodelling phase. 1 week -2 years.

During this final phase the granulation tissue is slowly converted to scar tissue. This phase overlaps with the proliferative phase and is predominantly facilitated by fibroblasts. They continue their ECM and collagen production. With maturity, collagen becomes orientated in line with local stresses. Type III collagen is replaced by the stronger Type I collagen. The goal of this process is to form a structure as similar as possible to parent tissue (S. Werner, R. Grose 2003f;B. J. Faler *et al.* 2006b;J. J. Tomasek *et al.* 2002d).

1.1.5.2 The role of fibroblasts and myofibroblasts in wound healing:

1.1.5.2 (a) The fibroblast:

Fibroblasts provide a structural framework for many tissues and play a critical role in wound healing. They secrete the precursors of all the components of ECM. Wound contraction is governed by two fibroblast-driven mechanisms: tractional forces generated by fibroblasts migrating into the wound, and differentiation of fibroblasts into highly contractile myofibroblasts (A. Desmouliere *et al.* 2005b).

1.1.5.2 (b) The myofibroblast:

The myofibroblast was initially discovered by means of electron microscopy in granulation tissue of healing wounds, which led to the suggestion that these cells have a role in granulation tissue contraction. Myofibroblasts are specialised mesenchymal cells with morphology and biochemical features between those of the fibroblast and the smooth muscle cell (G. Gabbiani *et al.* 1971). Myofibroblasts express αSMA and a complex array of stress fibres and adhesions. They are the key cells for the connective tissue remodelling that takes place during wound healing and

development of fibrosis (J. J. Tomasek *et al.* 2002c). They originate from three sources: resident mesenchymal cells, blood stream circulating fibrocytes and epithelial cells with the ability to undergo mesenchymal transiyion (Kalluri, Neilson 2003).

Morphologically, these cells are characterised by the presence of a contractile apparatus containing bundles of actin microfilaments in association with contractile proteins such as non-muscle myosin. Actin bundles terminate at the specialised area of the fibroblast - the fibro nexus - which acts to connect them to the extracellular fibronectin fibres via transmembrane integrins. The fibronexus serves to transmit the mechanical force generated by actin-myosin interaction to the ECM and carry extracellular signals back into the cells. Myofibroblasts are connected by gap junctions to form a multicellular lattice during granulation tissue contraction (J. J. Tomasek *et al.* 2002b).

It has been shown that initiation of granulation tissue contraction does not require the presence of myofibroblasts, and that it is the tractional forces generated by migrating fibroblasts that are important in the initiation of wound closure (P. J. Clements *et al.* 2000a). Following this initial migration, fibroblasts transform to proto-myofibroblasts. These cells express β- and γ-cytoplasm actins that are also normally found in resident fibroblasts. A crucial signal for the transformation to proto-myofibroblasts is mechanical tension. This was confirmed in experiments that used granulation tissue tension changes to measure markers of fibroblast activation. It was proposed that tractional forces that develop in the closing wound cause the fibroblasts and collagen fibres to align along lines of stress and thus acquire the proto-myofibroblast phenotype (B. Hinz *et al.* 2001b).

Proto-myofibroblasts, once formed, can be stimulated to develop into

myofibroblasts. It is proposed that this latter transformation does not occur before day four of wound healing, and requires both tension and additional growth factors such as TGF-β (P. Shephard *et al.* 2004a).

1.1.5.2 (c) In experimental environments:

Fibroblast populated three-dimensional collagen lattices are used in vitro to study reciprocal cell-matrix interactions (X. Shi-Wen *et al.* 2004c). There are three major FPCL models used: tension loaded (tethered or pre-stressed), stress-relaxed, and free floating. The difference is governed by whether the FPCL is restrained which determines the mechanical loading of the cells.

The free floating collagen matrix is released immediately upon polymerisation. The fibroblasts remain relatively spherical with fine protrusions to contract the matrix (F. Grinnell 2000).

The stress-relaxed system allows fibroblast pre-activation. Here FPCL remains attached to the container wall for 24 hours allowing morphological transformation and development of tractional force. During these first hours fibroblasts develop pseudopodial extensions. Contractional force on the lattice is then generated via cell attachment to fibronectin (which contains an ED-A domain) and traction without migration (M. Eastwood *et al.* 1996b). This is because as fibroblasts attach and pull on the substrate, the rear of the cell remains attached to the substrate. Fibroblasts tend to align themselves with the direction of maximal principle strain (M. Eastwood *et al.* 1998). In a circular 3D collagen model this means that the lattice is drawn in from 360 ˚, and the overall effect is centrifugal traction with a starburst appearance under electron microscopy (R. K. Sawhney, J. Howard 2002). Once the lattice is released, the mechanical tension together with a profibrotic stimulus such as TGF-β,

causes fibroblast differentiation to its active α-smooth-muscle actin containing contractile form - the myofibroblast (F. Grinnell 2003e). Since fibroblasts in restrained but not floating matrices can organize cell surface fibronectin matrix and develop fibronexus junctions, TGF-β only stimulates the differentiation of fibroblasts into myofibroblasts in restrained matrices. In addition matrix synthesis and cell proliferation can only occur in mechanically loaded lattices (F. Grinnell 2003d). The result of fibroblast contraction is a visible decrease in lattice diameter which can be measured and compared (P. D. Arora *et al.* 1999b;B. Hinz *et al.* 2001a).

The tension loaded system is similar to the pre-stressed one, except it is not released at any point and contraction is measeured by tension monitors (M. Eastwood *et al.* 1996a).

1.1.5.2 (d) Summary:

In summary, during normal healing, fibroblasts migrating into the wound area initiate wound contraction and lay down ECM. Tractional forces then confer the change to proto-myofibroblasts, following which the combination of traction and TGF-β cause their transformation to myofibroblasts (J. J. Tomasek *et al.* 2002a).

1.1.5.3 From normal wound healing to pathological fibrosis:

Physiological wound healing is spatially and temporally self-limiting. Normal fibroblasts can modulate their collagen production in response to tissue requirement, and once sufficient ECM is laid down and re-epithelialisation is complete they either revert to the quiescent state or undergo apoptosis. This way organ function is restored and cellular and collagen interactions are then remodelled gradually to strengthen the scar (S. O'Kane, M. W. Ferguson 1997). However, if fibroblast and

myofibroblast activity continues unabated, ECM secretion and tissue contraction will also persist. Pathological scar formation, and ultimately fibrosis will take place as a result. In support of this notion, myofibroblasts have been shown to persist in different fibrotic lesions including those of interstitial pulmonary fibrosis (IPF) and stromal tumours (H. F. Dvorak 1986;C. Kuhn, J. A. McDonald 1991). In addition, fibroblasts from SSc lesional skin grown in culture have been shown to produce more soluble collagen than the paired controls, and to have the capacity to synthesise collagen after up to fifteen subcultures (E. C. LeRoy 1974).

The important question is what causes the switch from normal regulated cells to persisting and autonomous ones capable of destructive fibrosis? Possible explanations include activation of fibroblasts by immune cells, inappropriate expression and/or persistence of fibrosis enhancing growth factors, or persistent fibroblast activation following environmental stress or cell injury.

1.1.6 Transforming growth factor β (TGF-β):

1.1.6.1 Background:

Since the early nineties investigators have been pursuing the role of TGF-β in fibrotic disease (G. C. Blobe *et al.* 2000). TGF-β is a multifunctional cytokine with proinflammatory as well as immunosuppressive activities (J. Massague 1990). TGF-β signalling controls a diverse set of cellular processes. Its expression serves in both autocrine and paracrine modes to cause cell proliferation, differentiation, apoptosis, and production of extracellular matrix proteins, as well as to determine cellular developmental fate during embryogenesis and in mature tissues. TGF-β is also known to affect nearly all aspects of wound repair.

Discovered in 1978 by Todaro and De Larco it was called the ‘sarcoma growth

factor' (SGF). The name was given because this factor, produced by virally transformed cells, caused phenotypic transformation of a normal reader cell into one able to grow in soft agar, a characteristic usually attributed to malignant cells only. It was also noted that SGF induced the growth of many large colonies of normal rat kidney cells, and was therefore re-named TGF-β. In 1980s TGF-β was purified from various different tissues including placenta, liver and heart. And in 1984 the first discovery of the inhibitory action of TGF-β was made (M. B. Sporn 2006b). In 1985 the complex nature of TGF-β was emerging in demonstration of its multifunctionality that depended on the local milieu. Thus, in the presence of PDGF, TGF-β caused stimulation of growth of reader cells, while in the presence of EGF, TGF-β functioned as a growth inhibitor (M. B. Sporn 2006a).

1.1.6.2 TGF-β structure:

Members of the TGF-β super-family are dimeric proteins with nearly 30 members in mammals that include TGF-β, bone morphogenic proteins (BMP), growth /differentiation factors (GDF), and anti-Mullerian hormone (AMH). All members have a conserved carboxyl terminal feature consisting of seven cysteine residues, six of which form a rigid cysteine knot. TGF-β exists in three isoforms: TGF-β 1, 2 and 3 which share 64-85% amino acid sequence homology and reside in the extracellular matrix of uninjured tissues (J. Massague 1990). All three isoforms exert similar effects grouped into three broad areas: inflammatory cell function regulation, growth inhibition and differentiation, and ECM production (J. S. Munger *et al.* 1999c). TGF-β is secreted in latent form noncovalently associated with latency associated peptide (LAP), which renders it unable to bind its receptor (i.e.TGF-β is latent). This small latent complex (SLC) of LAP and TGF-β is joined by latent TGF-β binding protein 1 (LTBP) which is a matrix proteins similar to fibrillin (S. Sinha *et al.*

1998a). Serum proteins intimately associated with the wound healing response catalyse TGF-β activation. Plasmin and thrombospondin-1 integrin have been shown to cleave the active form from the complex (J. P. Annes *et al.* 2003). Integrin $\alpha_v\beta_6$ is expressed in the epithelium. It has been shown that SLC is a ligand for this integrin, and their binding acts to change the conformation of the complex to allow TGF-β binding to TGF-β receptor (J. S. Munger *et al.* 1999b).

1.1.6.3 TGF-β signalling cascade:

Over the last ten years researchers have focused on delineating the TGF-β signalling pathways (A. Nakao *et al.* 1997). The intracellular messengers involved are of considerable interest as potential antifibrotic therapeutic targets.

The following signalling cascade is currently described (Figure 1.2).

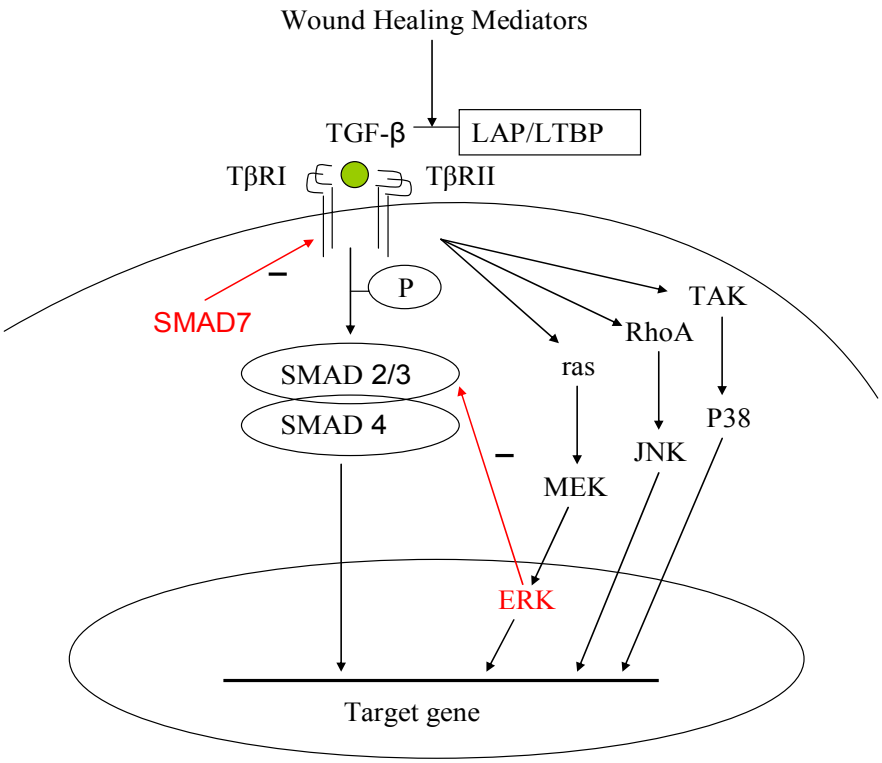


Figure 1.2: Simplified diagram of the TGF-β signalling cascade.

Most cells express two types of TGF-β receptor (TβR I and II). Both are transmembrane serine/threonine protein kinases. TβRI (or ALK5) cannot bind a free ligand, only a ligand that is bound to TβRII. Upon binding to TβRII (which is a constitutively active kinase), TβRI is activated and phosporylates downstream targets. These targets are a conserved family of intracellular signalling effector proteins called SMADs named after the genes Mad and Sma discovered in *Drosophila* and *Caenorhabdis elegans* respectively. The receptor activated SMADs transiently interact with the activated receptor complex and are C-terminally phosphorylated (R. Derynck *et al.* 1998c). After phosphorylation, receptor-activated SMAD2 and SMAD3 form a heterooligomeric complex with SMAD4 (a common mediator). The complex then moves to the nucleus (a key step in signal transduction) to activate transcriptional promoters directly or by combining with other transcription factors (J. Massague, R. R. Gomis 2006). SMAD7 is an intracellular antagonist of TGF-β signalling. It associates with activated TβRs and prevents SMAD2/3 binding and consequent phosphorylation (Y. Shi, J. Massague 2003). It is believed that fine-tuning of cellular responses to TGF-β is performed through the interaction of the SMAD cascade with other signalling molecules. In fact, TGF-β is capable of acting through pathways other than SMAD, including the MAP kinase pathway, in a cell and tissue specific fashion (A. Moustakas, C. H. Heldin 2005). TGF-β/SMAD signalling can be controlled by the ras/MEK/ERK MAPK cascade with ERK phosphorylation sites existing on SMAD3 and 4 linker regions (K. M. Mulder 2000). The MAP kinase pathway has different effects on SMAD signalling depending on the cell involved. In fibroblasts, the MAP kinase pathway is required for SMAD-mediated CTGF induction by TGF-β. A prostacyclin derivative iloprost blocks TGF-β-driven fibrosis by suppressing MAP kinase signalling in a protein

kinase A (PKA)-dependent fashion (R. Stratton *et al.* 2002l).

In kidney mesangial cells the MAP kinase system activation potentiates SMAD signalling (Y. Chen *et al.* 2002). In epithelial cells, on the other hand, this cascade is inhibitory of the SMAD pathway through blocking nuclear accumulation of SMADs (A. Leask, D. J. Abraham 2004). Since TGF-β is anti-proliferative in most cells, the latter could explain why in the context of oncogenesis ras activation causes uncontrolled cell proliferation (M. Kretzschmar *et al.* 1999).

In general it is clear that TGF-β signalling is highly complex. Its exact effect depends on the cell and tissue and on the interaction with signalling cascades activated by other molecules, as well as its own target gene activation.

1.1.6.4 Connective tissue growth factor (CTGF):

One of the most important targets of TGF-β signalling is the *CTGF* gene. CTGF is a 38kD heparin-binding cysteine-rich glycoprotein that functions as a downstream mediator of TGF-β-induced collagen deposition. It is a member of the CCN immediate early growth-responsive gene family which contains cyr61, nov, elm1, Cop1, and WISP-3 and 2 (E. E. Moussad, D. R. Brigstock 2000b).

It was originally identified in the human umbilical vein endothelial cells through its cross-reactivity with an antibody to PDGF. However, it was shown to be a separate moiety with mitogenic activity for endothelial cell and fibroblasts. It was then designated CTGF (D. M. Bradham *et al.* 1991). The same investigators demonstrated coordinate expression of CTGF and TGF-β in normal wound repair, and showed that TGF-β stimulated CTGF production in human foreskin fibroblasts (A. Igarashi *et al.* 1993). Around the same time CTGF was isolated from TGF-β-treated mouse cells (A. Brunner *et al.* 1991).

Epithelia of many normal tissues are also found to contain CTGF, where it is believed to be involved in regulation of cell differentiation and apoptosis (E. E. Moussad, D. R. Brigstock 2000a). The involvement of CTGF in wound repair is supported by the experiments showing activation of this growth factor in KCs following binding of factor VII to tissue factor (E. Camerer *et al.* 2000). Currently CTGF is thought to play an important role in wound healing and fibrotic reactions, and is implicated in pathogenesis of SSc. In fact, CTGF has been repeatedly identified in fibroblasts from sclerotic skin lesions of SSc patients. A positive correlation has been found between the level of skin sclerosis and CTGF mRNA content (A. Igarashi *et al.* 1995;A. Igarashi *et al.* 1996a). There is also a positive correlation between the level of skin sclerosis and the circulating levels of CTGF (S. Sato *et al.* 2000). Sampling of dermal interstitial fluid of SSc lesional skin demonstrated increases CTGF levels compared to healthy controls (R. Stratton *et al.* 2001a).

Various studies have been designed to confirm the role of CTGF as a downstream signal and a synergistic contributor to TGF-β activation and fibrotic action. It has been shown that in vitro, TGF-β-treated SSc fibroblasts produce greater amounts of CTGF than normal fibroblasts (K. Kikuchi *et al.* 1995). Recombinant CTGF and neutralising antibodies to this growth factor have been used to demonstrate that CTGF has a similar effect on fibroblast proliferation, ECM production, and granulation tissue formation as TGF-β (I. E. Blom *et al.* 2001;J. K. Crean *et al.* 2002;B. S. Weston *et al.* 2003). In vivo, subcutaneous CTGF injection into neonatal mice resulted in granulation tissue formation and ECM synthesis. In situ hybridisation confirmed that TGF-β could induce CTGF production in dermal fibroblasts at that injection site (K. Frazier *et al.* 1996). CTGF and TGF-β injection

into new-born mice established the synergistic role of these two growth factors. Separate applications induced transient fibrosis only. However, when injected together, a lasting fibrotic response could be elicited (T. Mori *et al.* 1999a). This finding was confirmed in vitro, when collagen matrix contraction by TGF-β was shown to be generated by CTGF. The same investigators later established that CTGF maintains the TGF-β induced skin fibrosis by sustaining COL1A2 collagen gene promoter activity and by increasing the number of activated fibroblasts (S. Chujo *et al.* 2005;A. Holmes *et al.* 2003a).

A recent study has confirmed that normal and SSc human skin fibroblasts produce CTGF after stimulation with TGF-β. However, the latter were much greater producers. The same study demonstrated an upregulation of the gene for CTGF in SSc fibroblasts, and showed that CTGF stimulated a two- to three-fold increase in proα1(I) collagen and fibronectin synthesis by dermal and lung fibroblasts. This produced significant contraction of FPCLs (X. Shi-Wen *et al.* 2000d). In an elegant experiment, the same investigators also attained evidence for the CTGF over-expression as a cause of increased collagen synthesis by fibroblasts. They did this by transfecting CMV-CTGF cDNA constructs into normal fibroblasts. This caused an increase in the transcription of Colα2 promoter-reporter construct to the level seen in SSc fibroblasts.

Normal dermal fibroblasts do not express CTGF unless stimulated by TGF-β, for example during normal wound healing (A. Igarashi *et al.* 1996b). However, fibroblasts from fibrotic lesions express CTGF genes constitutively, even in the absence of stimulation by TGF-β (X. Shi-Wen *et al.* 2000c). An increase in the basal promoter activity of the CTGF ccn2 gene has been found in dermal fibroblasts from SSc skin. Interestingly, CTGF promoter over-activity in lesional fibroblasts seemed

to be independent of the SMAD response element and the TGF-β response element which are responsible for CTGF expression in normal fibroblasts (A. Holmes *et al.* 2001a;A. Leask *et al.* 2001;A. Leask *et al.* 2003). Instead, a novel promoter element containing an Sp1 binding site has been shown to be involved in CTGF over-expression in SSc fibroblasts.

1.1.6.5 TGF-β in wound healing:

The presence of TGF-β is consistently found in the wound healing environment. Researchers have demonstrated TGF-β mRNA in incisional wounds of rats, as well as in activated macrophages harvested from wound chambers. Studies where TGF-β was added to wounds reported an accelerated rate of healing. When purified bovine TGF-β was injected into wire mesh chambers on the backs of rats, an accumulation of total protein, collagen, and DNA was observed (M. B. Sporn *et al.* 1983). McGee *et al.* reported that a single application of recombinant TGF-β1 accelerated incisional wound healing and increased tensile strength of rat wounds (G. S. McGee *et al.* 1989). Supporting the role of TGF-β in healing was the fact the exogenous application of this growth factor accelerated wound closure delayed by glucocorticoids (L. S. Beck *et al.* 1991;L. S. Beck *et al.* 1993;G. F. Pierce *et al.* 1989). It was demonstrated that the levels of TGF-β1 and 2 were decreased in the glucocorticoid-treated wounds. In a different model of impaired wounding -the elderly skin- TGF-β levels were also reduced (G. S. Ashcroft *et al.* 1995).
These observations led to the conclusion that dysregulation of TGF-β expression could be responsible for abnormal wound repair (S. Frank *et al.* 1996;P. Schmid *et al.* 1993). More recent experimental data supports this notion. In rat tissue transfected with TGF-β1 expression constructs, wound repair was markedly

improved (S. I. Benn *et al.* 1996).

TGF-β is important in all phases of wound healing. Released primarily from α granules of platelets and from the LAP-bound pool resident in the tissues, as well as from most inflammatory cells that migrate in, TGF-β is vital in the reactive phase of wound healing as a powerful chemoattractant (S. Sinha *et al.* 1998b;S. Werner, R. Grose 2003e). In fact, TGF-β is the most potent chemotactic factor described for human peripheral blood neutrophils (T. Parekh *et al.* 1994). In addition, TGF-β stimulates the influx of macrophages into the wounded area (C. P. Kiritsy *et al.* 1993). It has been demonstrated that SMAD3 null mice have significantly fewer inflammatory cells in their wounds compared to wild-type mice. Also, macrophages from SMAD3 null mice showed dramatically reduced migration to a TGF-β stimulus, with suppressed ability to digest ECM and debris (G. S. Ashcroft *et al.* 1999b).

For the purposes of this thesis, the role of TGF-β in the proliferative and remodelling phases of wound healing, which are marked by fibroblast proliferation and ECM deposition, as well as angiogenesis and re-epithelialisation is particularly notable. It is the uncontrolled processes of these stages of wound healing that potentially lead to fibrotic disease. When injected subcutaneously into newborn mice TGF-β activates fibroblasts and causes angiogenesis, and thus granulation tissue formation (A. B. Roberts *et al.* 1986a). A single TGF-β application accelerated healing of rat incisional wounds by 3 days, and elicited a 220% increase in wound strength after only 5 days. This was accompanied by increased fibroblast infiltration and collagen deposition (T. A. Mustoe *et al.* 1987). In addition, Quaglino *et al.* demonstrated the effect of TGF-β on excisional and incisional porcine wounds. After TGF-β application granulation tissue formation was significantly increased. In situ

hybridisation studies demonstrated upregulation of ECM protein expression, and downregulation of proteolytic enzyme expression. An auto-induction of TGF-β mRNA in the wounds was also noted (D. Quaglino, Jr. *et al.* 1990).

In summary, TGF-β accelerates wound healing processes of normal and impaired wounds.

1.1.6.6 TGF-β in fibrosis:

TGF-β is a good candidate for a fibrogenic growth factor. It is a potent chemotactic factor for fibroblasts, and stimulates the synthesis of ECM by explanted cultured fibroblasts (G. F. Pierce *et al.* 1991b;G. F. Pierce *et al.* 1991a;A. E. Postlethwaite, J. M. Seyer 1990;A. B. Roberts *et al.* 1986b). It was demonstrated back in 1979 that fibroblasts from SSc lesional skin do produce increased amounts of procollagen type I and II (J. Uitto *et al.* 1979). The same investigators later confirmed increased co-expression of type VII, type VI and type I collagen and TGF-β in the dermis of SSc patients (J. Peltonen *et al.* 1990e;L. Rudnicka *et al.* 1994a;J. Peltonen *et al.* 1990a). It has been shown that TGF-β stimulates the human promoter region of the Type I collagen gene (COL1A2) activity via SMAD signalling (S. J. Chen *et al.* 1999). The immunoreactivity of TGF-β in the skin of patients with diffuse SSc (as well as limited disease) was shown to be present prior to the onset of fibrosis and in its initial stages, indicating an early involvement of this factor in the disease (L. Rudnicka *et al.* 1994b).

In its additional pro-fibrotic role, TGF-β stimulates the secretion of proteinase inhibitors such as plasminogen activator inhibitor and tissue inhibitor of matalloproteinases, and inhibits production of proteinases, thus preventing ECM breakdown in preparation of the wound for the remodelling phase of healing (C. P.

Kiritsy *et al.* 1993).

Many experiments have highlighted the role of TGF-β in fibrotic responses in vivo. When foetal rabbit subcutaneous sponge implants containing TGF-β1 were analysed 7 days post wounding they were found to contain a large number of inflammatory cells and fibroblasts with marked collagen deposition (T. M. Krummel *et al.* 1988). In IPF, foci of activated fibroblasts resembling those found in healing wounds are responsible for the fibrotic process. Using in situ hybridisation and immunohistochemistry TGF-β was demonstrated adjacent to these foci and suggested to be the stimulus responsible for persistent expression of connective tissue genes (T. J. Broekelmann *et al.* 1991).

Lung treatment with bleomycin induces pulmonary fibrosis and TGF-β upregulation. Neutralising antibody to TGF-β reduces this fibrotic reaction (S. N. Giri *et al.* 1993). I has been shown that foetal wounds heal without scarring, and with a diminished inflammatory and cytokine response than adult wounds. When adult rat healing dermal wounds were injected with a neutralising antibody to TGF-β they healed without scar-tissue formation, less inflammation, and lower collagen and fibronectin content than the paired controls (M. Shah *et al.* 1992). In support of this finding, it has been shown that TGF-β mRNA can be induced by wounding adult skin, but not foetal skin. However, when TGF-β was added to the foetal skin, TGF-β mRNA expression was found in the fibroblasts, an adult-like inflammatory response occurred, and the skin healed with scar formation. This confirmed that TGF-β was an important modulator of fibrosis in vivo (R. Y. Lin *et al.* 1995).

Recently, a transgenic mouse model of fibrosis expressing a kinase-deficient type II TGF-β receptor on fibroblasts provided further evidence for the pivotal role of TGF-β over-activity in fibrosis. These mice developed both dermal and lung fibrosis, their

fibroblasts over-producing CTGF and type I collagen. In this model the fibroblast to myofibroblast differentiation was also increased (C. P. Denton *et al.* 2005).

1.1.6.7 TGF-β in fibroblast to myofibroblast transformation:

The presence of myofibroblasts has been described in every known fibrotic situation. Most of the SSc fibroblasts are known to be myofibroblasts expressing α smooth muscle actin (αSMA) (A. P. Sappino *et al.* 1990). It has been shown that fibroblasts from lesional (scarred) skin of patients with SSc persist as active myofibroblasts, and when cultured, have enhanced ability to produce, adhere to, and contract ECM (Y. Chen *et al.* 2005e).

TGF-β is responsible for the phenotypic change from fibroblast to myofibroblast (R. Abe *et al.* 2001;Y. Chen *et al.* 2005d). Desmouleir *et al.* demonstrated that in rats, subcutaneous injection of TGF-β caused an increase in αSMA-expressing granulation tissue fibroblasts. Moreover, TGF-β induced αSMA expression in growing and quiescent cultured fibroblasts, an effect partly inhibited by antibodies to TGF-β (A. Desmouliere *et al.* 1993).

1.1.6.8 TGF-β in SSc:

Uncontrolled ECM production as a consequence of disordered wound healing mechanisms, fibroblast and myofibroblast persistence, and ultimately fibrosis are the hallmarks of SSc. The evidence presented above implicates TGF-β in all these processes and thus in SSc pathogenesis. One of the first studies to demonstrate intense immunohistochemical staining for TGF-β in the dermis of SSc skin was by Sfikakis *et al.* in 1993. They asked the question as to the source of this growth factor, and proposed that it was unlikely to have been derived from the inflammatory

infiltrate as there was no perivascular inflammatory cell staining for TGF-β in their study. However, they did find positive staining in dermal fibroblasts, and the epithelial cells (P. P. Sfikakis *et al.* 1993c). This finding was in line with an earlier one by Gruschwitz *et al.*, who found increased levels of TGF-β in dermal and epidermal cells of SSc skin (M. Gruschwitz *et al.* 1990b).

Additional later experiments demonstrated increased TβR expression in fibroblasts from the dermis of SSc patients, with antibodies to TGF-β abolishing the increased mRNA expression as well as up-regulated transcriptional activity of the human alpha2(I) collagen gene (H. Ihn *et al.* 2001b). Chen *et al.* have demonstrated that enhanced matrix contraction by dermal fibroblasts from SSc patients required TGF-β and its signalling pathways (Y. Chen *et al.* 2005c). Also, a disordered signalling pathway, with increased SMAD2/3 phosphorylation and decreased expression of the inhibitory SMAD7 has been found in SSc fibroblasts (C. Dong *et al.* 2002). In murine models, SMAD7 deficiency has been shown to be associated with renal fibrosis (M. Schiffer *et al.* 2001;K. Uchida *et al.* 2000). However, two other studies failed to demonstrate a difference in SMAD7 expression between normal and SSc fibroblasts (A. Holmes *et al.* 2001b;Y. Mori *et al.* 2003f). A ligand-independent constitutive activation of the intracellular TGF-β/SMAD signalling axis in SSc fibroblasts has been shown, confirming the importance of disordered SMAD signalling in SSc pathogenesis and fibroblast autonomy (Y. Mori *et al.* 2003e).

Various animal models of SSc confirm the importance of TGF-β and its signalling pathways in pathogenesis of this disease. A mouse model of SSc has been established by repeated subcutaneous injections with bleomycin. Here, anti-TGF-β antibody caused a significant reduction in cutaneous sclerosis and inflammation (T. Yamamoto *et al.* 1999b). In the same model Takagawa *et al.* demonstrated

predominantly nuclear localisation of SMAD3 and intense staining for pSMAD2/3 in fibroblasts after bleomycin injection (S. Takagawa *et al.* 2003a). The role of SMAD3 and TGF-β in fibrosis was later confirmed in SMAD3 null mice injected with bleomycin. This transgenic model demonstrated a decreased fibrotic response, CTGF and collagen production, and αSMA expression. The SMAD3 null mice also had a decreased fibrotic response to TGF-β (G. Lakos *et al.* 2004).

A mouse graft-versus-host disease model of SSc has also been developed. It was shown to resemble the human condition more than the chemically-induced disease (M. C. Ruzek *et al.* 2004). This model was used to demonstrate that TGF-β inhibition with a neutralising antibody prevented the skin and lung fibrosis caused by TGF-β (L. L. McCormick *et al.* 1999).

1.1.6.9 TGF-β in SSc skin fibrosis:

Despite the evidence presented above for the role of TGF-β in SSc, some contradictory data exists regarding the role of TGF-β in the maintenance of SSc fibrosis. For example, Peltonen *et al.* found no elevation of TGF-β mRNA in SSc skin biopsy samples (J. Peltonen *et al.* 1990b), and TGF-β was unable to induce the SSc phenotype in normal fibroblasts, or to sustain this phenotype in vitro (A. McWhirter *et al.* 1994b). Kinase-deficient TβRI failed to down-regulate collagen synthesis by SSc fibroblasts (J. Pannu *et al.* 2004b). No correlation has been found between the circulating levels of TGF-β and SSc severity (M. Dziadzio *et al.* 2005a;N. Snowden *et al.* 1994). Also, as mentioned previously, the inflammatory cell infiltrate in SSc skin does not stain for the growth factor (P. P. Sfikakis *et al.* 1993b).

It has been shown that TGF-β1 and 2 are present in the inflammatory skin of SSc

patients but not in the established sclerotic lesions (C. Querfeld *et al.* 1999b). In addition, TGF-β mRNA expression in lesional tissues is early and transient (T. Yamamoto *et al.* 1999c)**.**

It has been suggested that dysregulated signalling downstream of TβR could account for SSc pathogenesis independently of continued stimulation by TGF-β (S. Takagawa *et al.* 2003b). SMADs are a possible molecular candidate for autonomous over-activation. When increased SMAD2/3 phosphorylation and nuclear translocation were demonstrated in SSc fibroblasts, withdrawal of TGF-β stimulus failed to reverse this effect (Y. Mori *et al.* 2003d).

Another candidate for an autonomous profibrotic agent is CTGF because its expression in SSc fibroblasts can be SMAD-independent (A. Holmes *et al.* 2001c). Since CTGF is consistently over-expressed in fibrotic lesions (M. Dziadzio *et al.* 2005c), its amount correlating well with the extent of fibrosis, and it seems to be able to function independently from TGF-β signalling, it has been suggested that TGF-β is only important transiently in the early inflammatory phase of SSc and not during the established fibrotic stage (A. Leask 2004). It is thought that TGF-β acts via the downstream CTGF expression and synergistically with it during the initiation of fibrosis, following which CTGF works to sustain the fibrotic response (A. Holmes *et al.* 2003b).

Another possibility, however, is that TGF-β is involved in the maintenance as well as initiation of fibrosis through the release of its active and latent forms from a source outside the dermis.

Most previous studies give emphasis to alterations of the dermis in SSc and show increased dermal thickness, ECM abundance with increased collagen deposition, and deficiency of microvessels. The epidermis, on the other hand, has received relatively

little attention.

1.1.7 The Epidermis:

1.1.7.1 Definition:

Epidermis is the outermost layer of the skin. It forms the waterproof protective cover for the body's surface and is made up of stratified squamous epithelium with an underlying basal lamina. The epidermis is divided into five sub-layers: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum germinatum from the superficial to deep layers respectively. The cells of the epidermis are predominantly keratinocytes (KCs), but also include dendritic cells and melanocytes. KCs move through the sub-layers towards the surface and change shape and composition, until eventually they are sloughed off in a process called desquamation (E. Fuchs 1993).

1.1.7.2 Epidermis is an active secretory organ:

For many years the epidermis was considered to be a passive mechanical barrier that retards entry of microbes, prevents fluid loss from the body, and forms the lining of body structures. Under normal homeostatic conditions these are indeed the functions of the epidermis. In disease states, it used to be presumed that the epidermis was a passive target for immunological attack from infiltrating inflammatory cells, participating only in so much as presenting the antigen via MHC class II cells. However, since 1970s it has been demonstrated that KCs are capable of producing numerous cytokines involved in active biological processes. These cytokines act on immune and inflammatory cells such as fibroblasts, endothelial cells and lymphocytes to induce a host response to external stimulae locally and within the

dermis. In other words the epidermis is an active participant in inflammation (J. N. Barker *et al.* 1991b).

To prove that the epidermis is active in inflammation, Nickoloff *et al.* showed that when the barrier function of the skin was abrogated by repeated tape stripping of healthy human volunteer skin, KCs became activated within six hours to produce various cytokines. These events preceded any inflammatory cell influx into the area and therefore reflected changes intrinsic to the epidermis (B. J. Nickoloff, Y. Naidu 1994).

Over the last two decades KCs have been shown to express the following factors and their receptors: IL-1, IL-6, IL-12, TGF, ET-1, EGF, FGF, VEGF , IL-21R, and GM-CSF to name but a few (H. Uchi *et al.* 2000a).

1.1.7.3 Epidermal-dermal cross-talk:

The idea of epidermal-dermal crosstalk is not new. Interactions between epithelial and mesenchymal cells play a crucial role in the regulation of tissue development, homeostasis and repair. In embryogenesis epithelial-mesenchymal interaction is recognised to be critical (J. M. Shannon, B. A. Hyatt 2004). For example, TGF-β is implicated in lung and palate formation through this type of cross-talk (V. Kaartinen *et al.* 1995).

In adult skin, this interaction was first demonstrated in the 1970s by Rheinwald and Green. They showed that normal human epidermal KCs require the presence of fibroblasts for efficient growth in tissue culture (J. G. Rheinwald, H. Green 1975). Later, a fibroblast-derived growth factor was identified and shown to stimulate the proliferation of a KC cell line. Owing to its unique activity, the new mitogen, which was shown to belong to the fibroblast growth factor (FGF) family, was termed

keratinocyte growth factor (KGF) (J. S. Rubin *et al.* 1995). Granulocyte–macrophage colony stimulating factor (GM-CSF), interleukin 1 (IL-1), IL-6, PDGF and a new member of the TGF-β superfamily activin were next shown to be important in paracrine stimulation loops between the epidermis and fibroblasts (G. Hubner *et al.* 1999;H. Smola *et al.* 1993b;S. Werner *et al.* 2007f).

TGF-β has been demonstrated to be co-expressed in all four layers of the differentiating epidermis as well as in the basal lamina and mesenchymal cells during excisional wound repair. This led to the suggestion that TGF-β had a role in epidermal-dermal interaction during wound healing (J. H. Levine *et al.* 1993). In addition, when SSc skin biopsies were examined for the presence of TGF-β, TGF-β3 was found subepidermally (C. Querfeld *et al.* 1999a). This finding supported the notion of TGF-β diffusion through the basal lamina into the dermis.

In a transgenic mouse model over-expressing TGF-β in the epidermis, type I collagen mRNA and hydroxyproline content were significantly increased in the wound area. This result also supported the idea that when TGF-β was released from KCs after wounding it penetrated to the underlying dermis and stimulated fibroblasts to produce collagen (L. Yang *et al.* 2001).

The epidermal-dermal interaction has been studied in co-cultures of normal human KCs and dermal fibroblasts. When normal explanted fibroblasts were co-cultured with a normal epidermal cell line, ECM and smooth muscle cell associated gene transcripts were upregulated in fibroblasts, suggesting their differentiation into myofibroblasts. In addition IL-1 responsive genes such as the genes for KGF, IL-6, ET-1, heparin binding EGF, GM-CSF were upregulated in fibroblasts(S. Werner *et al.* 2007e). The effect required the presence of TGF-β. αSMA production did not occur until day four despite the presence of TGF-β. This was attributed to IL-1-

mediated NF-Kb induction and thus TGF-β inhibition from days one to three (P. Shephard *et al.* 2004g). A reciprocal paracrine loop between KCs and fibroblasts was proposedn where IL-1 released from KCs caused production of KGF by fibroblasts, which in turn stimulated KC proliferation and differentiation(N. Maas-Szabowski *et al.* 1999g).

Several experiments have demonstrated the importance of epidermal-dermal cross-talk in fibrotic disease. Recent studies have proposed that the lung scarring condition IPF is caused by multiple cycles of epithelial injury and activation. The authors suggested that the resulting active alveolar epithelial cells (AECs) induce migration, proliferation and activation of the underlying mesenchymal cells. The formation of fibroblastic/ myofibroblastic foci and the exaggerated accumulation of ECM that ensues, mirrors abnormal wound repair, and accounts for the disease pathogenesis (M. Selman 2001;M. Selman, A. Pardo 2002). In pulmonary fibrosis epithelial cells initiate the fibrotic response via integrin-$\alpha_V\beta_6$ dependent activation of TGF-β, showing that epithelial cells can be involved in the induction of fibrotic response (R. G. Jenkins *et al.* 2006). $\alpha_V\beta_6$ knockout mice develop an enhanced inflammatory response, but no fibrosis (J. S. Munger *et al.* 1999a).

It has been demonstrated that the receptor for IL-21 (IL-21R) is over-expressed in SSc skin KCs, accompanied by vascular endothelial growth factor (VEGF) over-expression in KCs, fibroblasts and endothelial cells. This led to the suggestion that IL-21R induction in KCs may be causing dermal VEGF activation and thus the disturbed blood vessel morphology typically seen in SSc (J. H. Distler *et al.* 2005c).

In support of TGF-β's involvement in epidermal-fibroblast crosstalk in fibrotic disease are the experiments performed with keloid scar cells. Keloids represent a pathological wound healing response. Co-culture of keloid-derived KCs with

fibroblasts from normal or keloid skin increased the fibroblast proliferation rate. Further studies demonstrated that these KCs expressed more of the TGF-β and its receptor than normal KCs, and addition of TGF-β antibody slowed the fibroblast proliferation in co-culture. In addition, the fibroblasts co-cultured with keloid KCs produced more ECM proteins and had increased levels of TGF-β activity (W. Xia *et al.* 2004a).

Insummary, ample evidence exists for KC-fibroblast interactions both in wound healing and fibrotic disease, potentially resulting in fibroblast activation and pathological fibrosis.

1.1.7.4 Epidermis in SSc:

Perhaps the most idea leading to the current study is that the epidermis in SSc is fundamentally different from normal epidermis, with its structure and function significantly disordered. Relatively, little is published about the epidermis in SSc. A new technique of confocal laser scanning microscopy (CLSM) has been used to image the skin of patients with SSc non-invasively and in real time. The epidermis was found to be hypertrophied with a large number of melanocytes and an increased melanin content (K. Sauermann *et al.* 2002a). Changes in skin pigmentation which can be severe and widespread imply that the epidermis is also abnormal in the disease (H. Tabata *et al.* 2000a)]. ET-1 (H. Tabata *et al.* 2000b), TGF-β (L. Rudnicka *et al.* 1994c), monocyte chemo-attractant protein-1 (MCP-1) (O. Distler *et al.* 2001a), VEGF (C. A. Davies *et al.* 2006b) and IL-21 receptor (IL-21R) (J. H. Distler *et al.* 2005b) have all been shown to be up-regulated in the epidermis in SSc. Recent results from this laboratory demonstrate that the epidermis in SSc is thickened and has an expanded nucleated cell layer. Proteomic analysis revealed

altered abundance of proteins involved in extracellular matrix production, myofibroblast contractility, energy metabolism and response to oxidative stress. In addition, proteins specific to the epidermis and involved in epidermal cell differentiation were altered in abundance in the disease (N. Aden *et al.* 2008a).

1.1.8 Regulation of KC migration and proliferation:

1.1.8.1 TGF-β in KC migration:

TGF-β has already been discussed as being important in wound healing. In the epidermis this growth factor has a pro-migratory, but an anti-proliferative effect on KCs.

Cell migration is the rate-limiting step in wound healing. In the process of re-epithelialisation KCs migrate from the wound edge across the wound bed to cover the defect. It is felt that the suprabasal KCs are the ones actively participating in this process (M. L. Usui *et al.* 2005b).

The pro-migratory effect of TGF-β has been confirmed in several experiments. Epidermal cell outgrowth from partial-thickness porcine skin explants was used as an in vitro model of re-epithelialisation. TGF-β produced earlier initiation of outgrowth by 1-2 days compared to controls through facilitating KC migration (P. A. Hebda 1988f).

In addition, exposure of KCs to serum rather than plasma has been found to promote epidermal cell migration, a phenomenon attributed to the presence of TGF-β (B. Bandyopadhyay *et al.* 2006f). In a transgenic model, mice over-expressing TGF-β1 had an increased rate of closure of partial thickness wounds due to faster KC migration (E. B. Tredget *et al.* 2005b).

KC migration is influenced by fibronectin and to a lesser extent thrombospondin.

TGF-β (at 10 ng/ml) induced a six fold increase in the secretion of fibronectin and a two-fold increase in thrombospondin production by human KCs. It was proposed that TGF-β may exert its effect on KC motility via endogenous production of ECM proteins by the cells themselves (N. E. Wikner *et al.* 1988). In line with this, more recent studies demonstrated that TGF-β increased the affinity of KC promigratory integrin $\alpha_3\beta_1$ for an ECM protein βig-h3, which is known to be involved in KC adhesion and migration (H. W. Jeong, I. S. Kim 2004).

1.1.8.2 TGF-β in KC proliferation:

In addition to being a promigratory factor, TGF-β is also a potent inhibitor of cellular proliferation. This effect is noted even at concentrations as low as 1 ng/ml (Y. Choi, E. Fuchs 1990).

An important observation is that TGF-β promotes the type of KC differentiation normally associated with wound healing. A temporally and spatially regulated expression of TGF-β has been shown to correlate with physiological changes during epidermal wound healing. TGF-β was rapidly induced in the epidermis within 5 minutes following injury and progressed outward from the injury site. Initially TGF-β was expressed in all KC layers, which corresponded to a transient block in the KC proliferation rate. However, after day three basal proliferating KCs were spared this inhibition (C. J. Kane *et al.* 1991d). This experiment provided conclusive evidence for the anti-proliferative effect of TGF-β in wound healing.

To further investigate the effect of TGF-β on KC proliferation, a transgenic mouse model expressing a dominant negative type II TGF-β receptor in KCs was created. This loss of TGF-β signalling led to accelerated re-epithelialisation of full thickness excisional wounds, which was found to be due to increased KC proliferation and a

decreased rate of apoptosis (C. Amendt *et al.* 2002e).

It is felt that the mechanism through which TGF-β inhibits KC proliferation is by prevention of cell cycle progression into S phase (K. Munger *et al.* 1992). In fact, this is also true for other cell types. For example, TGF-β maintains haematopoietic stem cells in a quiescent slow cycling state by decreasing their cell cycle entry (N. O. Fortunel *et al.* 2003).

1.1.8.3 TGF-β - the overall effect:

The overall physiological response to TGF-β signalling is dependent on the target cell and its extracellular milieu. As described previously, TGF-β is pro-proliferative in fibroblasts, but anti-proliferative and pro-migratory in KCs.

SMAD2 has been shown to be responsible for re-epithelialisation of incision wounds through the induction of suprabasal KC migration, whereas SMAD3 is implicated in the anti-proliferative action of TGF-β (R. Hosokawa *et al.* 2005;G. S. Ashcroft *et al.* 1999a). These data indicate that 'natural' wound healing might involve suppression of SMAD3 and upregulation of SMAD2 levels.

The SMAD signalling pathway is under modulation at multiple levels. For example, SMAD1 (which has similar phosphorylation sites to SMAD2 and 3) receives opposing regulatory inputs by the fibroblast growth factor and bone morphogenic protein (member of the TGF-β family) via protein kinases, and it is this balance that determines the level of SMAD1 activity in the nucleus, and so possibly the cell fate (M. Kretzschmar *et al.* 1997). IL-1 alleviates the inhibitory effect of TGF-β on the proliferation of haematopoietic progenitors. Via its downstream mediator TAK1, IL-1inhibits SMAD3-mediated TGF-β target gene activation (G. F. Benus *et al.* 2005).

In addition, the inhibitory SMAD7 can be stimulated by numerous agents, including TNFα, IFNγ, UV light, and norepinephrine (J. Varga 2002). In doing so, these stimuli influence the overall outcome of TGF-β signalling.

Previous data from this laboratory showed that TGF-β signalling could be modified by prostanoids (R. Stratton *et al.* 2002k). Since a member of the prostanoid family PGE_2 is induced during wound healing and KCs express PGE_2 receptors (see below), I went on to study the effect of PGE_2 on TGF-β driven KC activities.

1.1.9 Prostaglandin metabolism in KCs:

Human KCs are known to produce prostaglandins and to express all 4 types of PGE_2 receptor (E-series PG receptors: EP1, EP2, EP3 and EP4) (S. Narumiya 1995). However, the role of PGs in KC function is not clear.

Prostaglandins are ubiquitous molecules. They are important epidermal cytokines. Just like TGF-β they influence wound healing and KC proliferation. Prostaglandin production is catalysed by cyclo-oxygenase (COX) from arachidonic acid. Two isoforms of COX have been identified: COX I and COX II. COX I is constitutively expressed in many tissues and cell types. It is presumed to be involved in cell homeostasis, whereas COX II is transiently induced after mitogenic or inflammatory stimulation.

Studies have suggested that prostaglandins are pro-proliferative autocoids for the epidermis. Intradermal injection of PGE_1 and PGE_2 increases epidermal proliferation (C. B. Bentley-Phillips *et al.* 1977a).

NSAIDs (pharmacological prostaglandin inhibitors) reduce the proliferation rate of KCs. Topical addition of PGI_2 to indomethacin-treated non-confluent KC cell cultures restored their normal proliferation rate (A. P. Pentland, P. Needleman

1986a). The same investigators also confirmed that PGE_2 is synthesised only by non-confluent KCs to enhance their proliferation rate. This synthesis stopped as soon as KCs became confluent.

In vivo studies support the role of prostaglandins in KC proliferation. Prostaglandins are required for skin tumour development, with indomethacin and colecoxib inhibiting the UV-induced tumourgenesis (S. M. Fischer *et al.* 1999a).

There is conflicting evidence regarding the influence of PGE_2 on wound healing. Partial thickness burns in mice devoid of fatty acid precursors for prostaglandin synthesis take longer to heal (T. K. Hulsey *et al.* 1980). On the other hand, there is a five-fold increase in epidermal cell proliferation in fatty acid-deficient rats, with topical PGE_2 application restoring the slower normal rate of proliferation (J. L. McCullough *et al.* 1978). The differences in these results could be related to experimental design variations (which include animal models, intervals of treatment, doses and prostaglandin types used) as well as the fact that total prostaglandin content was measured without differentiating between different prostaglandin subtypes. More recent data reports that the presence of PGE_2 or active COX II enzyme is beneficial to the healing process in various models of tissue repair. This has been demonstrated by exogenously adding the stable PGE_2 analogue 16, 16-dimethyl prostaglandin E2-methyl ester (T. Brzozowski *et al.* 1993b), COX II inhibitors (L. E. LeDuc *et al.* 1993b), or agents that increase plasma PGE_2 levels (B. Gonul *et al.* 1993a).

It has previously been found that prostanoids are able to antagonise TGF-β signalling in human cells. Prostaglandins elevate cellular cAMP and protein kinase A (PKA) levels (R. Stratton *et al.* 2002j). Studies have shown that agents which

increase intracellular cAMP production, such as isoprenaline and PGE_2, cause fibroblasts to suppress the amount of collagen they produce (L. E. Saltzman *et al.* 1982). It has also been found that iloprost (a prostanoid derivative) suppresses TGF-β signalling in human fibroblasts via PKA and cAMP production (R. Stratton *et al.* 2002i).

Prostaglandins have an important antifibrotic role. In line with this, it appears that their levels are decreased in IPF due to reduced expression of COX II by the fibroblasts (J. Wilborn *et al.* 1995), and COX deficient knockout mice develop more severe lung fibrosis following exposure to bleomycin compared to the wild type controls (J. C. Bonner *et al.* 2002). In addition, reduction in alveolar epithelial cell (AEC) prostaglandin production has been linked to interstitial lung fibrosis (M. Selman, A. Pardo 2002).

Overall, PGE_2 is an important wound healing regulator with TGF-β inhibitory properties in fibroblasts. It is found in epithelial cells and implicated in epithelial-mesenchymal antifibrotic functions.

This thesis will explore the influence of PGE_2 on TGF-β dependent effects on human KCs (migration and proliferation).

1.1.10 Endothelin:

The endothelins are peptides of 21 amino acids that are produced in a wide variety of cells. Isoforms ET-1, ET-2 and ET-3 have been identified (P. Teder, P. W. Noble 2000). Endothelins are synthesized from precursors known as preproendothelins (ppET), comprising 212 amino acid residues, which are cleaved by membrane-bound metalloendopeptidases.

Most tissues contain more ET-1 than ET-2 or ET-3, with the highest levels of ET-1 found in the lung. It is secreted by endothelial cells, epithelial cells, alveolar macrophages, polymorphonuclear leukocytes, and fibroblasts. ET-1 was initially characterised as a potent smooth-muscle spasmogen, but accumulating evidence is supporting its role as a pro-fibrotic cytokine. ET-1 has been shown to be an endogenous modulator of myofibroblast-mediated granulation tissue and ECM contraction (I. Appleton *et al.* 1992), and a fibroblast mitogen (C. Guidry, M. Hook 1991). Later experiments confirmed that ET-1 was able to enhance fibroblast proliferation and collagen synthesis in a dose-dependent manner (M. B. Kahaleh 1991a).

In vivo studies support the role of in ET-1 fibrosis. Transgenic mice over-expressing ET-1 together with its promoter had accelerated glomerulosclerosis and lung fibrosis (B. Hocher *et al.* 1997). In addition, bile duct ligation induced liver fibrosis was accompanied by a rise in ET-1 expression by liver parenchyma (D. C. Rockey *et al.* 1998).

Patients with IPF have increased ppET-1 messenger RNA expression and intense immunoreactivity in airway epithelial cells, proliferating type II pneumocytes, and endothelial and inflammatory cells. In patients with pulmonary fibrosis associated with systemic sclerosis, bronchoalveolar lavage fluid contains 5-fold greater ET-1 levels than those of controls (A. D. Cambrey *et al.* 1994).

Recent evidence also suggests that ET-1 plays a significant role in SSc fibrotic processes. Studies have shown increased plasma and tissue ET levels in SSc patients (R. Vancheeswaran *et al.* 1994d;R. Vancheeswaran *et al.* 1994a). ET-1 was found to induce a fibrogenic phenotype in normal dermal fibroblasts which resembled that seen in fibroblasts grown from lesional SSc skin (S. Xu *et al.* 1998). In addition,

when ET-1 was applied to normal lung fibroblasts it activated expression of pro-contractile proteins such as αSMA and moesin and enhanced fibroblast ability to contract FPCL. The ability of SSc lung fibroblasts to express pro-contractile proteins and contract FPCL was greatly reduced by antagonising ET-1 signalling (S. W. Xu *et al.* 2004e).

Interestingly, antifibrotic agents prostacyclins have been found to inhibit the production of ET-1 through a common mechanism involving the generation of cyclic guanosine monophosphate (E. R. Levin 1995).

Diffuse pigmentation is one of the main skin signs in systemic sclerosis. The presence of binding sites for ET-1 in the epidermis and hair follicles does suggest a possible mitogenic function for this factor in human skin (G. A. Knock *et al.* 1993). It has been shown that human KCs produce ET-1 which works as an intrinsic mitogen for human melanocytes, especially under UV stimulation (G. Imokawa *et al.* 1992). A correlation has been found between the amount of KC derived ET-1 and epidermal pigmentation in SSc (H. Tabata *et al.* 2000c).

It is reasonable to suggest that ET-1 secreted by human KCs could contribute to dermal fibroblast activation.

1.1.11 IL-1:

IL-1 was one of the first cytokines to be described. Its initial discovery was as a factor that could induce fever and regulate lymphocytes (M. J. Kluger 1991). IL-1 is now known as a pivotal pro-inflammatory cytokine involved in local and systemic immune responses. There are two isoforms of IL-1: IL-1α and IL-1β. They are similar in structure and bind the same receptors, which results in activation of transcription factors including NFkB and AP1. Among the genes induced by IL-1

are growth factors and cytokines involved in the response to tissue injury, including KGF, GM-CSF, TNF-α, IL-6, IL-18 and more IL-1. In addition, IL-1 causes KCs to express promigratory cytokeratins K6 and K16(P. Angel, A. Szabowski 2002). Below is a simplified diagram of IL-1 signalling (Figure 1.3) (H. E. Barksby ***et al.*** 2007a).

KC at the wound edges respond immediately with the release of pre-formed IL-1, which acts in an autocrine and paracrine fashion to activate surrounding KCs and dermal cells respectively (I. M. Freedberg ***et al.*** 2001g).

It is known that IL-1 activates dermal fibroblasts causing them to migrate, proliferate and secrete ECM (A. Mauviel ***et al.*** 1993c;A. Mauviel ***et al.*** 1991a). IL-1 is recognised to complete the paracrine epithelial-mesenchymal activation loop by feeding back to activate KCs, partly through stimulating keratinocyte growth factor (KGF) production by these cells (N. Maas-Szabowski ***et al.*** 1999f).

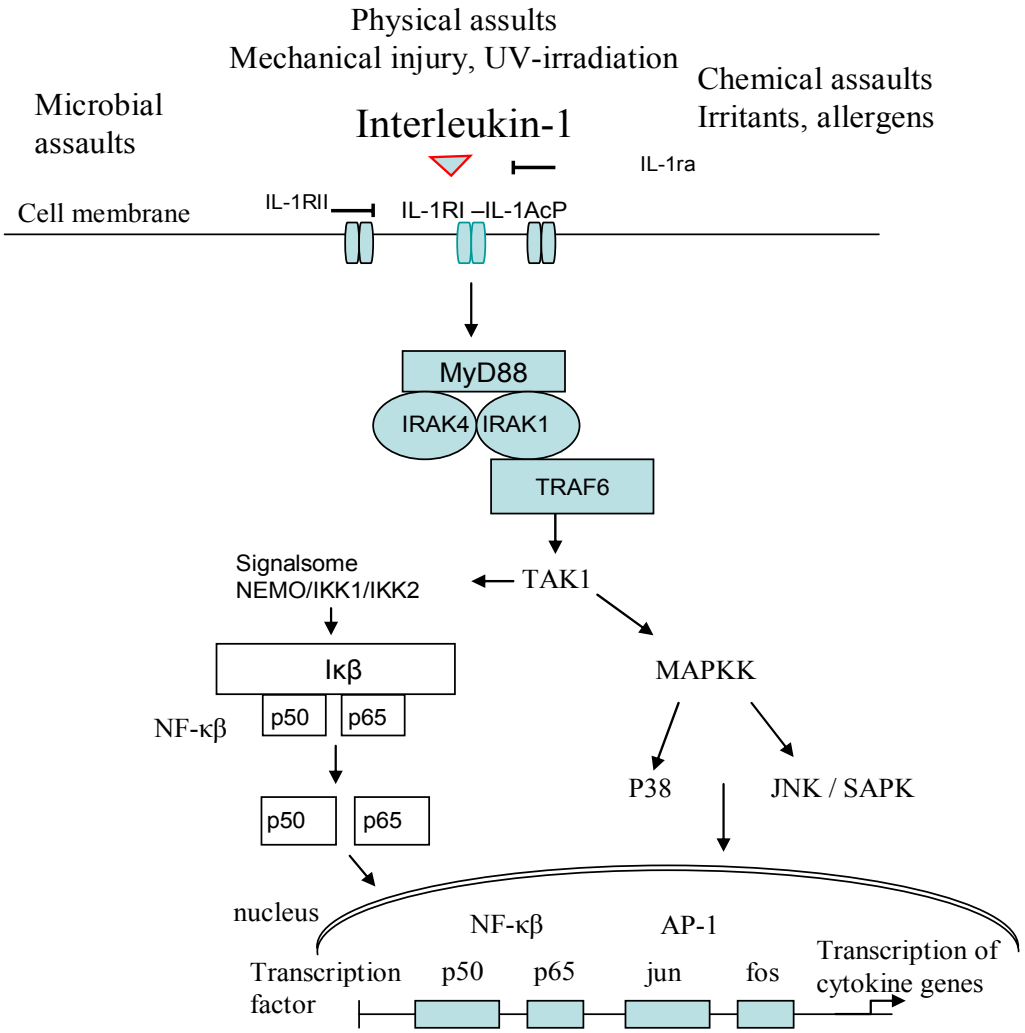


Figure 1.3: IL-1 cytokine signalling cascade.

1.1.12 Summary, Hypothesis and Aims:

SSc is a systemic disorder of unknown aetiology, characterised by uncontrolled fibrosis of the skin and internal organs. Fibrosis is a pathological process that occurs when the normal wound healing events continue unabated with excessive deposition and contraction of ECM. Fibroblast activation is thought of as key to the pathogenesis of SSc. But what causes the switch from regulated cells involved in normal wound healing to persisting autonomous ones capable of destructive fibrosis?

Most previous studies give emphasis to alterations in the dermis of SSc skin. The epidermis, on the other hand, has received relatively little attention. SSc epidermis undergoes pigmentary change, it is capable of secreting pro-fibrotic mediators for dermal fibroblast activation, and, most importantly, it has an abnormal phenotype with persistence of wound-healing type characteristics.

Hypothesis: SSc epidermis is abnormal and could be contributing to fibroblast contraction via release of pro-fibrotic mediators.

The hypothesis will be addressed under the following three experimental aims.

Aims:

1. To determine whether the SSc epidermis has an altered structural and signalling phenotype.
2. To determine whether the SSc epidermis activates normal human fibroblast in vitro.
3. To determine whether PGE_2 modifies the responses of KCs to TGF-β.

CHAPTER 2

Materials and Methods

2.1 Effect of SSc epidermis on FPCL contraction:

2.1.1 Patient selection:

Scleroderma was defined according to internationally agreed guidelines (American Rheumatism Association 1980a). Patients selected for inclusion in the study were from the diffuse SSc subset and were within 2 years of the onset of skin changes. Local ethical committee approval was obtained for the study from the ethical committee of the Royal Free Hospital.

2.1.2 FPCL contraction studies:

FPCLs were set up in 24 well plates as follows. 24-well tissue culture plates were pre-coated with bovine serum albumin (BSA). Trypsinised normal fibroblasts were suspended in DMEM medium and mixed with collagen solution (1 part of 0.2 M HEPES, pH 8.0, 4 parts rat dermal collagen type I (First Link, UK), and 5 parts of DMEM), yielding a final concentration of 80,000 cells/ml and 1.2 mg/ml collagen. Collagen/cell suspension (1 ml) was added to each well.

Skin punch biopsies (4 mm) were obtained from the forearms of patients with recent onset active diffuse SSc (within 24 months), and normal control subjects. The epidermis was separated after incubation with 0.05 mg/ml trypsin and 10 ml of PBS at 37°C for 120 minutes. The epidermal sheets were removed using fine forceps and allowed to settle in wells containing FPCLs. Control wells were left untreated. The other wells were exposed to either SSc or normal epidermal discs, or TGF-β (10 ng/ml) (R&D Systems, 240-B-002). TGF-β was used at a concentration of 10 ng/ml

because we have previously found this concentration to efficiently contract FPCL. Maximal stimulation is known to be achieved between 7.5-15 ng/ml, with higher doses causing contraction inhibition (R. A. Brown *et al.* 2002). Further FPCLs containing the neutralising anti-TGF-β antibody 1D11 (1μg/ml) (R&D Systems), or bosentan Ro 47-0203 (10 μM), or IL-1 Receptor antagonist (100 ng/ml) (Imgenex, IMR- 245) were also co-cultured with control and SSc epidermis. Gel contraction was determined by photography and measurement of maximum diameter, and expressed as a percentage of the original diameter (well size).

At the end of the assay some FPCLs were lysed in Western lysis buffer (laemmli buffer) (see section II.1.3); and others were fixed in formalin and then sectioned at 5 μm prior to staining for CTGF using immunohistochemistry (see section II.1.4).

2.1.3 Western blot assay:

Western blots of the fibroblast gels were performed to detect levels of TGF-β, CTGF, ET-1, αSMA, and IL-1. The FPCLs were lysed with 200 μl of Western lysis buffer (laemmli buffer), and then heated for 5 minutes at 95°C and spun for 5 minutes to remove the sediment. Samples were loaded on 4-12% Tris-glycine gels (Invitrogen, Paisley, UK) at 12 μl per well and run with broad range protein markers. Proteins were electrophoretically transferred to nitrocellulose (Hybond-C extra; Amersham Pharmacia Biotech), which was then left in PBS with 5% milk protein and 0.05% Tween 20 for 2 hours, in order to block non-specific binding. The nitrocellulose filters were stained with primary antibodies overnight at 1:1000 dilution in blocking buffer (PBS with 0.05% Tween 20 and 5% milk powder) (see Appendix 1).

The nitrocellulose transfers were then washed three times in 0.05% Tween 20 in PBS and stained with biotinylated species-specific secondary antibody (Vector, anti-mouse BA-9200, anti-rabbit BA-1000, anti-goat BA-9500) for 1 hour and again washed three times before staining with biotin substrate ABC (VECTASTAIN; Vector Laboratories, Peterborough, UK), then chemiluminescent substrate (Amersham Pharmacia Biotech), and developed against photographic film (Hyperfilm ECL; Amersham Pharmacia Biotech).

An additional Western blot assay of SSc and normal epidermal lysates was performed for pSMAD2/3. Sample preparation as per ELISA (Section II.1.5), Western blot assay performed as above. Primary pSMAD2/3 antibody was obtained from Santa Cruz Biotechnology (Appendix 1) and used at 1:200 dilution.

2.1.4 Immunohistochemistry:

Relevant slides (with tissue or cell material) were prepared and stained using the following method.

- Dewaxing sections:

Slides were immersed in xylene for 10 minutes and then immersed in 100% alcohol to remove the wax solvent. The slides were dipped in and out of the alcohol several times to allow thorough rinsing. They were subsequently immersed in a trough of 95% alcohol for 5 minutes, and then in a trough of 70% alcohol for 5 minutes. Lastly, the slides were immersed in distilled water (DH_2O) for 5 minutes to remove all traces of alcohol.

Once dry, the sections were circled using a hydrophobic pen and allowed to dry for a few minutes. The sections were then re-hydrated by immersing in PBS 3 times for 5 minutes each time. The slides were boiled in citrate for 10 minutes.

- Adding serum:

25% secondary antibody species-specific serum was made up (horse serum for pSMAD 2/3, LAP TGF-β and CTGF) to block non-specific binding. 40µl of the serum was aliquoted onto each of the sections, the section slides then placed in the humidity chamber for 10 minutes.

- Adding the antibodies:

The primary antibody was made up: LAP-TGF-β (1:100 dilution), pSMAD (1:100 dilution), pERK1/2 (1:100 dilution), CTGF (1:50 dilution) as before (see section 2.1.3), ET-1 from Abcam (1:100 dilution), ET-A Receptor (1:100 dilution), ET-B Receptor (1:100 dilution), COX II (1:100 dilution), IL-1α (1:100 dilution), IL-1α Receptor (1:100 dilution) (Appendix 1); and 40µl aliquoted onto each section covering each entire section. Tissue slides were placed back into the humidity chamber for 1 hour.

The slides were then rinsed with PBS 3 times for 5 minutes each time.

The secondary species specific antibody was made up, 40 µl aliquoted onto each section. Tissue slides were placed into the humidity chamber for 30 minutes.

The slides were then rinsed with PBS 3 times for 5 minutes each time.

- Antibody detection by fluorescence:

Sections were incubated in FITC-Avidin D (1:500 Vector Labs) in PBS for 30 minutes at room temperature. Slides were protected against UV light with aluminium foil. They were washed in PBS three times for 2 minutes each time.

- Antibody detection by colour change:

The ABC (Vectastain Elite) was made up 30 minutes before usage by adding 1.5 ml of PBS to 1 drop of *reagent A* and 1 drop of *reagent B*. 40µl of the ABC was aliquoted until all the sections were totally covered, the slides then left for 30 minutes. After 30 minutes the slides were rinsed with PBS 3 times for 5 minutes each time.

- Mounting:

The sections were mounted with Vector-shield DAPI mounting solution, covered with Vector aqueous Anti-fade fluorescent mounting medium, and sealed with nail polish. The slides were stored in the dark at 4°C.

2.1.5 ELISA:

Commercially obtained Enzyme-Linked Immunosorbent Assays were used to quantify PGE_2, TGF-β, ET-1 and IL-1 content of prepared mouse or human tissue (all R&D Systems).

Human skin tissue:

4 mm tandard forearm skin punch biopsies from six SSc patients and six normal subjects were taken. Epidermal layers were separated surgically and snap frozen in liquid nitrogen. Once thawed, the epidermal discs were lysed in buffer containing 10 mM Tris buffered saline, 0.1%SDS, 1% Nonidet p40, 5 mM EDTA and one tablet of complete mini protease inhibitor (Roche). A microhomogeniser was used to break down the samples on ice. The samples were centrifuged for 5 minutes.

ELISA amounts were normalised per total biopsy rather than being expressed as ng/mg of tissue. The results are are per unit area of epidermis so they relate to total body burden and local fibroblast exposure.

- TGF-β ELISA:

20 µl of sample supernatant was mixed with 180 µl Assay Dilutent (RD1-21) provided for a 1:10 dilution. Half was used for the standard ELISA protocol to detect free TGF-β. The other half was activated using acidification/neutralisation to release TGF-β bound to LAP. The samples were then used in the ELISA assay. The activated sample TGF-β concentrations were then multiplied by 1.4 (the dilution factor). All TGF-β concentrations were then multiplied by the dilution factor of 10 to achieve the final result.

- ET-1/IL-1 ELISA:

10 µl of sample supernatant was mixed with 90 µl sample Dilutent provided for a 1:10 dilution. R&D Systems ELISA kit was then used to assay ET-1 and IL-1 concentrations.

All ET-1/IL-1 concentrations were then multiplied by the dilution factor of 10 to achieve the final result.

Mouse wound tissue:

All animal protocols were approved by the local animal ethics committee at University College London. 6- to 8-week-old female mice were anesthetized with avertin (500 mg/kg). The dorsum was shaved and cleaned with alcohol. Four equidistant 4-mm full-thickness excisional wounds were made on either side of the midline of the mouse. Wounds were harvested daily after animal sacrifice on days 0-14. Tissue was fixed in formalin, embedded in wax and sectioned prior to staining for COX II. Whole wound tissue was snap frozen in liquid nitrogen and homogenized in a lysis buffer containing 10mM Tris Buffered saline, 0.1%SDS, 1% Nonidet p40, 5mM EDTA and one tablet of Complete mini protease inhibitor (Roche). After adding 2 ml of ice-cold ethanol the proteins were pelleted by

centrifugation (10 min at 3000*g* and 4°C) and then dissolved in 8M urea. The sample was mixed 50:50 with the ELISA kit sample buffer (R&D Systems). PGE_2 was quantified using the standard ELISA kit (R&D Systems).

2.1.6 Phosphoprotein microarray:

Four SSc and four normal control forearm skin biopsies were obtained. The samples were immediately frozen in liquid nitrogen in order to prevent the decay of phosphorylation events. Epidermal cell layers were then separated surgically. Samples were shipped to Kinexus Bioinformatics (Canada), where they were lysed and processed using phospho-site specific antibodies. This service uses the KAM-1.2 chip with two samples analyzed at a time utilising 500 pan-specific antibodies (for protein expression) and 300 phospho-site-specific antibodies (for phosphorylation) in duplicate for at least 248 different phospho-sites, 193 protein kinases, 24 protein phosphatises and 150 regulatory subunits of these enzymes and other cell signalling proteins that regulate cell proliferation, stress and apoptosis.

2.2 Effects of TGF-β and PGE_2 on KC migration:

2.2.1 KC cell culturing:

Normal human epidermal KCs (Invitrogen) were cultured in Keratinocyte Serum Free Medium (K-SFM) (Invitrogen) supplemented with growth factor concentrate and gentamicin (5 μg/ml). Cryo-preserved cells were initially cultured in 25 ml flasks. When 70-80% confluent, KCs were detached from adhesion by the addition of trypsin/EDTA (0.025% trypsin/0.01% EDTA,) obtained from GIBCO. After 2 minutes at 37^0C in a 5% CO_2 incubator, cells were detached by a blow to the flask. Once 90% of the cells were dislodged, the trypsin reaction was stopped by adding

10-15 ml of DMEM with 10% FCS. The cells were then transferred into a 50 ml centrifuge tube and spun at Speed-1500 rpm, 5 min, Temp-20 ^{0}C. Once centrifuged, trypsin was removed and the cells washed in K-SFM twice by re-suspension and re-spinning. The cells were then seeded into labelled T75- flasks or the appropriate welled plates, passage number marked, and placed in the 37 ^{0}C 5% CO_2 incubator to grow. Media were exchanged every 48 hours.

2.2.2 Scratch Assay:

Normal human KCs were grown to confluence on 6 well plates. Four plates were treated with TGF- β (4 ng/ml), one left as control, and one treated with 10 μmol solution of PGE_2 only (Calbiochem 538904). Dose response was tested by adding PGE_2 of increasing concentrations to the three remaining TGF- β containing wells (0.1; 1; and 10 μM). A scratch was then induced by scoring with a pipette. KCs were maintained in K-SFM with mitomycin C (10 μg/ml) for 48 hours to suppress proliferation. Migration of KCs into the defect and defect closure were determined by photography at 12 hour intervals. The width of the scratch defect was measured.

2.2.3 Inhibition of the promigratory effect of TGF-β on KCs by the MEK 1/2 inhibitor U0126:

Human KCs were grown to 80% confluence on a 6 well plate. Prior to the experiment cells were washed in K-SFM. Cells were then treated for 1 hour with three different concentrations of U0126 solution (Invitrogen) in SFM: 0 μM (Control), 5 μM (Solution B), 10 μM (Solution A), and 12 μM (Solution C) at 2 ml total solution per well (Y. Imamichi *et al.* 2005a;R. Stratton *et al.* 2002h;R. Stratton *et al.* 2002g;S. W. Xu *et al.* 2004d). After 1 hour the cells were treated with 4 ng/ml

of TGF-β. A scratch wound was then induced by scoring with a pipette. KCs were maintained in tissue culture with mitomycin C (10 μg/ml) for 48 hours to suppress proliferation. Migration of KCs into the defect and defect closure were determined by photography at 12 hour intervals for 48 hours. The width of the scratch defects was measured.

2.3 Effect of PGE_2 on ERK phosphorylation in human KCs:

2.3.1 Timecourse of pERK1/2 induction after treatment of KCs with TGF-β and PGE_2:

Passage 2-3 sub-confluent primary human KCs were trypsinised and seeded into Lab-Tec chamber slides and allowed to adhere overnight.

The cells were then pre-treated with 10μM PGE_2 for 30 minutes in chamber slides. TGF-β (4ng/ml) was then added to the chambers and left for 15, 30, and 60 minutes. In other experiments cells were treated with 4ng/ml TGF-β only for 15, 30, and 60 minutes. After treatment, the cells were washed twice in PBS-Tween and fixed with 4% formalin for 10 minutes at room temperature. They were then permeabilised with 0.5% of Triton X-100 (in PBS-Tween) for 5 minutes at -20^{o}C. Immunohistochemistry protocol (section 2.1.4) was then followed to stain for pERK1/2.

2.3.2 Effect of TGF-β and PGE2 on total cellular ERK phosphorylation in KCs:

Human KCs were grown to 80% confluence on a 12 well plate. Prior to the experiment cells were washed in re-warmed K-SFM. KC wells were treated with TGF-β (4ng/ml) and lysed in laemmli buffer at time points as follows: baseline, 5 minutes, 15 minutes, and 30 minutes. The remaining KC wells were pre-treated with

PGE_2 at 10 μM 30 minutes prior to addition of TGF-β (4ng/ml). The cells were then collected and lysed in laemmli buffer at time points as above. Control well was exposed to 2 ml of medium only and lysed in laemmli buffer. All lysates were heated at 95°C and subjected to Western blot analysis for ERK/pERK1/2 (see section 2.1.3). Solution of PGE_2 in SFM medium was added to 6 wells and left for 30 minutes to achieve pathway inhibition. 1 ml of pure K-SFM was added to the other 6 wells. 4ng/ml of TGF-β solution was the added to all 12 wells. Each time frame at 5 min, 15 min, and 30 minutes saw TGF-β added to the paired wells: one containing medium only, and one containing medium and PGE_2. The KCs were then collected from each well with 100 μl of laemmeli buffer added to each for lysis. Western blot for ERK1/2 and pERK1/2 was then performed (see Western blot protocol and Appendix 1)

2.4 KC proliferation assays:

Normal human epidermal KCs were cultured to confluence on 6 well plates. Cell numbers were quantified at baseline by Coulter counting. Cells were cultured for a further 48 hours in K-SFM with or without the addition of TGF-β 10 ng/ml. At the end of 48 hours the cells were removed by trypsinisation and counted using a Coulter counter. Subsequently, PGE_2 at 3 different concentrations (0.1 μM, 1 μM, and 10 μM) was co-added with TGF-β at the start of the experiment in order to measure its effects on TGF-β driven inhibition of KC proliferation.

2.5 Statistical methods:

In general the studies presented in this thesis are based on small numbers of biopsy specimens taken from SSc patients and healthy controls. The total number of biopsies available is constrained by the relatively low incidence of the disease with approximately one new diffuse SSc patient per month presenting to the clinic, and not all patients willing to undergo skin biopsy. Immunohistochemistry and gel contraction experiments were performed based on biopsies derived from 6 patients with diffuse SSc and 6 healthy control individuals. Studies with these sample sizes are designed to detect large biologically important differences in outcome between disease and control groups.

Data for mean gel diameter were expressed as means and standard error of means, and the means of different groups compared by parametric analysis. Student's t test was used for comparison of mean gel diameter between treatment groups eg. untreated FPCL vs SSc epidermis treated FPCL, using a threshold of $p<0.05$ to establish the statistical significance of changes seen (Y. Chen *et al.* 2005b).

Phosphorylation arrays (Kinexus) were used to make multiple comparisons between phosphoprotein abundance in SSc and control epidermal biopsy samples (both n=4). In total 632 phosphorylation sites were assayed in SSc and control biopsies. There are a number of issues regarding the statistical analysis of this sort of array data. One problem is to decide what threshold of increase is of biologic importance. Our approach to this has been to look at the preliminary results generated in the present thesis to see if there is a suitable cut off threshold above or below which a reasonable number of potential targets for further studies are generated. In this instance we found that a threshold of 2 fold increase or decrease in phosphorylation

site abundance gave a reasonable cut off with 19 increased phosphorylation sites and 22 decreased.

The other issue in interpreting the array data is the problem of multiple comparisons. Because we are making 632 comparisons between SSc and control samples one would expect a large number of false positives. For example if a p value of <0.05 is used for statistical significance of comparison of means then one would expect 0.05 X 632 = 32 positive results by chance alone, even if there were no important differences between disease and control samples. The traditional approach to this problem was to use a correction for the effect of multiple comparisons (Bonferroni's correction) reducing the threshold for statistical significance in proportion to the number of comparisons made. In this instance it would mean reducing the p value to $0.05 \div 632 = 0.00008$. The problem with this approach when applied to array data is that it is overly stringent and the majority of potential positive findings are greatly diminished i.e. there are many false negatives. For example when we applied the Bonferroni's correction to the array data in the present study we obtained no positive results with a twofold change in abundance and a statistical significance at $p< 0.00008$.

Because of these problems more modern statistical models have been developed for the interpretation of array data and these have been recently reviewed (P. de Winter 2009). The technique described compares a property such as t or the mean of the observed data for each gene or protein assayed against multiple random permutations of the data (permutation test). The observed mean value for each phosphoprotein is compared against a frequency distribution generated by the multiple permutations.

In the present thesis permutation testing of each array data point was performed using an Excel add-in, called Significance Analysis of Microarray Data (SAM) (V. G. Tusher *et al.* 2001). The False Discovery Rate was then calculated using a method described in 1990 (Y. Hochberg, Y. Benjamini 1990).Using Benjamini and Hochberg's False Discovery Rate procedure the p value for each comparison is made as follows. First rank all the p values obtained from the significance test from lowest to highest, then starting with the lowest p value divide its rank by the total number of tests (632 in this instance) and multiply by 0.05. Compare this result with the original p value, and if the result is greater than the original value then there is a significant difference, and if the calculated value is less than the original value then the result is considered non significant. The technique has been refined further and the SAM software calculates a refined p value termed the q value.

The data were analysed using a two-class unpaired test with 5000 permutations and Student's t as the computed statistic. A twofold change in phosphorylation site abundance was selected as the threshold for biological relevance with a false discovery rate (FDR) of 15%.

CHAPTER 3

Scleroderma Epidermis Characteristics

Introduction

The SSc epidermis has previously been found altered in terms of its structure and function. In a previous study confocal laser scanning microscopy (CLSM) was used to image the skin of patients with SSc non-invasively and in real time. The epidermis was found to be hypertrophied with a large number of melanocytes and an increased melanin content (K. Sauermann *et al.* 2002b). SSc skin is often hyperpigmanted and a correlation has been found between the amount of KC derived ET-1 and epidermal pigmentation in the disease (H. Tabata *et al.* 2000d). Another group has shown interleukin 21 receptor levels to be increased in SSc epidermis, although the functional significance of these changes is not clear (J. H. Distler *et al.* 2005a).

Recent results from this laboratory used proteomic analysis followed by mass spectroscopy to separate and define the high abundance proteins present in unfractionated skin biopsy material from SSc patients and healthy controls. Proteins of altered abundance in SSc of recent onset were identified. Prominent among these were proteins specific for the epidermis and involved in the differentiation of epidermal cells, such as cytokeratins 1, 14 and Galectin 7.

Protein	Gene ontology molecular function	Gene ontology biological process	Abundance in control n=12 (mean, sem)	Abundance in SSc n=12 (mean, sem)
Cytokeratin 1	Structural constituent of cytoskeleton	Epidermis development	47 (1.14)	144 (18.0) P=0.0002
Cytokeratin 14	Structural constituent of cytoskeleton	Epidermis development	507 (93.9)	1814 (295) P=0.0012
Cytokeratin 5	Structural constituent of cytoskeleton	Epidermis development	2095 (364)	615 (105) P=0.0021
Caspase 14 precursor	Caspase activity	Epidermis development	347 (35.4)	660 (92.3) P=0.0081
Galectin 7	Sugar binding	Heterophilic cell adhesion	13928 (1370)	4469 (1280) P=0.0003
Heat-shock protein beta-1 (HSP 27)	Protein binding	Response to unfolded protein	254 (23.0)	1498 (323) P=0.0023
40S ribosomal protein SA	Laminin receptor activity	Cell adhesion	116 (3.90)	1753 (201) P<0.0001

Table 3.1: Proteins of altered abundance in SSc skin biopsy material vs healthy control. For full details see Appendix 3 article **'**Proteomic analysis of scleroderma lesional skin reveals activated wound healing phenotype of epidermal cell layer' (N. Aden *et al.* 2008b).

Following on from these findings I began to study the epidermis in SSc.

Normal human interfollicular epidermis contains postmitotic differentiating KCs, whose fate is to become suprabasal (spinous) cells and undergo terminal differentiation. These differentiating KCs can be distinguished by the pattern of cytokeratin expression (M. L. Usui *et al.* 2005a). The basal progenitor cells, which are mitoticaly active, appear relatively undifferentiated and express cytokeratins 5,

14 and 15. They renew a stratified epithelial layer adapted to protection from mechanical injury and microbial invasion from the environment (W. G. Nelson, T. T. Sun 1983a). Differentiating and migrating upwards these cells switch to expression of cytokeratins 1 and 10, progressively flatten, cross link envelope proteins via the action of transglutaminase, and extrude nuclei and organelles culminating in a phenotype adapted to mechanical strength and barrier function (E. Fuchs 1993). This process is greatly altered following injury to the skin so that KCs at the wound edge withdraw from terminal differentiation and take on an activated phenotype characterised by loss of peripheral cytoplasmic cytokeratin filaments which become restricted to a perinuclear distribution, loosening of desmosomal attachment to adjacent cells, and increasing motility (F. Grinnell 1992a). The activation of KCs adjacent to wounds is associated with a change in the pattern of cytokeratin expression with persistence of cytokeratin 14 into suprabasal layers, and expression of inducible cytokeratins 6, 16 and 17 normally absent in the interfollicular epidermis (M. L. Usui *et al.* 2005c). The switch to suprabasal cytokeratin 16 expression disrupts and shortens intermediate filaments so that peripheral cytoplasmic cytokeratin filaments are largely lost and replaced by cytokeratin 16 positive perinuclear filaments (R. D. Paladini *et al.* 1996a). These changes are presumed to allow for greater motility of the cells required for their migration over the wound.

This chapter is aimed at exploring the SSc epidermal characteristics. Differences between normal and SSc epidermis structure and signalling content are examined. Comparisons between the SSc epidermis and an epidermis activated after wounding are made.

Study of the epidermis in SSc is a new area and because of this I have taken a broad

screening approach using biopsy material from new onset diffuse SSc patients and normal subjects. With the small number of subjects used (due to the rare nature of the disease) I was looking for strong biological effects and large expression differences between disease and control.

Results

3.1 SSc epidermis structural changes:

3.1.1 KC Differentiation Program:

Skin biopsy material from the forearm of healthy controls (n=5) and from SSc patients (n=5) involved forearm skin were stained for DAPI and cytokeratins. Figure 3.1 shows that the SSc epidermis is thickened in comparison to the healthy control. A similar nucleated cell expansion can also be found in wound healing, and represents KC proliferation and differentiation necessary in the re-epithalialisation process (M. L. Usui *et al.* 2005d).

Normal epidermis SSc epidermis

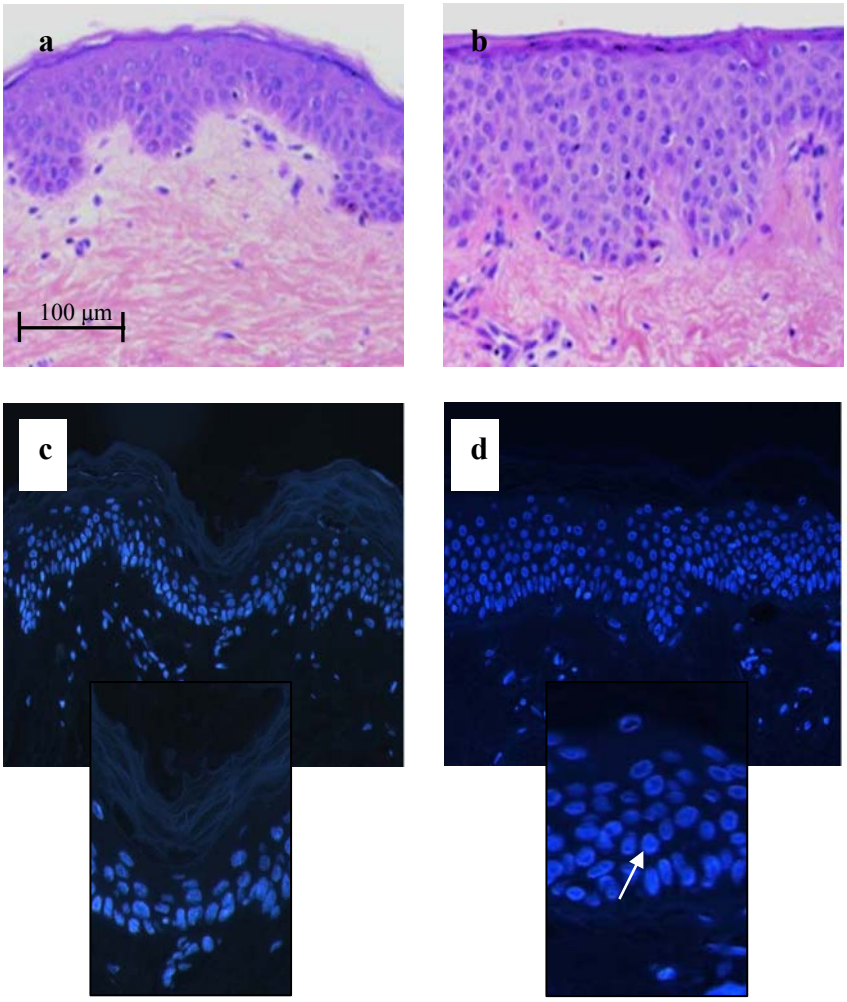


Figure 3.1: Staining of normal and SSc skin. Panels **a** and **b** represent H&E staining; panels **c** and **d** represent DAPI immunohistochemical staining of epidermal cell nuclei (arrow). Panels **a** and **c** show normal epidermis; panels **b** and **d** show SSc epidermis. Inserts in panels **c** and **d** highlight the differences between normal and SSc epidermis cell thickness (n=2).

3.1.2 Cytokeratin Differentiation Program:

Skin biopsy sections were subsequently stained for the cytokeratins which define KC position and differentiation in the epidermis. In SSc epidermis cytokeratin 14 expression was no longer confined to the basal layer but expressed in the superficial cell layers as well. A more superficial expression of differentiation markers cytokeratins 1 and 10 was also noted (Figure 3.2). This suggested a delay in KC maturation and terminal differentiation as they migrated to the epithelial surface, i.e. the cells maintained their basal phenotype.

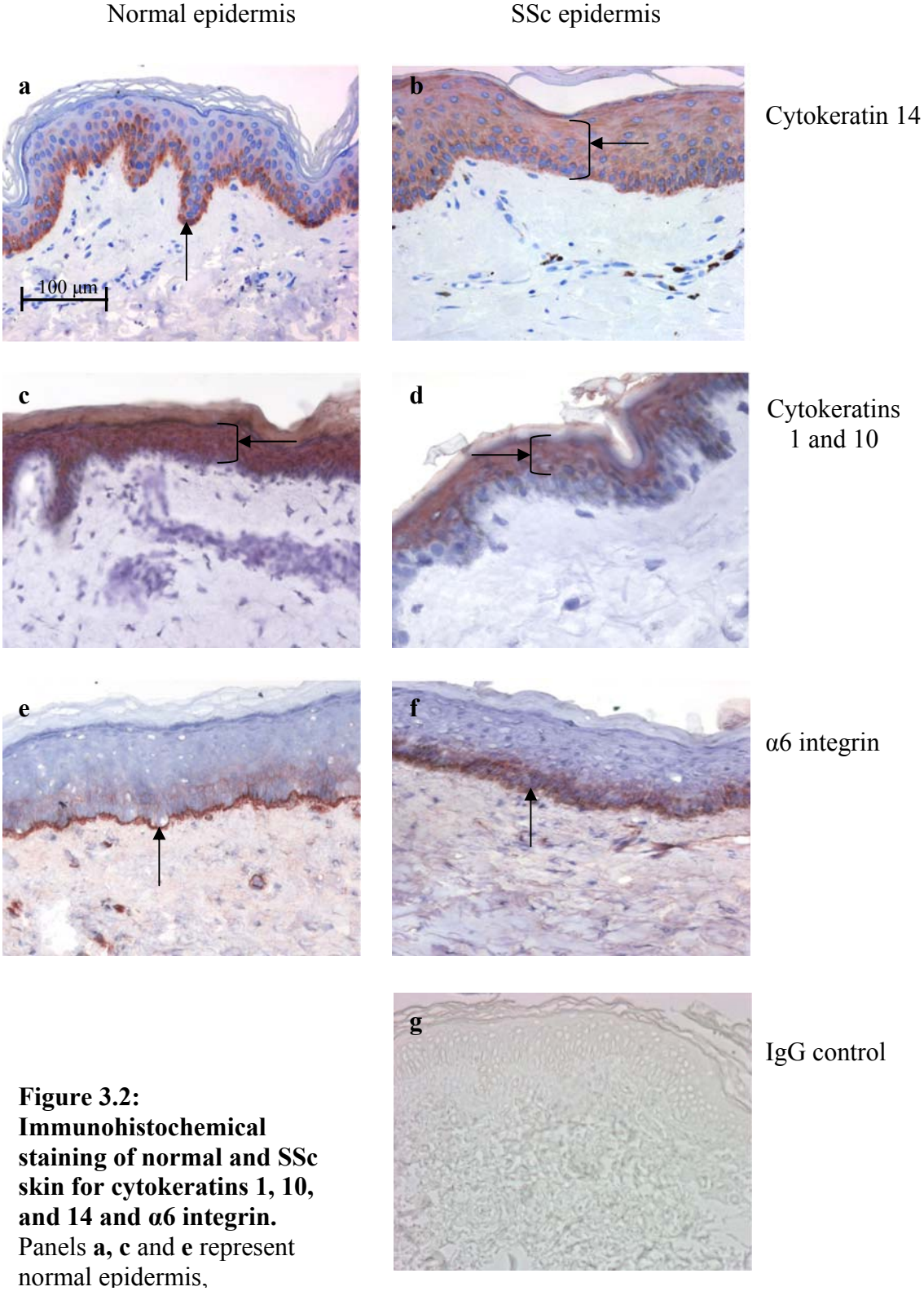


Figure 3.2: Immunohistochemical staining of normal and SSc skin for cytokeratins 1, 10, and 14 and α6 integrin. Panels **a, c** and **e** represent normal epidermis, panels **b, d** and **f** represent SSc epidermis. Panels **a** and **b** show cytokeratin 14 staining; panels **c** and **d** show cytokeratin 1 and 10 staining; panels **e** and **f** show alpha6 integrin staining; panel **g** shows IgG control (n=3). Arrows and brackets show areas of interest.

3.1.3. Cytokeratins defining wound healing phenotype:

Because of these findings, and the suspicion that basal cell layer characteristics were being displayed by suprabasal cells in the disease, the expression of α6 integrin was studied. This integrin is normally concentrated at the basement membrane zone in association with β4 integrin, which is in turn associated with hemidesmosomes and the intermediate filament system (T. Tennenbaum *et al.* 1996a). In healthy control specimens a sharply demarcated basal expression adjacent to the basement membrane is seen, consistent with the role of α6 as an anchor integrin between epidermal basal cells and hemidesmosomes. In SSc the expression of the α6 integrin was no longer confined to the basal layer (Figure 3.2). Previous studies have shown that suprabasal expression of α6 integrin is highly abnormal, and that it is associated with the hyperproliferative phenotype of the epidermis in situations of wound healing response (M. D. Hertle *et al.* 1992f). These findings lead to the initial comparison between the changes in the SSc epidermis and those described during wound healing when the epidermis becomes expanded and shows persistence of the basal phenotype cells into superficial layers (M. L. Usui *et al.* 2005e).

Because of this I went on to explore whether SSc epidermis is taking on an activated wound healing phenotype by staining for cytokeratin 16 and 6, markers of KC activation during wound healing.

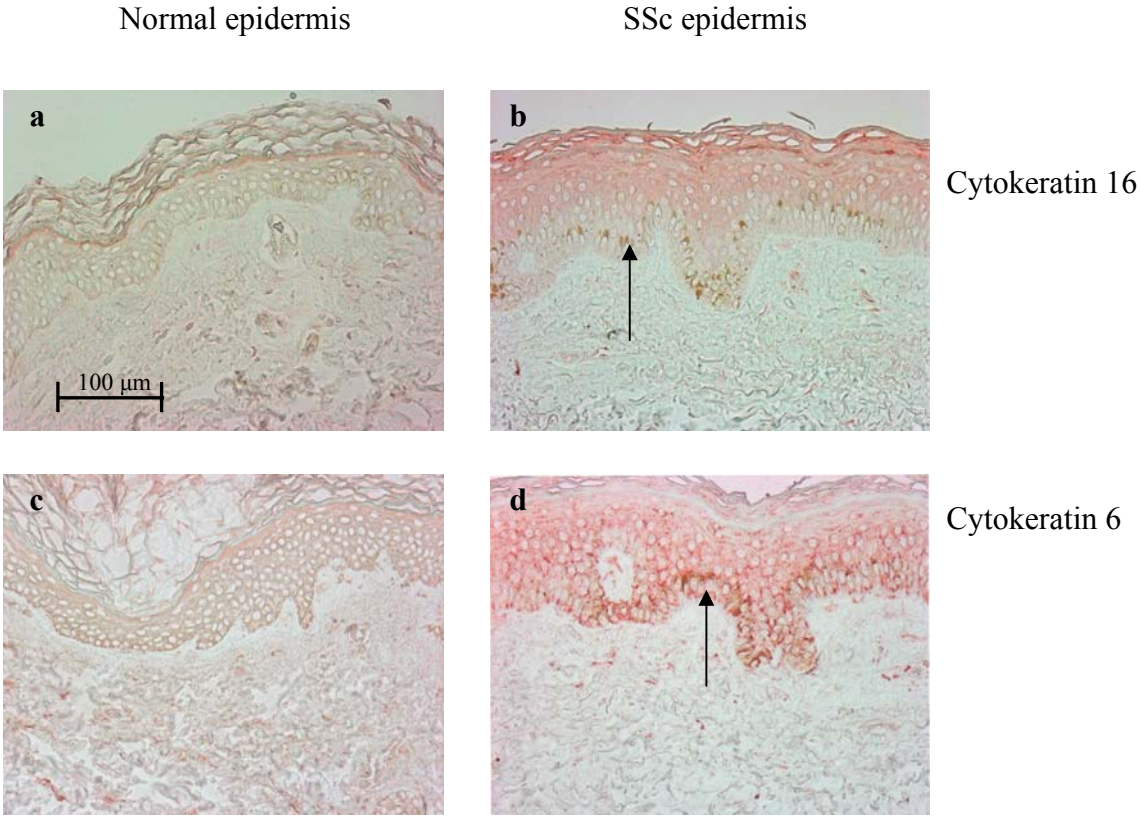


Figure 3.3: Immunohistochemical staining of normal and SSc skin for cytokeratins 6 and 16. Panels **a** and **c** show normal epidermis; panels **b** and **d** show SSc epidermis. Panels **a** and **b** show cytokeratin 16 staining; panels **c** and **d** show cytokeratin 6 staining (n=3). Arrows show areas of interest.

Expression of cytokeratins 6 and 16 was found in SSc but not in healthy control epidermis (Figure 3.3). These cytokeratins are usually induced during wound healing and are not expressed in uninjured forearm skin (M. L. Usui *et al.* 2005f). Collectively these data are supporting the idea that the epidermis in SSc is altered with changes resembling those seen during wound healing, displaying delayed cell maturation, abnormal stratification, and persistence of the basal cell layer markers throughout the epidermis.

3.2 SSc epidermis signalling changes:

3.2.1 Kinexus microarray:

Because of the above findings I became interested in determining which signalling pathways were altered in SSc epidermis.

Kinex™ antibody microarray was used for detection of phosphorylation of more than 240 protein kinases, 28 phosphatases and 90 other cell signalling proteins that regulate cell proliferation, stress and apoptosis. While direct functional implications of protein phosphorylation cannot be made using this assay alone, phosphoprotein analysis proved very useful in assessing large differences between SSc and control epidermis phosphorylation activities, thus directing further studies into specific proteins and receptors that may be important in disease pathogenesis (Tables 3.2, 3.3).

Statistical analysis and correction for multiple testing were performed using Significance Analysis of Microarrays (SAM) software for 628 sites detected by the array. The data were analysed using a two-class unpaired test with 5000 permutations and t as the computed statistic. A fold change of two was selected as the threshold for biological relevance with delta at 0.592 and a false discovery rate (FDR) of 15%.

3.2.2 Phosphoproteins upregulated in SSc epidermis:

Phosphoprotein Functional Class	Phosphoprotein Site	Fold Increase	Full Target Protein Name
Receptor Kinases	Y1003	2.89	Hepatocyte growth factor (HGF) receptor-tyrosine kinase
	Y1189/Y1190	2.15	Insulin receptor/Insulin-like growth factor 1 receptor
Transcription Factors	S63	2.37	Jun proto-oncogene-encoded AP1 transcription factor
	Y705	2.18	Signal transducer and activator of transcription 3
Signal Transduction	Pan-specific	2.63	Protein-serine phosphatase 4 - regulatory subunit (PPX/A'2)
	S674	2.57	Protein-serine kinase C eta
	T514	2.23	Protein-serine kinase C gamma
	Pan-specific	2.18	Dual specificity protein kinase
	T180+Y182	2.13	Mitogen-activated protein-serine kinase p38 alpha
	Pan-specific	2.10	PCTAIRE-1 protein-serine kinase
ECM/Integrins/ Cytoskeletal components	Pan-specific	2.17	Integrin-linked protein-serine kinase 1
	S605	2.62	Synapsin 1 isoform Ia
Cell cycling/metabolism	S70	5.55	Tyrosine hydroxylase isoform a
	Pan-specific	2.43	Male germ cell-associated protein-serine kinase
	Pan-specific	2.38	Wee1 protein-tyrosine kinase
	Pan-specific	2.29	NIMA (never-in-mitosis)-related protein-serine kinase 7
	Pan-specific	2.09	NIMA (never-in-mitosis)-related protein-serine kinase 4

Table 3.2: Upregulation of signalling molecule phosphorylation in SSc epidermis. Phosphoprotein microarray data demonstarating the mean fold increase in signalling protein phosphorylation in SSc epidermis biopsies versus normal controls. The twofold increase has been used as a cut off. Phosphoproteins have been subdivided according to class, the fold change shown in descending order for each class (control epidermis n=4, SSc epidermis n=4).

3.2.2 Phosphoproteins downregulated in SSc epidermis:

Phosphoprotein Functional Class	Phosphoprotein Site	Fold Decrease	Full Target Protein Name
Apoptosis	Pan-specific	3.2	Diacylglycerol kinase zeta
	Pan-specific	3	Bcl2-like protein 1
	Pan-specific	2.9	Pro-caspase 3
	Pan-specific	2.77	caspase 5
Signal Transduction	Pan-specific	2.56	Janus protein-tyrosine kinase 2
Cell Cycling	Pan-specific	2.56	Cyclin-dependent protein-serine kinase 4
	Pan-specific	2.5	Cell division cycle 25B phosphatase
	Pan-specific	2.4	Cell division cycle 2-like protein-serine kinase 5
	Pan-specific	2.38	Cyclin-dependent protein-serine kinase 5
	Pan-specific	2.32	p21-activated serine kinase 5 (Serine/threonine-protein kinase PAK 7)
	Pan-specific	2.12	Large tumor suppressor 1 protein-serine kinase (WARTS)
Metabolism	Pan-specific	2.5	Cyclo-oxygenase 2 (prostaglandin G/H synthase 2 precursor)
	Pan-specific	2.5	Heme oxygenase 2
	Pan-specific	2.5	Calcium/calmodulin-dependent protein-serine kinase 2 alpha
	S36	2.27	Caveolin 2
	Pan-specific	2.32	Calcium/calmodulin-dependent protein-serine kinase 2 beta
	Pan-specific	2.22	Calcium/calmodulin-dependent protein-serine kinase 1 delta
	S789	2.08	Caldesmon
Receptor Kinases	Y612	2.5	Insulin receptor substrate 1
	Pan-specific	2.27	Leukocyte common antigen CD45 receptor-tyrosine phosphatase
Transcription Factors	S129+S133	2.4	cAMP response element binding protein 1
Other	Pan-specific	2.17	Heat shock 70 kDa protein 1

Table 3.3: Downregulation of signalling molecule phosphorylation in SSc epidermis. Phosphoprotein microarray data demonstarating the mean fold decrease in signalling protein phosphorylation in SSc epidermis biopsies versus normal controls. The twofold decrease has been used as a cut off. Phosphoproteins have been subdivided according to class, the fold change shown in descending order for each class (control epidermis n=4, SSc epidermis n=4).

For a False discovery rate of 15%, nineteen differentially phosphorylated sites were identified by SAM (Table 3.4). Of these, three would be expected to be false positives. Eleven sites exhibited greater phosphorylation in SSc compared with normal control subjects and 8 sites exhibited less phosphorylation in SSc.

Target protein name	Phospho site	Fold change	q-value (%)
Increased phosphorylation in SSc			
Hepatocyte growth factor (HGF) receptor-tyrosine kinase	Y1003	2.89	15.5
Protein-serine phosphatase 4 - regulatory subunit (PPX/A'2)	Pan-specific	2.63	0
Synapsin 1 isoform Ia	S605	2.62	15.5
Protein-serine kinase C eta	S674	2.57	15.5
NIMA (never-in-mitosis)-related protein-serine kinase 7	Pan-specific	2.29	0
Signal transducer and activator of transcription 3	Y705	2.18	15.5
Dual specificity protein kinase	Pan-specific	2.18	12.3
Protein-serine kinase C gamma	T674	2.18	15.5
Integrin-linked protein-serine kinase 1	Pan-specific	2.17	15.5
Mitogen-activated protein-serine kinase p38 alpha	T180+Y182	2.15	15.5
PCTAIRE-1 protein-serine kinase	Pan-specific	2.10	12.3
Decreased phosphorylation in SSc			
Pro-caspase 3 (apopain, cysteine protease CPP32)	Pan-specific	0.31	8.2
Caspase 5 (ICH3 protease, ICE(rel)-III)	Pan-specific	0.36	8.2
Cyclin-dependent protein-serine kinase 4	Pan-specific	0.39	12.3
Cyclo-oxygenase 2 (prostaglandin G/H synthase 2 precursor)	Pan-specific	0.40	0
Cell division cycle 25B phosphatase	Pan-specific	0.40	0
Heme oxygenase 2	Pan-specific	0.40	0
Cell division cycle 2-like protein-serine kinase 5	Pan-specific	0.41	0
Leukocyte common antigen CD45 receptor-tyrosine phosphatase (LCA, T200)	Pan-specific	0.44	8.2

Table 3.4: Differentially phosphorylated sites in epidermis from SSc patients compared with normal control subjects (n=4, $P<0.05$).

Out of over 500 phosphoproteins screened by Kinexus 78 are found to be over-expressed and 61 are under-expressed in SSc (see full list in Appendix 2). I used a twofold cut off to demarcate a biologically important change (X. Shi-Wen *et al.* 2000b).

The phosphoproteins over-expressed in SSc represent those involved in signal transduction pathways from receptors to transcription factors, as well as cell cycling and cell metabolism participants. Many are involved in wound healing and fibrotic processes.

For instance, phosphorylation of a member of the AP 1 family of transcription factors c- Jun is more than doubled in SSc epidermis. c-Jun is the name of a gene and protein which, in combination with c-Fos, forms the AP-1 early response transcription factor. It is activated through double phosphorylation by the JNK pathway but has also a phosphorylation-independent function (H. J. Rahmsdorf 1996b) (Figure 1.3). c-Jun is phosphorylated in response to UV radiation, and is now recognised as a regulator of cell proliferation, cell death, DNA repair and metabolism (M. Karin, E. Gallagher 2005b). It is known that TGF-β acts via c-Jun to activate fibronectin production (B. A. Hocevar *et al.* 1999b). Fibronectin is an ECM component important in both wound healing and fibrosis (D. Greiling, R. A. Clark 1997a;G. Serini *et al.* 1998a). Thus c-Jun over-expression in SSc epidermis is possibly a marker of TGF-β activity which could be contributing to skin fibrosis in the disease.

Tyrosine hydroxylase phosphorylation is raised more than five times in SSc epidermis. It is an intermediate enzyme in ET-1 driven melanogenesis, the latter well described in SSc skin pigmentation (G. Imokawa *et al.* 1995c;X. Shi-Wen *et al.* 2001d).

Integrin linked kinase (ILK) is a serine-threonine kinase that couples integrins (primarily the β1 integrin) to downstream signalling pathways that regulate a variety of cellular functions including cell viability. I found that phosphorylated ILK is increased two-fold in SSc epidermis. ILK is required for TGF- β dependent epithelial-mesenchyme transition in the epidermis as well as KC migration (Y. I. Lee *et al.* 2004b;Y. Yang *et al.* 2008a). It is interesting that this kinase is known to inhibit fibroblast apoptosis in response to ECM contraction in FPCLs thus leading to persistence of fibroblasts in this environment. Knockout studies showed that hyperactive ILK and wild type ILK protected fibroblasts from collagen matrix contraction-induced apoptosis compared to knockout animals. Thus fibroblasts continue to be active where they are normally removed in the setting of controlled tissue repair (Y. Li *et al.* 2003a;R. S. Nho *et al.* 2005b).

In addition HGF receptor tyrosine kinase phosphorylation at the site normally activated by HGF itself (Y1003) is almost tripled in SSc epidermis. HGF is released in response to tissue injury. In the epidermis it is involved in fibroblast-epithelial cross talk thus controlling epithelial cell recovery (R. Zarnegar 1995). The role of HGF is SSc is also well described. It is interesting that in fibroblasts its role is antifibrotic (T. Iwasaki *et al.* 2006;R. Sherriff-Tadano *et al.* 2006). It may be that in the epidermis HGF has different role to the one in the fibroblasts, alternatively it is there as a feedback mechanism to try and prevent excessive fibrosis.

In conclusion, the results highlight changes in signalling pathways responsible for fibrosis and wound healing.

3.3 Assays of growth factors and cytokines in SSc epidermis:

Since both the structural and some of the signalling properties of the SSc epidermis resemble those of a healing wound, I looked for any alterations in soluble mediators that would confer a wound healing phenotype in the disease. I looked at three mediators: IL-1, ET-1, and TGF-β.

IL-1, because it is one of the most important cytokines involved in the wound healing process. KCs are known to contain cytoplasmic reservoirs of IL-1, which get rapidly released upon cell injury and activate surrounding KCs and dermal cells (A. Mauviel *et al.* 1993b;A. Mauviel *et al.* 1991b). In addition, IL-1 is known to act via PKC and to induce p38 and c-Jun signalling, which are notably raised in SSc epidermis (Table 3.2).

TGF-β is also involved in all aspects of wound repair, from inflammation to re-epithelialisation and remodelling (S. Werner, R. Grose 2003d). In addition, several molecules over-expressed in SSc epidermis are involved in TGF-β signal transduction pathways (eg. HGF and c-Jun).

ET-1 is primarily involved in fibroblast activation and contraction post wounding, and has been noted to be important in SSc fibrosis (S. Xu *et al.* 1998). In addition Tyrosine Hydroxylase, which is highly raised in SSc epidermis, is involved in ET-1 driven melanogenesis (G. Imokawa *et al.* 1995b).

Four SSc patient and four normal control epidermal discs were lysed after surgical separation from the dermis and examined using ELISA for the presence of the above mediators. Quantification of the average concentrations of IL-1, ET-1, and TGF-β in healthy control and SSc epidermal samples is presented below.

Signalling molecule	Normal epidermis (mean and range)	SSc epidermis (mean and range)
TGF-β (active)	168.09 (139.64-194.68)	135.25 (100.56-153.16)
LAP-TGF-β	1222.76 (1101.44-1404.74)	1436.12 (1291.01-1499.77)
ET-1	668.89 (651.44-686.12)	687.79 (672.03-705.06)
IL-1	2.99 (0.5-10.5)	74.2 (4.66-170.54)

Table 3.5: **Results of ELISA immunoassay of normal and SSc epidermal lysates for active and LAP-TGF-β, ET-1 and IL-1.** Signalling molecule concentrations were measured by ELISA of epidermal biopsy material from SSc patients (n=6) and healthy controls (n=6).

3.3.1 TGF-β content in SSc epidermis:

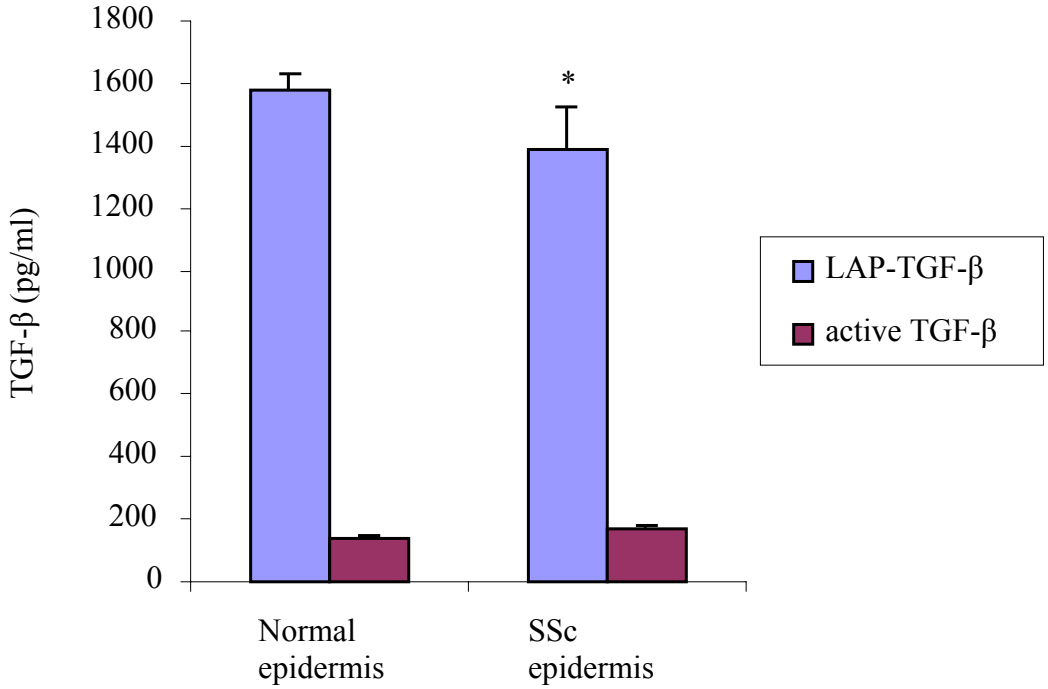


Figure 3.4: Histogram to show TGF-β (active and LAP) content of normal and SSc epidermal lysates as determined by ELISA. Lower level of detection is 11 pg/ml. *p=0.044

3.3.2 ET-1 content in SSc epidermis:

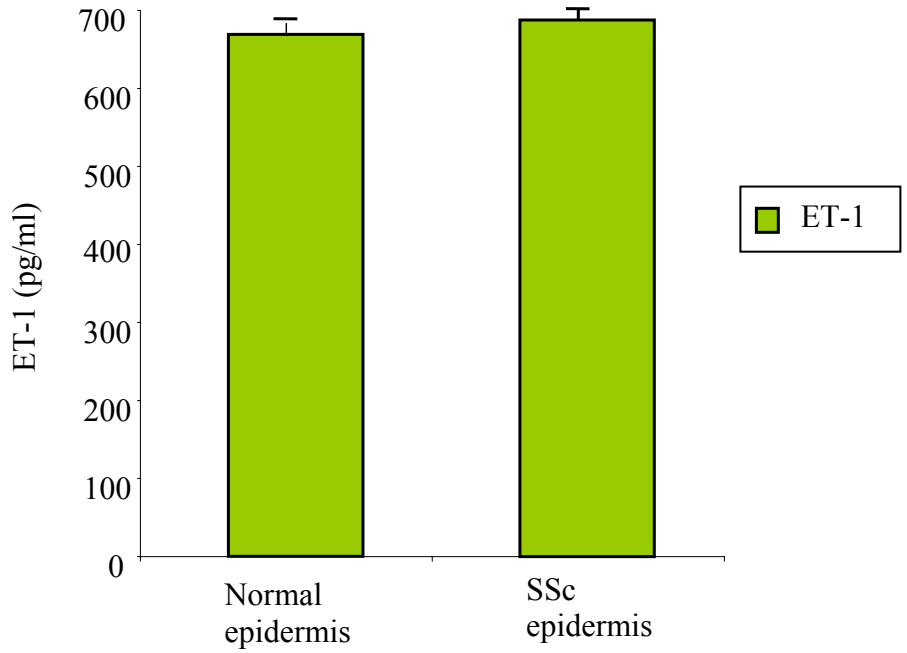


Figure 3.5: Histogram to show ET-1 content of normal and SSc epidermal lysates as determined by ELISA. Lower level of detection is 600 pg/ml. p=0.11

3.3.3 IL-1 content in SSc epidermis:

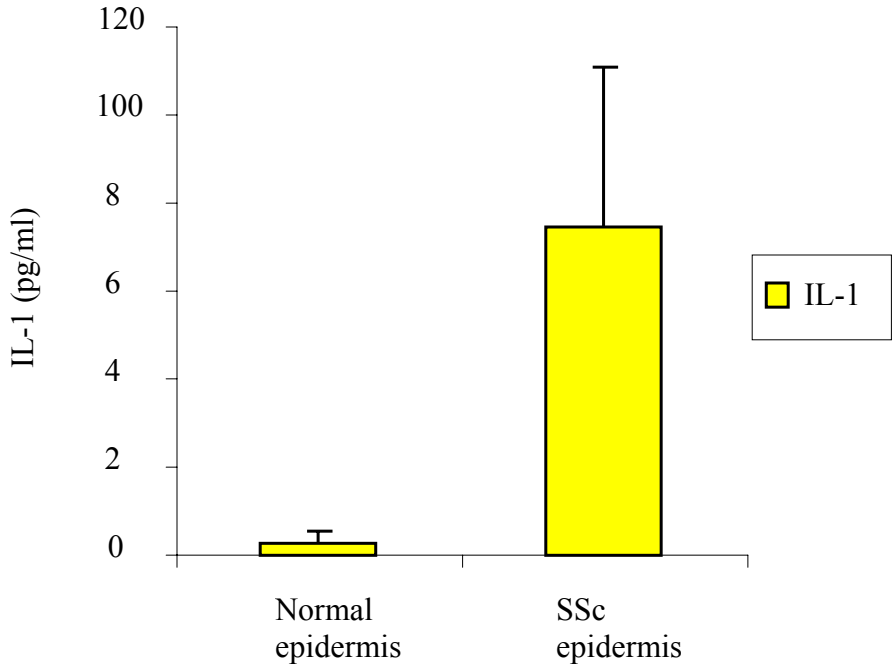


Figure 3.6: Histogram to show IL-1 content of normal and SSc epidermal lysates as determined by ELISA. Lower level of detection is 0.4 pg/ml. $p<0.05$

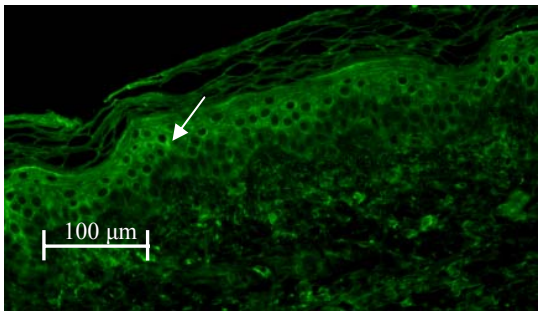
ELISA immunoassay was used to measure the active TGF-β and LAP-TGF-β content in SSc and normal epidermal lysates (Figure 3.4, Table 3.4).

There was no significant difference between the total TGF-β content of the four normal and four SSc epidermial samples taken from eight different subjects ($p=0.069$). There was no significant difference in the active TGF-β content of the samples ($p=0.129$). LAP-TGF-β was only marginally increased in SSc epidermis compared to control (SSc epidermis LAP-TGF-β concentration 1436 pg/ml, normal epidermis LAP-TGF-β concentration 1222.7 pg/ml; $p=0.044$).

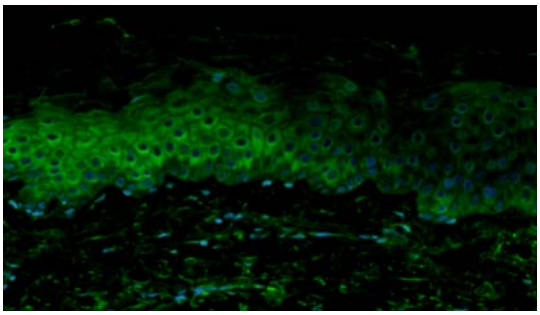
ELISA immunoassay to measure ET-1 concentrations in the SSc and normal epidermal lysates was also performed (Figure 3.5, Table 3.4). No significant difference between these concentrations was found (normal epidermis mean ET-1 concentration 668.9 pg/ml, SSc epidermal mean ET-1 concentration 687.8 pg/ml; p=0.11). It is important to note that the levels of ET-1 were very low on the ELISA detection curve making it difficult to interpret the differences between the samples. IL-1 concentration of SSc epidermal lysates was significantly higher than that of normal controls as measured by ELISA (SSc epidermal mean ET-1 concentration 74.2 pg/ml versus normal epidermis mean IL-1 concentration 2.98 pg/ml; p=0.05) (Figures 3.6, Table 3.5).

3.4 Assays of normal and SSc epidermis for TGF-β, ET-1 and IL-1 activity:
3.4.1 TGF-β axis:
3.4.1.1 Immunohistochemistry for TGF-β:

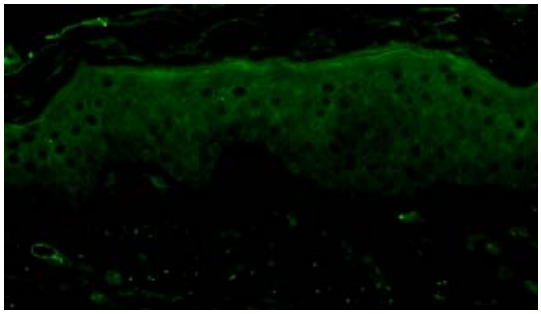
Immunohistochemical staining was used to visualise LAP-TGF-β and ET-1 levels in the disease and control epidermal samples (Figures 3.7, 3.11). Sections were also stained for pSMAD2/3 as a marker of TGF-β signalling activation in KCs (Figure 3.8, 3.9). Western blotting of two normal and two SSc epidermal lysates was used to further quantify pSMAD2/3 expression (Figure 3.10).



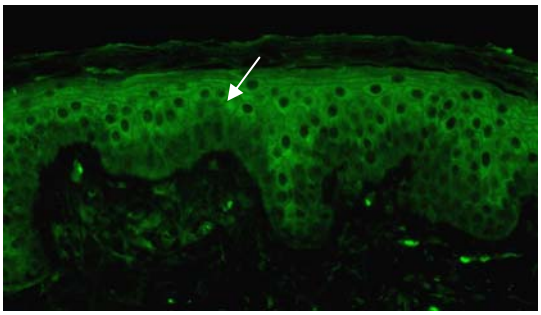
Normal epidermis active TGF-β



SSc epidermis active TGF-β



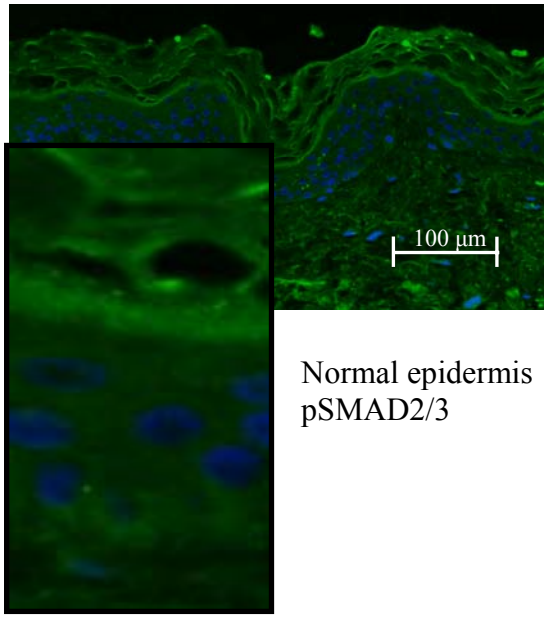
Normal epidermis LAP-TGF-β



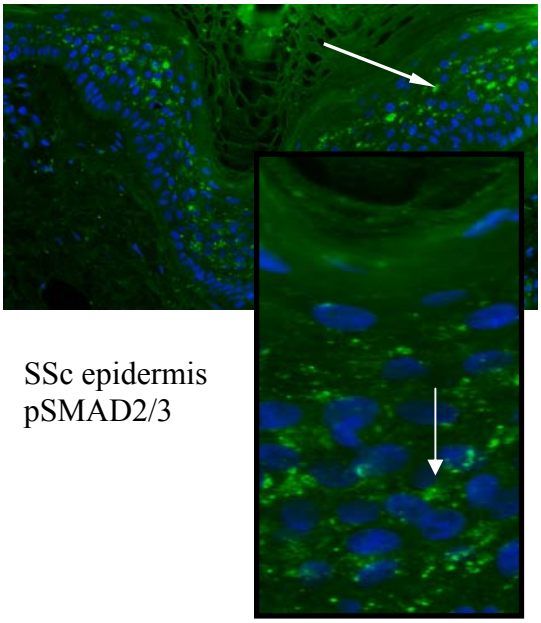
SSc epidermis LAP-TGF-β

Figure 3.7: Immunohistochemical staining of normal and SSc epidermis for active and LAP- TGF-β (n=3).

3.4.1.2 Immunohistochemistry for pSMAD:

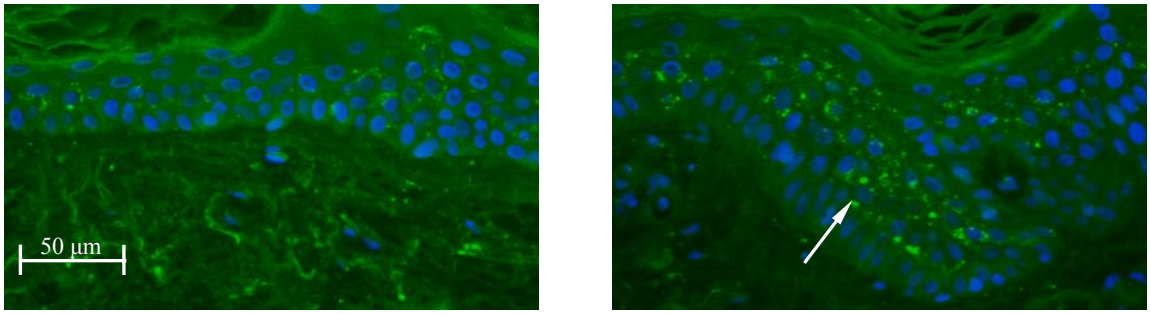


Normal epidermis pSMAD2/3



SSc epidermis pSMAD2/3

Figure 3.8: Immunohistochemical staining of normal and SSc epidermis pSMAD2/3 (arrow). (x20 magnification). The inserts show magnified areas of the epidermal sections (n=3).



Normal epidermis pSMAD2/3 SSc epidermis pSMAD2/3

Figure 3.9: Immunohistochemical staining of normal and SSc epidermis pSMAD2/3 (arrow). (x40 magnification) (n=3).

Immunohistochemical staining of SSc epidermis versus normal control highlights the elevated expression of LAP-TGF-β (Figure 3.7) and pSMAD2/3 (Figures 3.8, 3.9) in the disease. pSMAD2/3 expression appears elevated in SSc sections in the epidermal layers as well as the adjacent epidermal-dermal junction. Of note is also the increased thickness of the SSc epidermis compared to control.

3.4.1.3 Western blot for pSMAD:

Western blotting confirms increased pSMAD2/3 production in SSc epidermal lysate compared to normal control (Figure 3.10), indicating a more active TGF-β signalling pathway. It is notable that Kinexus phosphoprotein array has highlighted an increase in SMAD phosphorylation in SSc epidermis 1.64 fold over the control (Appendix 1).

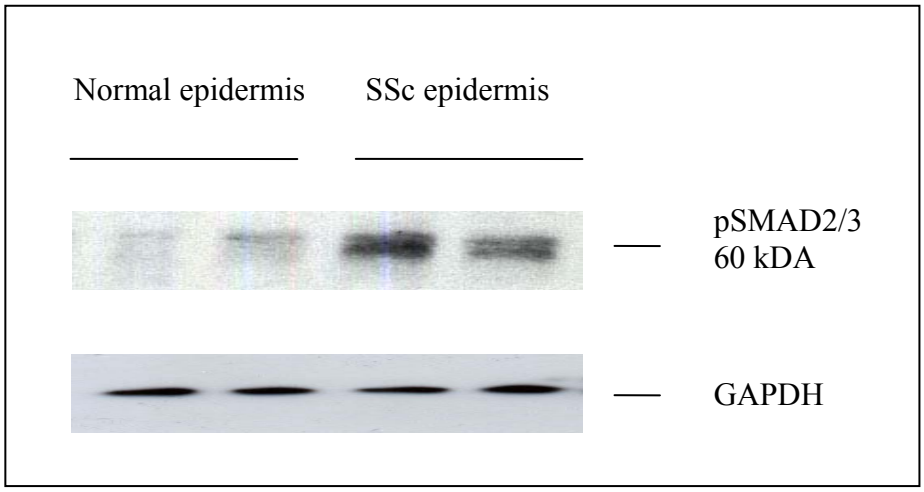
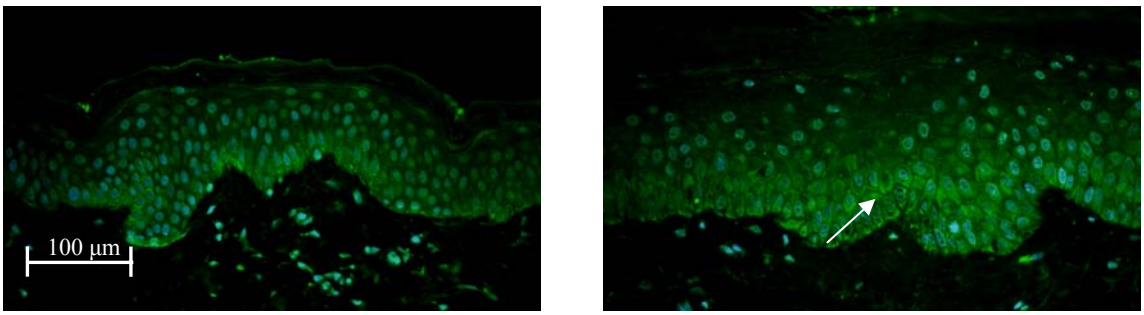


Figure 3.10: Western blot of 2 normal and 2 SSc epidermises for pSMAD2/3. GAPDH protein loading control shown.

3.4.2 Immunohistochemistry for ET-1 and ET receptors:

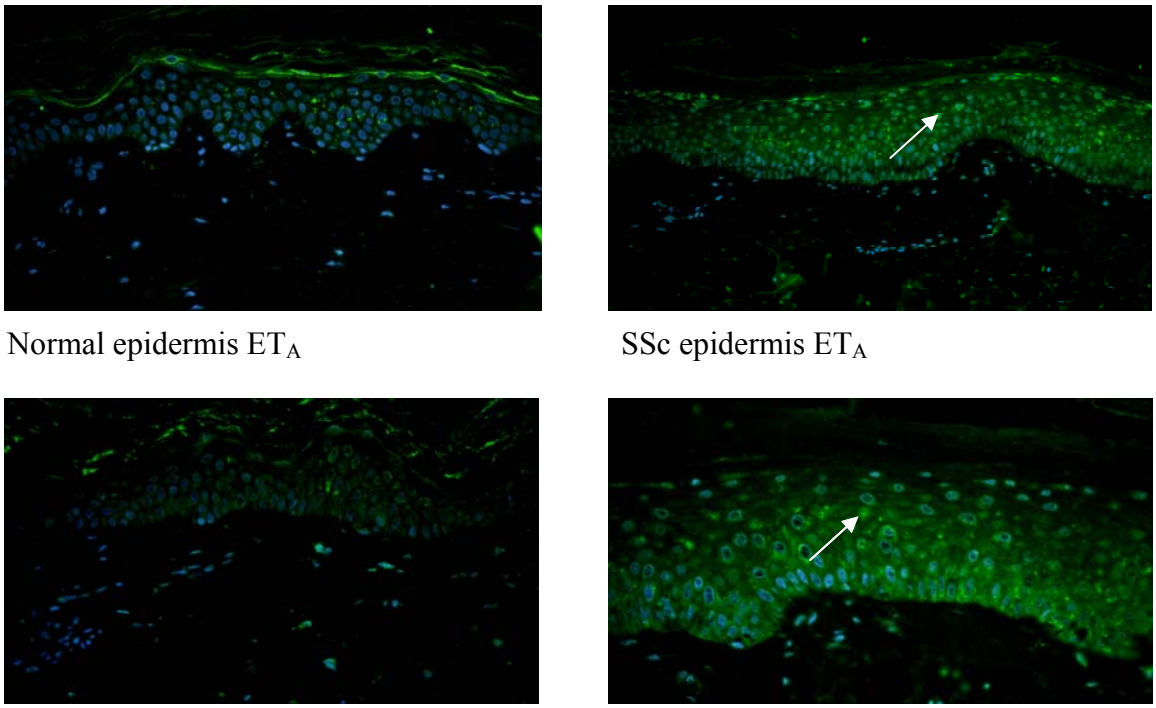
Immunohistochemical staining for ET-1 and its receptors ETA and ETB in SSc and normal epidermal biopsies was also performed (Figure 3.12, 3.13).



Normal epidermis ET-1

SSc epidermis ET-1

Figure 3.11: Immunohistochemical staining of normal and SSc epidermal biopsies for ET-1 (X20 magnification) (n=3).



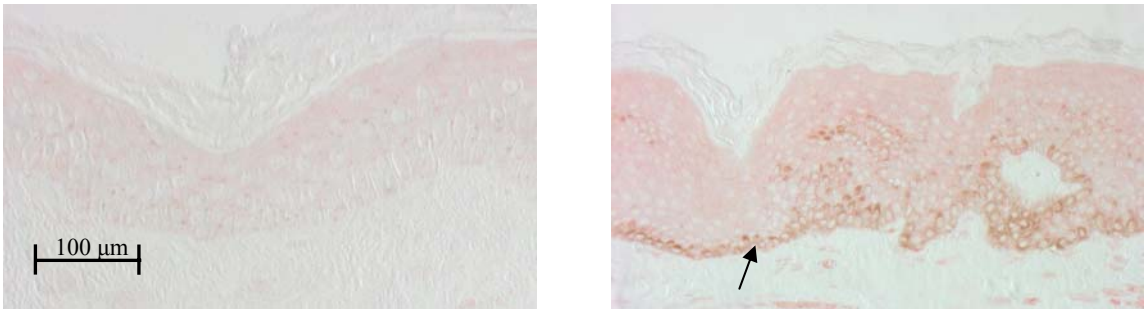
Normal epidermis ET_A SSc epidermis ET_A

Normal epidermis ET_B SSc epidermis ET_B

Figure 3.12: Immunohistochemical staining of SSc and normal epidermal biopsies for ET-A and ET-B receptors (X20 magnification) (n=3).

ET-1 expression was seen in the cytoplasm of basal cells of both the disease and normal epidermis, and appears to be increased in the disease (Figure 3.11). Staining for the ET-1 receptors A and B also demonstrates an increase in the disease epidermal biopsy compared to the control (Figure 3.12). However both ET-1 and its receptor expression is patchy in the samples. Therefore it is not surprising that ELISA data from epidermal lysates did not yield significantly differences between disease and control samples (Figure 3.5).

3.4.3 Immunohistochemistry for IL-1 receptor:



Normal epidermis IL-1R SSc epidermis IL-1R

Figure 3.13: Immunohistochemical staining of SSc and normal epidermal biopsies for IL-1 receptor (IL-1R) (X20 magnification) (n=3).

Staining for IL-1 receptor shows increased expression in SSc epidermis (Figure 3.13).

Discussion

The SSc epidermis has been noted to be altered in the past. Pigmentary changes, which can be severe and widespread, are a common feature and imply abnormalities of the epidermis in the disease (H. Tabata *et al.* 2000e). The studies presented in the current thesis are the first studies, however, that closely examine changes in its structure and signalling capacity, and note the similarities with a wound healing epidermis.

1. Altered cytokeratin expression in SSc epidermis:

The results of staining SSc and normal epidermal biopsies show a pattern of delayed and abnormal KC maturation and terminal differentiation with expansion of the nucleated cell layer and abnormal persistence of the basal cytokeratin 14 into suprabasal layers. There is little variation between samples with cytokeratin 14 consistently basal in controls. Cytokeratins 1 and 10 also move to a more superficial location in SSc, suggesting that their expression is delayed (Figure 3.2). These are robust and consisitent findings present in all of the biopsies studied. These findings lead to a comparison between the changes in SSc epidermis and those described during wound healing during which the epidermis becomes expanded and shows persistence of the basal phenotype into superficial layers (M. L. Usui *et al.* 2005g;G. K. Patel *et al.* 2006a). I went on to confirm that in SSc KCs are taking on an activated wound healing phenotype with suprabasal expression of the inducible cytokeratins 16 and 6, which are normally absent from an intact epidermis (Figure 3.3) (M. D. Hertle *et al.* 1992e). These changes were seen in all the SSc biopsies and none of the control samples (6 out of 6 SSc biopsies, 0 out of 6 control biopsies, chi-square=12, p=0.001, 1 degree of freedom).

Other indirect evidence supports the comparison between SSc and a wounded epidermis. The basal α6 integrin lost its clear basement membrane demarcation in the disease showing strong expression into the suprabasal layers consistent with a wound healing response (Figure 3.2) (M. D. Hertle *et al.* 1992d).

I therefore went on to determine whether the signalling pathways activated in the disease also resemble those activated after normal epidermal wounding in order to gain insite into activation of the epidermis in SSc.

2. Altered protein phosphorylation in SSc epidermis:

2.1 Upregulation of signalling molecule phosphorylation in SSc epidermis:

Phosphoprotein microarray analysis of SSc and normal epidermal samples was performed and has highlighted several differences (Tables 3.1 and 3.2).

2.1.1 Upregulation of enzyme phosphorylation in SSc epidermis:

The signalling molecule whose phosphorylation is most noticeably raised in SSc epidermis (over a five fold increase) is Tyrosine Hydroxylase (TH). This enzyme is responsible for the first step in the biosynthesis of catecholamines in human KCs, which is in turn responsible for melanocyte pigmentation via beta2 adrenergic receptors (K. U. Schallreuter *et al.* 1992). TH is also required for melanogenesis within melanocytes themselves via L-Dopa production (L. K. Marles *et al.* 2003). This finding is not surprising as pigmentation of SSc skin is well recognised clinically. ET-1 is known to be important in pigmentation in general and hyper-pigmentation of SSc skin in particular. It has also previously been shown that ET-1 acts via TH to cause UV-induced melanocyte stimulation (G. Imokawa *et al.* 1995a). It is possible, therefore, that some of the pigmentary action of ET-1 in SSc epidermis is via activation of TH.

Phosphorylation of the Hepatocyte growth factor (HGF) receptor-tyrosine kinase is increased 2.89 fold in SSc epidermis compared to normal controls. The phosphorylation site Y1003 is the same as that activated by HGF binding itself. HGF was originally identified as a potent mitogen in hepatocytes (E. Gohda *et al.* 1988). However, it has also been identified in platelets and is thought to be important in wound healing regulation (T. Nakamura *et al.* 1986). In line with its importance in epidermal healing processes, HGF has been shown to stimulate both migration and proliferation of normal human KCs (K. Matsumoto *et al.* 1991). HGF is also found to stimulate matrix metalloproteinase (MMP-1) production by KCs. This collagenase is normally expressed by migrating cells at the front of the re-epithelialising edge in chronic wounds. Persistent HGF-driven MMP-1 over-expression may be important, therefore, in wound chronicity (S. E. Dunsmore *et al.* 1996).

In addition to its role in wound healing, HGF seems to be important in preventing cell death. HGF has potent anti-apoptotic properties, which have been confirmed in developing hepatocytes, and are known to act via a Bcl-2-like protein BAG-1 (A. Bardelli *et al.* 1996). Interestingly, Bcl-2-like protein, known to be an apoptotic signal, is found to be reduced three fold in SSc epidermis.

Thus HGF may be a significant player in confirming the pro-proliferative as well as persistent wound healing phenotype of the SSc epidermis.

TGF-β is known to stimulate HGF production, which in turn has inhibitory anti-TGF-β and anti-fibrotic properties in SSc fibroblasts (T. Iwasaki *et al.* 2006;M. Jinnin *et al.* 2005). We need to reconcile the fact that HGF is anti-fibrotic in nature with increased presence of its receptor tyrosine kinase in SSc epidermis. One possibility is that HGF is indeed activating its epidermal receptors in a natural inhibitory feedback mechanism in order to counteract the action of the pro-fibrotic

surrounding environment of the SSc epidermis. On the other hand, HGF production by SSc fibroblasts may be feeding back to the epidermis to induce its migratory and proliferative wound healing-type phenotype. In fact it is believed that following tissue injury mesenchymal cells release HGF which in turn regulates epithelial cell repair. HGF synthesis is induced by IL-1 (R. Zarnegar 1995). In this case, a loop between epidermis and dermis would be established. The outcome of this feedback would depend on the overall environment and duration of the disease.

Phosphorylation of Protein kinase C (PKC) is found to be more than doubled in SSc epidermis compared to normal control. It is a kinase involved in transmembrane signal transduction, and is thought to be important in epidermal function. PKC is known to be involved in promotion of epidermal proliferation, its over-expression implicated in psoriasis pathogenesis (L. Hegemann *et al.* 1991;L. Hegemann *et al.* 1994). Along with HGF kinase, PKC may be responsible for the increased activation of disease KCs.

Phosphorylation of integrin-linked protein-serine kinase 1 (ILK) is doubled in SSc versus normal epidermis. This kinase is known to be important in epithelial integrin-dependent cell cycle progression regulation as well as inhibition of apoptosis (G. E. Hannigan *et al.* 1996;S. Persad *et al.* 2000;G. Radeva *et al.* 1997). In addition, phosphorylation of NIMA kinases is more than doubled in SSc epidermis suggesting that these cells are more mitoticaly active and have increased survival kinetics compared to normal cells.

2.1.2 Upregulation of transcription factor phosphorylation in SSc epidermis:

Phosphorylation of the members of the transcription factor activator cascade is also increased in SSc epidermis. Signal Transducers and Activators of Transcription (STATs) are transcription factors that are phosphorylated by JAK kinases in

response to cytokine activation of cell surface receptor tyrosine kinases. Phosphoprotein microarray has highlighted doubling of STAT3 phosphorylation in SSc epidermis. Upon activation, the STATs dimerise and are localised to the nucleus where they activate transcription of cytokine-responsive genes. Cytokines and growth factors that activate STAT3 include growth hormones such as HGF, IL-6 family cytokines, and G-CSF. STAT3 induces progression through the cell cycle, prevents apoptosis, and regulates cell migration (M. Kira *et al.* 2002). In the epidermis, STAT3 is essential for wound healing, and is found in the leading wound edge. Transgenic mice over-expressing STAT3 are found to have abnormal KC activation which results in the development of psoriasis-like lesions (S. Sano *et al.* 2005). Epithelial tubule formation required for cell differentiation, is also regulated by HGF through STAT3 signalling (C. Boccaccio *et al.* 1998).

Phosphorylation of c-Jun AP-1 transcription factor is increased 2.3 fold in SSc epidermis. This transcriptional factor is widely responsible for gene regulation in response to a variety of stimuli which activate the MAPK cascade. It is found in the epidermis, and is thought to be important in KC differentiation and cell cycle progression, as well as cell proliferation. AP-1 is activated by IL-1 via MAPK signalling that involves JNK and p38. The products of this activation include KGF and GM-CSF, which are thought to be involved in epithelial-mesenchymal crosstalk (J. Ninomiya-Tsuji *et al.* 1999;H. E. Barksby *et al.* 2007b;L. Florin *et al.* 2004b). I find that phosphorylation of p38, which is upstream of c-Jun AP-1, is also increased in SSc epidermis (Table 3.1). IL-1 and its signalling cascade are activated after epidermal injury (Figure 1.3), and the above data supports the notion that SSc epidermis has wound healing characteristics.

Another group of proteins with increased phosphorylation prophiles in SSc epidermis are cell-cycling promoters.

Several other biologically important proteins have increased phosphorylation profiles in SSc versus normal epidermis and are worth mentioning even though their fold increase falls below the twofold cut off. These are: TGF-β activated protein serine kinase1, MAPK/ERK kinase kinase 2 and inhibitor of NF-kB protein serine kinase β (Appendix 1). The first is clearly involved in TGF-β signalling cascade, and the latter two are important in IL-1 signalling (Figure 1.3). This supports the idea of SSc epidermis having activated TGF-β and IL-1 pathways.

2.2 Downregulation of signalling molecule phosphorylation in SSc epidermis:

2.2.1 Downregulation of cell cycling protein phosphorylation in SSc epidermis:

The phosphoproteins with reduced phosphorylation in SSc epidermis represent inhibitors of cell cycle progression. For example Diacylglycerol kinase (DGK), with three fold reduced phosphorylation in SSc versus normal control epidermis, is known to both interfere with cell cycle progression and has been found to be anti-fibrotic in cardiac muscle (C. Evangelisti *et al.* 2007;M. Harada *et al.* 2007). Under-expression of DGK would therefore encourage cell proliferation in the epidermis and could potentially have pro-fibrotic consequences.

In addition, phosphorylation of p21 is found to be reduced in SSc epidermis.

The picture is a little more complicated, however, since we find that certain cell cycling promoters are actually under-expressed in SSc epidermis: cell division cycle kinases and phosphatases, as well as cyclin-dependent protein kinases are more than halved, highlighting the abnormal cycling dynamics of the disease cells.

2.2.2 Downregulation of apoptosis related protein phosphorylation in SSc epidermis:

Further proteins with reduced phosphorylation include those involved in stimulation of apoptosis: Bcl-2 like protein (see above), heat shock protein, Capsase-5, and Calcium/calmodulin-dependent protein kinases (K. M. McGinnis *et al.* 1999;T. R. Soderling 1999;L. Zhuang *et al.* 2000). Their reduced function in SSc epidermis would promote cell survival and thus abnormal proliferative dynamics.

2.2.3 Downregulation of COX II phosphorylation in SSc epidermis:

Phosphoprotein array demonstrates that phosphorylation of COX II is suppressed in SSc epidermis. Chapter 5 of this thesis addresses the role of prostaglandins in epidermal function. Importantly, prostaglandins are known to inhibit TGF-β signalling in KCs, and are also known to be antifibrotic through inhibition of CTGF in human fibroblasts (R. Stratton *et al.* 2002f). Reduction of prostaglandin synthesis in SSc epidermis would be consistent with the profibrotic state of this organ.

In summary, phosphoprotein microarray data indicates that the SSc epidermis differs from normal in its protein expression pattern with pro-proliferative, wound healing-like features.

In view of these findings, changes in soluble mediator expression by SSc epidermis were addressed.

2.3 Immunohistochemistry of normal and SSc epidermis for TGF-β, ET-1, and IL-1 axis:

Immunohistochemical staining showed that the active TGF-β levels in SSc epidermis are not significantly different from those of the normal control group (Figure 3.8). This free active TGF-β form is patchy in the epidermis and may reflect the variable need for this growth factor in every day epidermal functions. This correlates with the

fact that previous published experimental evidence involving SSc skin biopsy staining produced highly varied results with regards to TGF-β presence and quantity in the epidermis (M. Gruschwitz *et al.* 1990a;A. McWhirter *et al.* 1994a;J. Pannu *et al.* 2004a;J. Peltonen *et al.* 1990c;P. P. Sfikakis *et al.* 1993a). In line with these results are the findings in the serum of patients affected by SSc. It has been demonstrated that active TGF-β is actually reduced in early disease, and the quantity is inversely correlated with skin score (M. Dziadzio *et al.* 2005b).

LAP-TGF-β and pSMAD levels, on the other hand, were increased in SSc epidermis (Figures 3.8-3.11). The LAP-TGF-β result was confirmed by ELISA performed on the homogenised epidermal samples from normal and SSc affected subjects (Figure 3.5). The mean active TGF-β concentration was slightly reduced in the SSc epidermal samples compared to the healthy controls, although the difference was not statistically significant ($p=0.129$). However, when LAP-TGF-β was measured by subtracting the active from the total TGF-β concentration, the difference between the means was statistically significant with higher LAP-TGF-β concentration in SSc epidermis ($p=0.044$). This may be a significant finding as TGF-β has an important role in wound healing in general and epithelial re-surfacing in particular (C. Amendt *et al.* 2002d;S. Werner, H. Smola 2001d;S. Werner, R. Grose 2003c).

ET-1 has a well established role in fibrosis (M. B. Kahaleh 1991b;S. Xu *et al.* 1998). It is found in epithelial cells and fibroblasts. ELISA immunoassay demonstrated no difference between normal and SSc epidermal ET-1 content (Figure 3.6). One explanation could be the limitation of the ELISA method in detecting very low levels of the factor. Another explanation could be that the nature of ET-1 expression in the epidermis is patchy and disease duration sensitive. Immunostaining of SSc

epidermis for ET-1 and its receptors demonstrates an increased expression compared to normal controls (Figures 3.12, 3.13). This result confirms previous published experimental data where increased ET-1 production in SSc epidermis was linked to increased pigmentation of the diseased skin (H. Tabata *et al.* 2000f). Previous published work describes the role of ET-1 in epithelial wound healing and fibrosis (Y Hamada 1999), thus supporting my hypothesis of a wound healing phenotype in SSc epidermis.

ELISA immunoassay and immunostaining demonstrated greatly increased IL-1α and its receptor content in SSc epidermis (Figures 3.7, 3.14). IL-1 is of course vital in driving the early stages of wound healing, its increased expression in SSc epidermis supporting the idea of a wound healing epidermal phenotype in the disease.

Conclusions

- SSc epidermis has a wound healing phenotype of cytokeratin, integrin and signalling molecule expression.
- SSc epidermis also has an increased expression of soluble mediators known to be important in wound healing and fibrosis.

CHAPTER 4

Scleroderma Epidermis has Fibroblast Activating Properties

Introduction

It has previously been shown that the SSc epidermis has an abnormal structure and growth factor content (K. Sauermann *et al.* 2002c;S. Werner *et al.* 2007d;H. Tabata *et al.* 2000g). Results from the previous chapter showed that the SSc epidermis has an altered phenotype with persistence of wound-healing type characteristics. Epithelial-mesenchymal interactions are a well recognised phenomenon in scarring conditions and wound environments (N. Maas-Szabowski *et al.* 1999e;P. Shephard *et al.* 2004f). In the latter the KC-fibroblast co-activation loop is thought to be that of double paracrine signalling, with KCs instructing fibroblasts to produce and secrete growth factors which are in turn capable of KC activation (I. M. Freedberg *et al.* 2001f). I hypothesised that the abnormalities of the epidermis in SSc are responsible in part for fibroblast activation and fibrosis in the adjacent dermis.

The study of fibroblast behaviour under the influence of various factors has been made possible by the FPCL system which mimics the complex interactions between cells and their surrounding matrix found in real tissues (F. Grinnell 2003c). The tension loaded restrained system was chosen for this study since I was interested in fibroblast activation and contraction under the influence of pro-fibrotic factors which include/induce TGF-β. TGF-β has a stronger impact on the differentiation of fibroblasts into myofibroblasts in restrained matrices where fibroblasts organise cell surface fibronectin and develop fibronexus junctions (P. D. Arora *et al.* 1999a;F. Grinnell 2003b). Restrained matrices are allowed to develop tension overnight

before they are released. In doing so fibroblasts are encouraged to align along stress axis promoting a contractile phenotype.

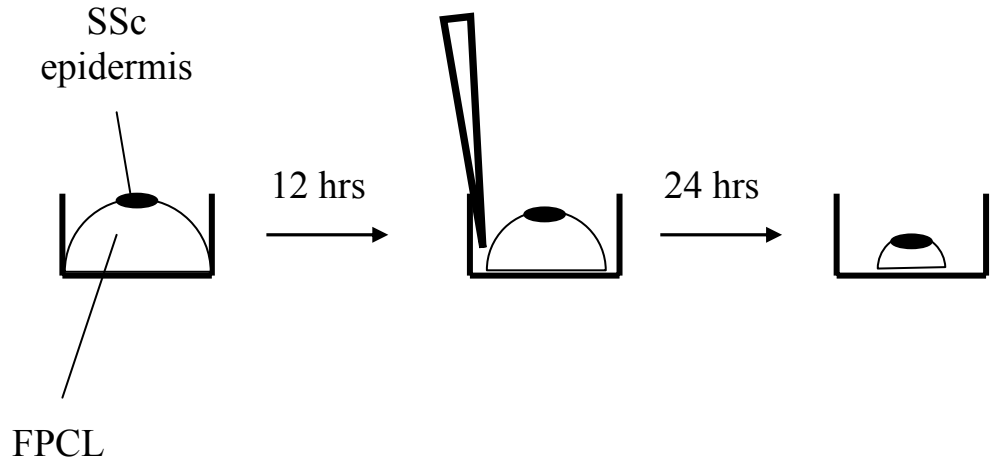


Figure 4.1: FPCL contraction in a tension loaded system. FPCL was treated with an epidermal disc and left for 12 hours. The lattice was then released with a pipette and allowed to contract.

Three mediators are known to be involved in fibrotic processes as well as in epidermal-fibroblast cross-talk: TGF-β, ET-1, and IL-1.

IL-1 is one of the most important cytokines involved in wound healing. KCs are known to contain cytoplasmic reservoirs of IL-1, which get rapidly released upon cell injury and activate surrounding KCs and dermal cells (A. Mauviel *et al.* 1993a;A. Mauviel *et al.* 1991c). It is known that IL-1 activates dermal fibroblasts and participates in paracrine epithelial-mesenchymal signalling. IL-1 is believed to initiate KC-fibroblast interaction and lead to induction of gene expression in fibroblasts including KGF and G-CSF (N. Maas-Szabowski *et al.* 1999d;Y. Chen *et al.* 2005a).

TGF-β is a well known fibrotic factor involved in SSc pathogenesis (T. Yamamoto *et al.* 1999a). Chen *et al.* have demonstrated that enhanced matrix contraction by dermal fibroblasts from SSc patients required TGF-β and its signalling pathways (W.

Xia *et al.* 2004b). In a mouse model of SSc anti-TGF-β antibody caused a significant reduction in cutaneous sclerosis and inflammation (R. Vancheeswaran *et al.* 1994c;R. Vancheeswaran *et al.* 1994b). In support of TGF-β involvement in epidermal-fibroblast crosstalk in fibrotic disease are the experiments performed with keloid scar cell co-culture, which demonstrated increased fibroblast proliferation rate and ECM production after exposure to keloid KCs (S. Xu *et al.* 1998).

ET-1 is also important in SSc fibrosis. Studies have shown increased plasma and tissue ET-1 levels in SSc patients (S. W. Xu *et al.* 2004c). ET-1 was found to induce a fibrogenic phenotype in normal dermal fibroblasts which resembled that seen in fibroblasts grown from lesional SSc skin. The contribution of ET-1 to KC-fibroblast cross-talk was also demonstrated in co-culture experiments (P. Shephard *et al.* 2004b).

Results from the previous chapter demonstrate significant alterations in ET-1, TGF-β and IL-1 content and signalling components of SSc epidermis.

I therefore examined whether normal human fibroblast are activated by SSc epidermis, proposing that ET-1, TGF-β and IL-1 are involved.

Results

4.1. Contraction of fibroblast collagen lattices by SSc epidermis:

FPCLs were treated for 48 hours with SSc or normal epidermial biopsy discs (Figure 4.2). In the same experiment FPCLs were treated with different concentrations of TGF-β. Contraction was seen with 8/8 SSc epidermis samples and 0/8 control samples (Figure 4.3).

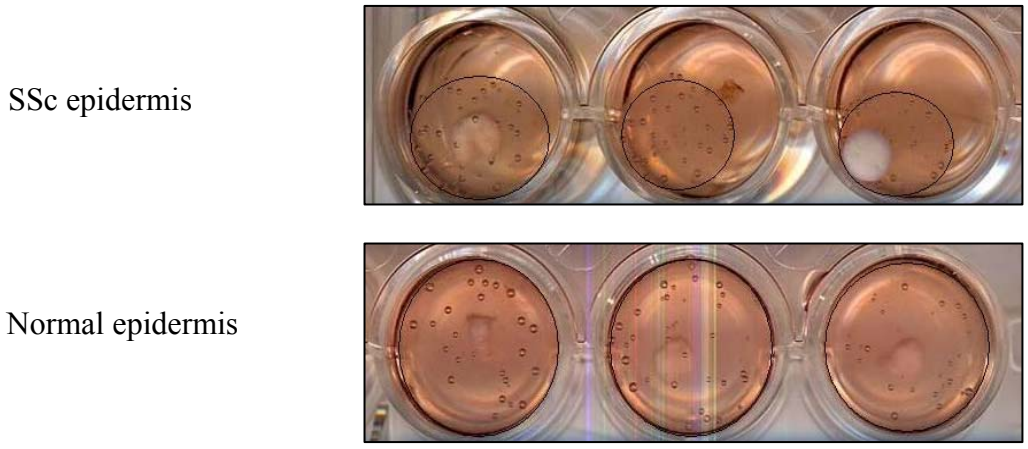


Figure 4.2: FPCL contraction after exposure to TGF-β, normal epidermis, and SSc epidermis. Normal dermal fibroblasts in FPCL were co-cultured with SSc epidermal cell layer (n=8), healthy control epidermis (n=8), TGF-β (4 ng/ml), or with medium only for 48 hours. Gel diameter was measured by photography. Co-culture with SSc epidermal cell layer and treatment with TGF-β lead to FPCL contraction. Contracted gels are highlighted with a black line for ease of visualisation.

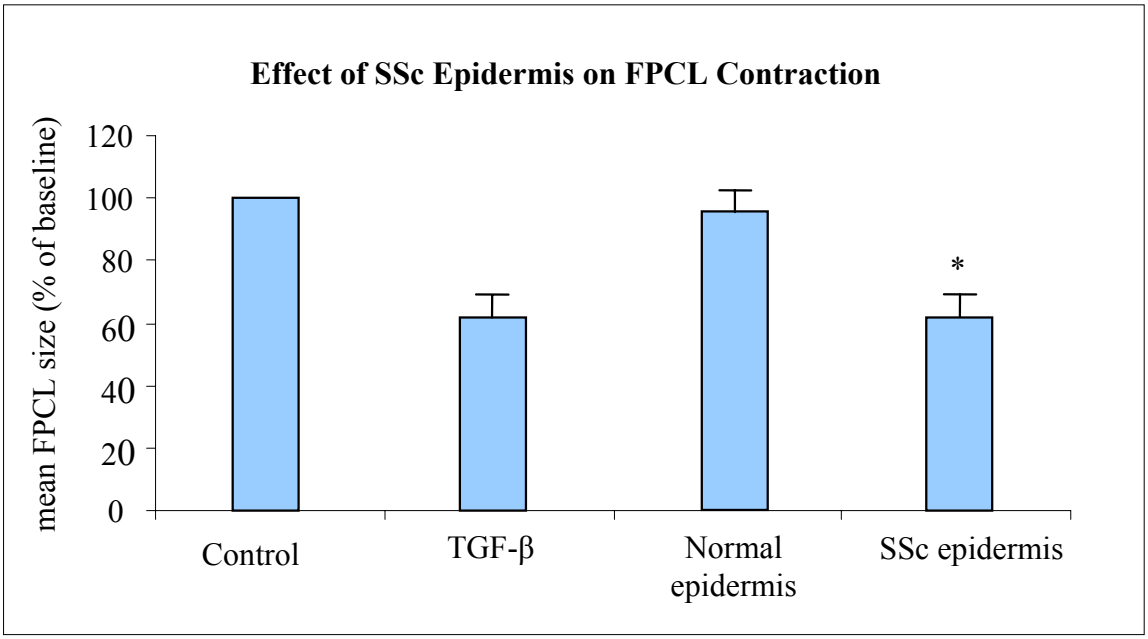


Figure 4.4: Histogram of the mean FPCL sizes. Contraction means were expressed as percentage (%) of baseline for each well type after treatment with control medium, TGF-β, normal epidermis and SSc epidermis. n=8 *p<0.01

4.1.1 CTGF expression in FPCLs:

Since I observed activation of normal fibroblasts to a contractile state, I hypothesised that treatment with SSc epidermis was causing the induction of CTGF, a marker of fibroblast activation in SSc. I went on to measure CTGF expression in the fibroblast lattices by Western blot analysis and by immunohistochemistry.

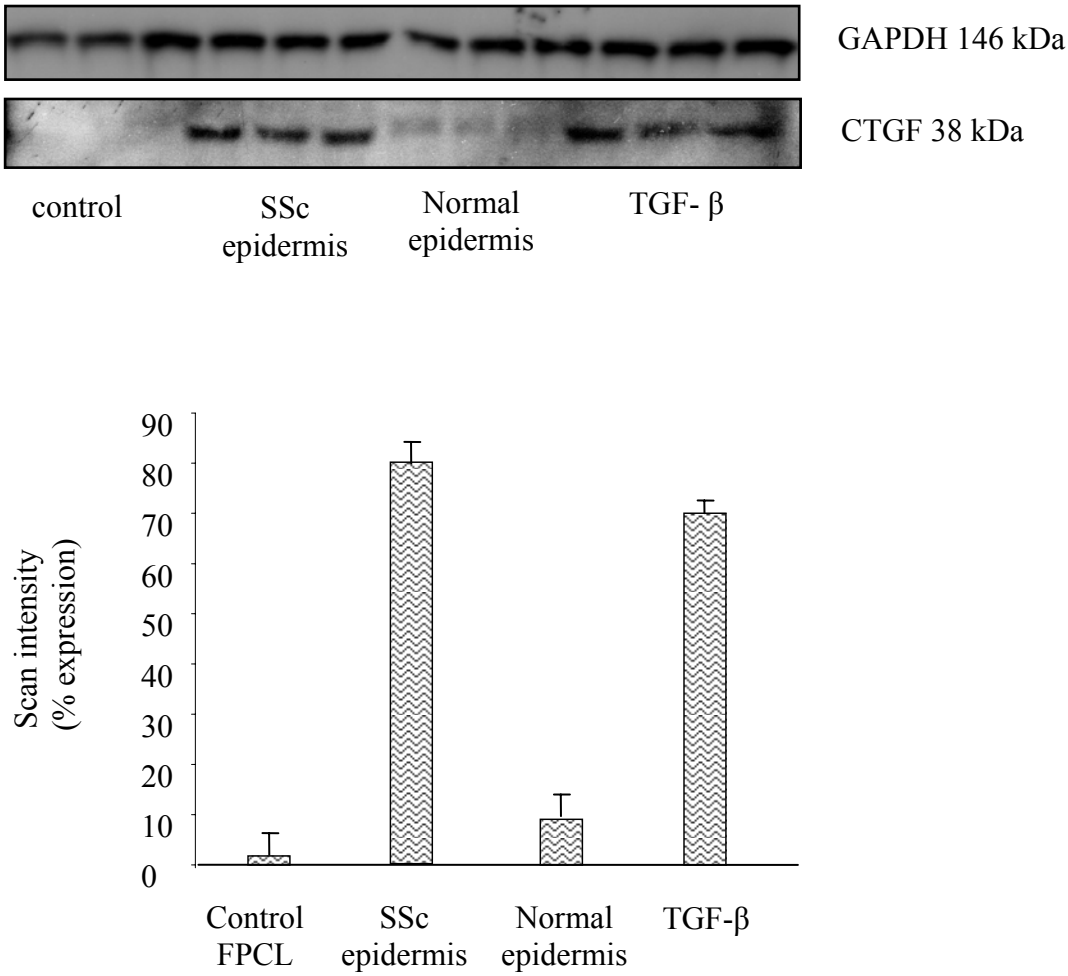


Figure 4.4: CTGF content of FPCLs. Western blot assay and histogram of CTGF content of FPCLs (n=3) that had been exposed to: medium only (control), SSc epidermis, normal epidermis, and TGF-β.

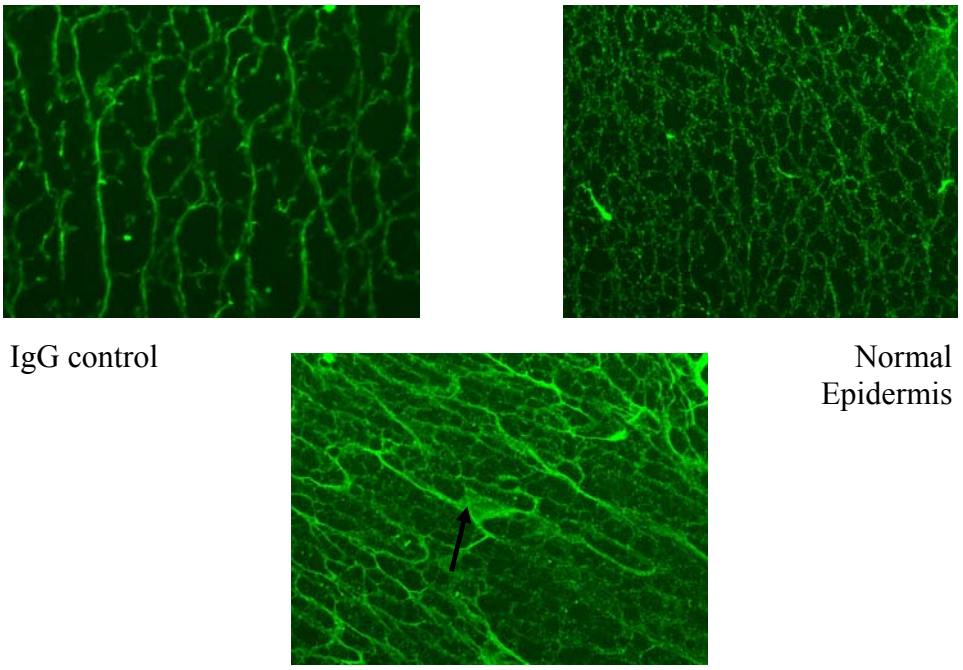


Figure 4.5: CTGF content of FPCLs - immunostaining. Immunohistochemical staining for CTGF of FPCLs that had been exposed to normal and SSc epidermis for 48 hours (n=4).

Western blot for CTGF (Figure 4.4) was used as a surrogate marker for TGF-β signalling activation in treated fibroblasts. The normal epidermis caused a rise in CTGF that was little more than the background expression in the untreated FPCL. As expected, TGF-β caused a significant rise in CTGF expression. The SSc epidermis caused a rise in CTGF expression to over and above the level seen with TGF-β treatment.

Immunohistochemical staining of treated FPCLs was used to confirm the above Western blot results (Figure 4.5). Staining of the untreated FPCL demonstrated a weak background staining for CTGF only, similar to that of IgG control. Normal epidermis FPCL treatment did not increase the lattice CTGF staining. SSc epidermis treatment, however, caused a pronounced increase in the intensity of CT

4.2 Contraction of fibroblast collagen lattices by SSc epidermis is TGF-β, ET-1, and IL-1 dependent:

4.2.1 Contraction of fibroblast collagen gels by SSc epidermis is TGF-β dependent.

I was interested in the mechanism by which SSc epidermis activates normal fibroblasts in vitro. Therefore further studies were performed using inhibitors of growth factors and cytokines involved in paracrine signalling between epithelial cells and fibroblasts (T. Krieg *et al.* 2007). I began by studying the effect of a neutralising anti-TGF-β antibody 1D11.

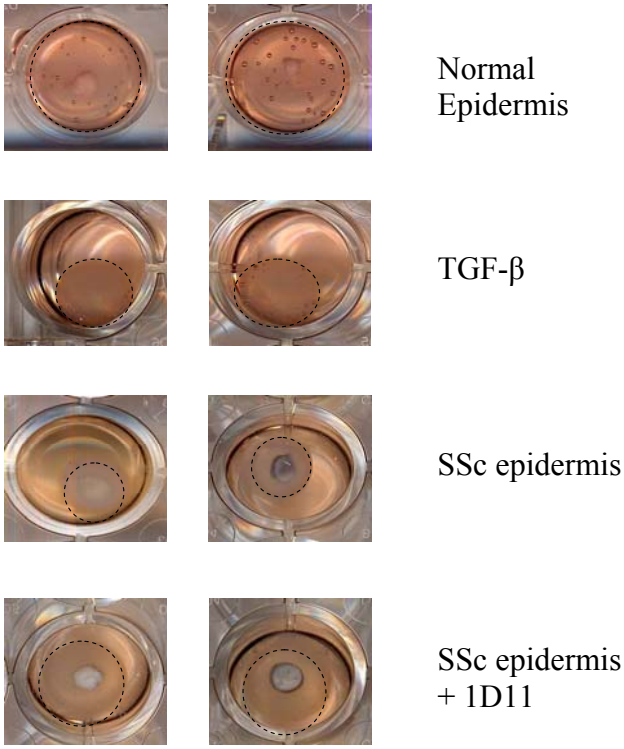


Figure 4.6: 1D11 inhibits SSc epidermis driven FPCL contraction. As before FPCL contraction was induced by co-culture with SSc epidermal cell layer and TGF-β for 48 hours. Addition of TGF-β antagonist 1D11(1 μg/ml) at baseline antagonised FPCL contraction by SSc epidermal cells (n=3). Summary figure of two representative contraction experiments is shown. Epidermal discs from the same patients were used to treat standard and 1D11 impregnated FPCLs.

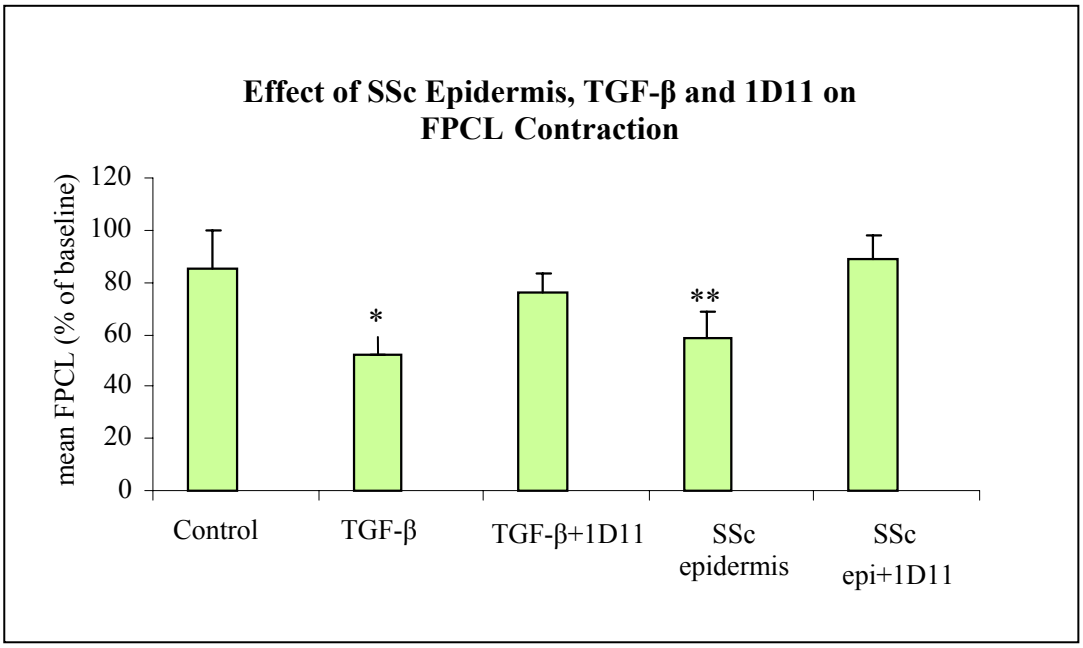


Figure 4.9: Histogram of SSc epidermis driven FPCL contraction after treatment with 1D11. Histogram of mean sizes of FPCLs after exposure to medium only (control), TGF-β, and SSc epidermis with and without 1D11 for 48 hours expressed as percentages of the baseline size. n=3; *p<0.01, **p<0.01

In these experiments two punch biopsies were taken from each subject's forearm. One was embedded in the standard fibroblast collagen gel, and the other was embedded in a gel containing the TGF-β neutralising antibody 1D11 (1μg/ml). The SSc epidermis produced contraction of the non-1D11 treated lattices as observed previously, down to 58.65% of the original gel size. However, pre-treatment with 1D11 prevented this contraction, with the lattice remaining at 88.47% of the baseline size. The contraction of the 1D11 pre-treated lattices exposed to the SSc epidermal disc was in fact similar to the contraction of the control untreated FPCLs (84.8%) and those of the 1D11-treated ones exposed to TGF-β (75.92%) (Figsure 4.6). The experiment was perfomed with three replicates (Figure 4.7). The difference

between the contraction produced by the SSc epidermal discs in the untreated FPCLs and ones pre-treated with 1D11 was statistically significant ($p<0.01$).

4.2.2 Contraction of fibroblast collagen gels by SSc epidermis is ET-1 dependent.

A further experiment was performed to determine the role of ET-1 in SSc epidermis-driven fibroblast activation. Two biopsies were taken from each of the forearms of three patients with early onset diffuse SSc and three control subjects. One epidermal disc from the biopsy was embedded in the standard FPCL. The other one from the same individual was embedded in the lattice containing ET-1 inhibitor bosentan. A concentration of bosentan (10 μM) known to inhibit ET-1 A and B receptors in cell culture experiments was used (S. Xu *et al.* 1998). The FPCL contraction elicited by the SSc epidermis was significantly inhibited by ET-1 inhibitor bosentan The experiment was repeated three times and the average SSc epidermis induced FPCL contraction was 56.26% , versus 90.7% with bosentan treatment ($p=0.01$) (Figures 4.8-4.11).

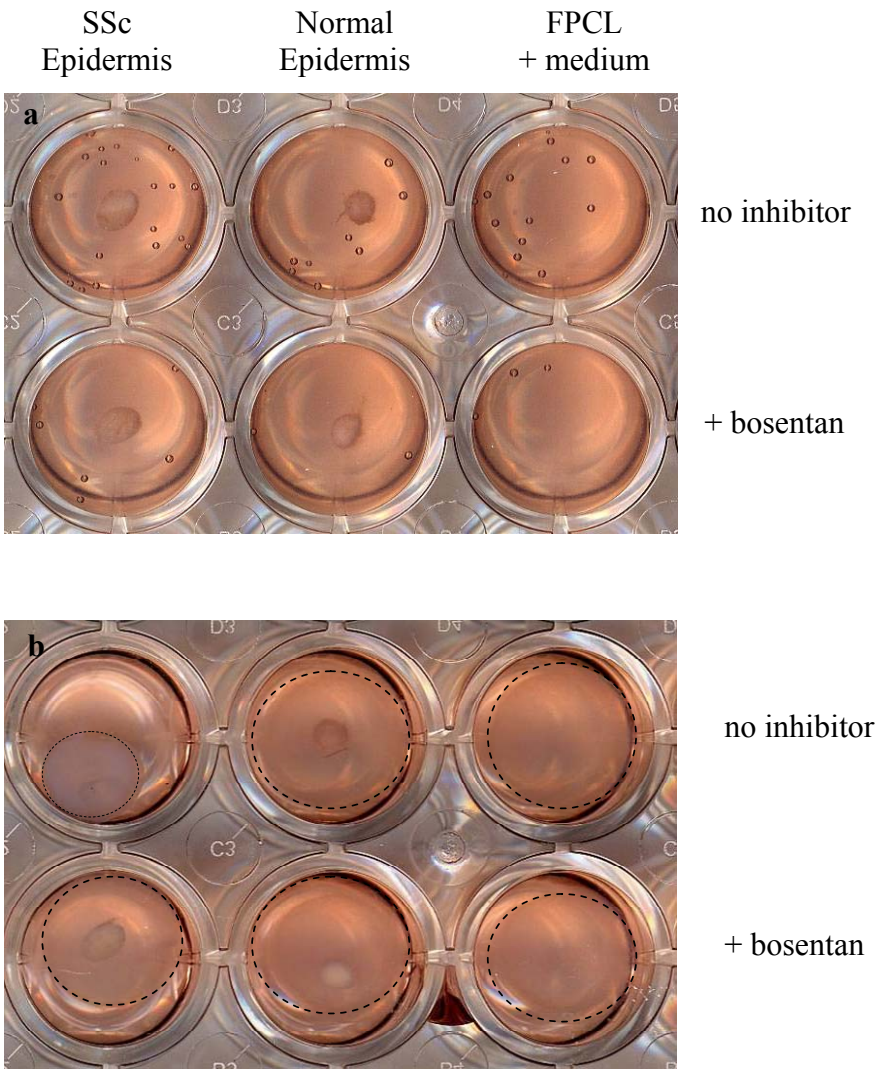


Figure 4.8 (a,b): Bosentan inhibits SSc epidermis driven FPCL contraction. As before FPCL contraction was induced by co-culture with SSc epidermal cell layer for 48 hours. Addition of the endothelin receptor antagonist bosentan (10 μM) at baseline antagonised FPCL contraction by SSc epidermal cells (n=3). Pictures were taken at **(a)** baseline and **(b)** 48 hours.

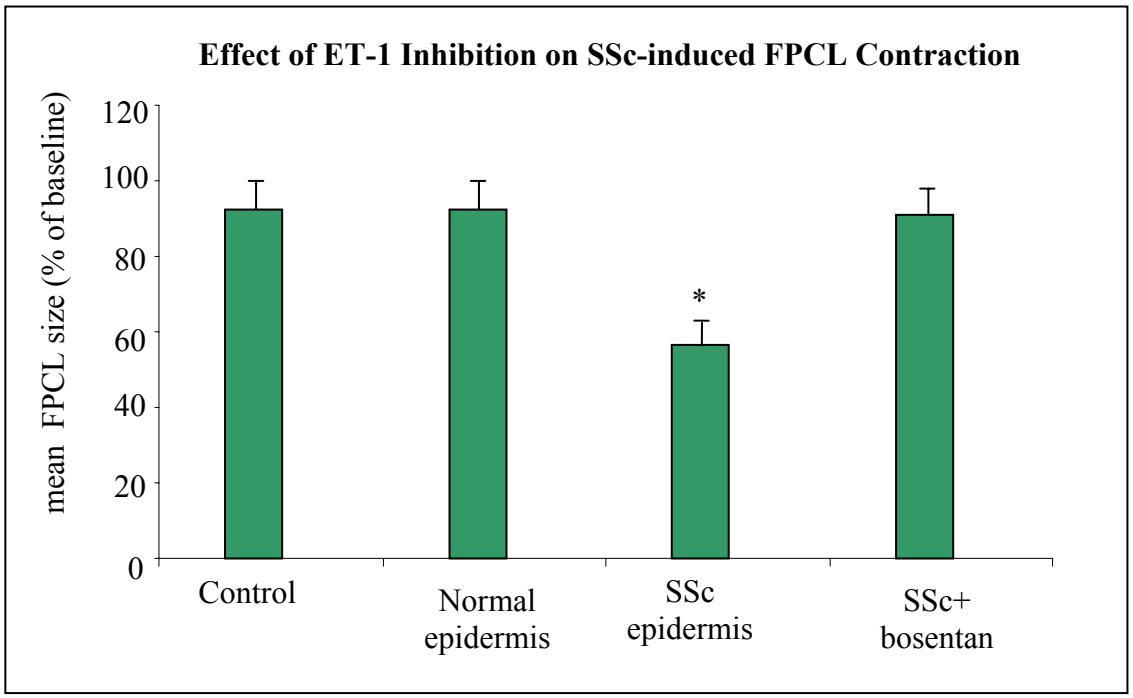


Figure 4.9: Histogram of SSc epidermis driven FPCL contraction after treatment with bosentan. Histogram showing mean FPCL sizes as percentage (%) of baseline size after 48 hours of treatment with SSc epidermal discs with and without the presence of bosentan in the lattice, as well as control and normal epidermis treated FPCL sizes. n=3, *$p<0.01$

A further experiment was performed to determine whether ET-1 was involved in TGF-β driven FPCL contraction as suggested in the past (X. Shi-Wen *et al.* 2001c) (Figure 4.10).

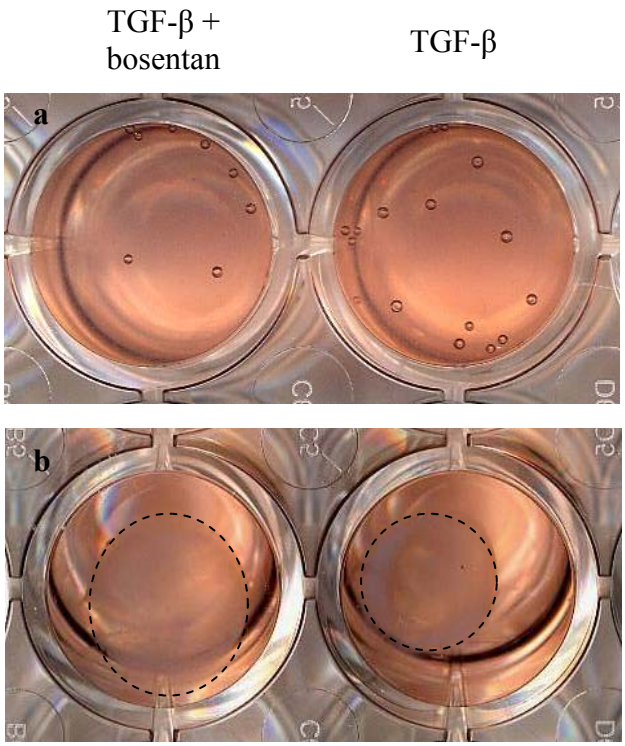


Figure 4.10 (a,b): FPCL contraction after exposure to TGF-β (4 ng/ml) with and without bosentan. Addition of the endothelin receptor antagonist bosentan (10 μM) at baseline antagonised FPCL contraction by TGF-β.
Pictures were taken at **(a)** baseline and **(b)** 48 hours. n=3 per well.

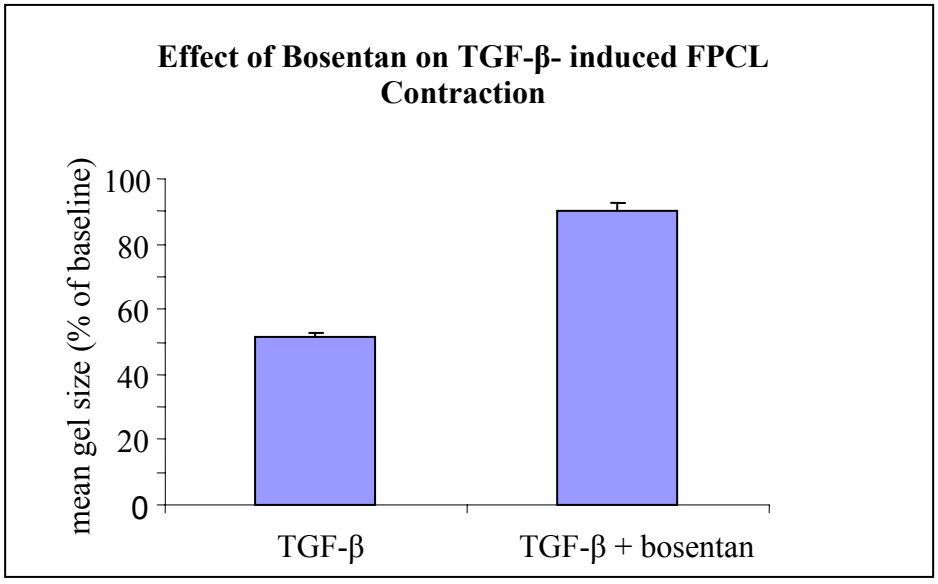


Figure 4.11: Histogram of FPCL contraction after treatment with TGF- β the presence and absence of bosentan. n=3; $p<0.001$

TGF-β driven FPCL contraction was inhibited by bosentan suggesting that a pathway involving ET-1 is important downstream of TGF-β stimulation.

4.2.3 Contraction of fibroblast collagen gels by SSc epidermis is IL-1 dependent.

A further experiment was used to determine the importance of IL-1 in SSc epidermis-driven FPCL contraction.

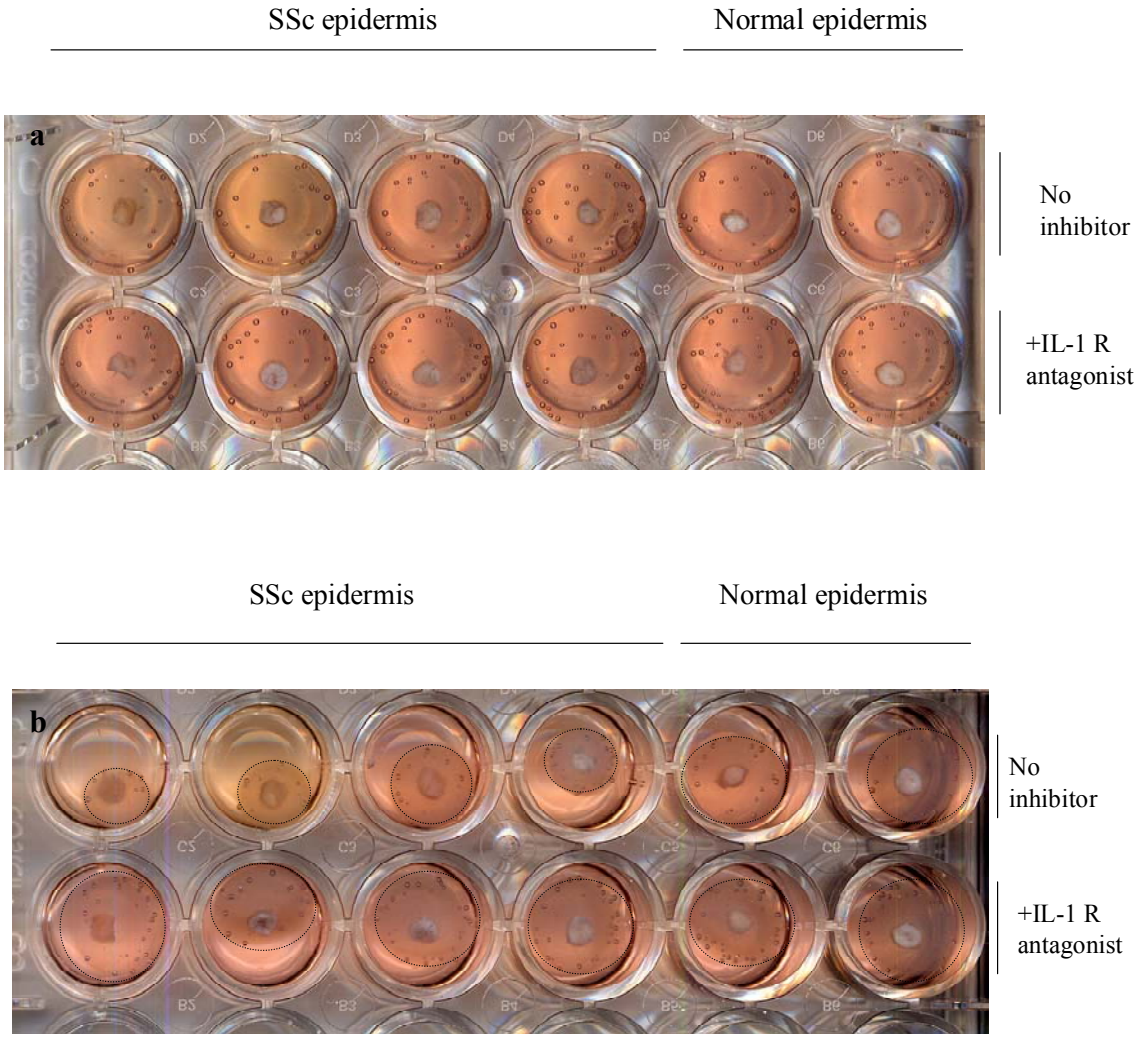


Figure 4.12 (a,b): IL-1 R antagonist inhibits SSc epidermis driven FPCL contraction. Normal dermal fibroblasts were cultured within FPCL in the presence of SSc epidermal layer (n=4) or normal epidermis (n=2). Paired biopsies were taken from the same subjects. As before FPCL contraction was seen following 48 hours co-culture. Addition of IL-1 Receptor antagonist (100 nM) at baseline antagonised FPCL contraction.
Pictures were taken at **(a)** baseline and **(b)** 48 hours.

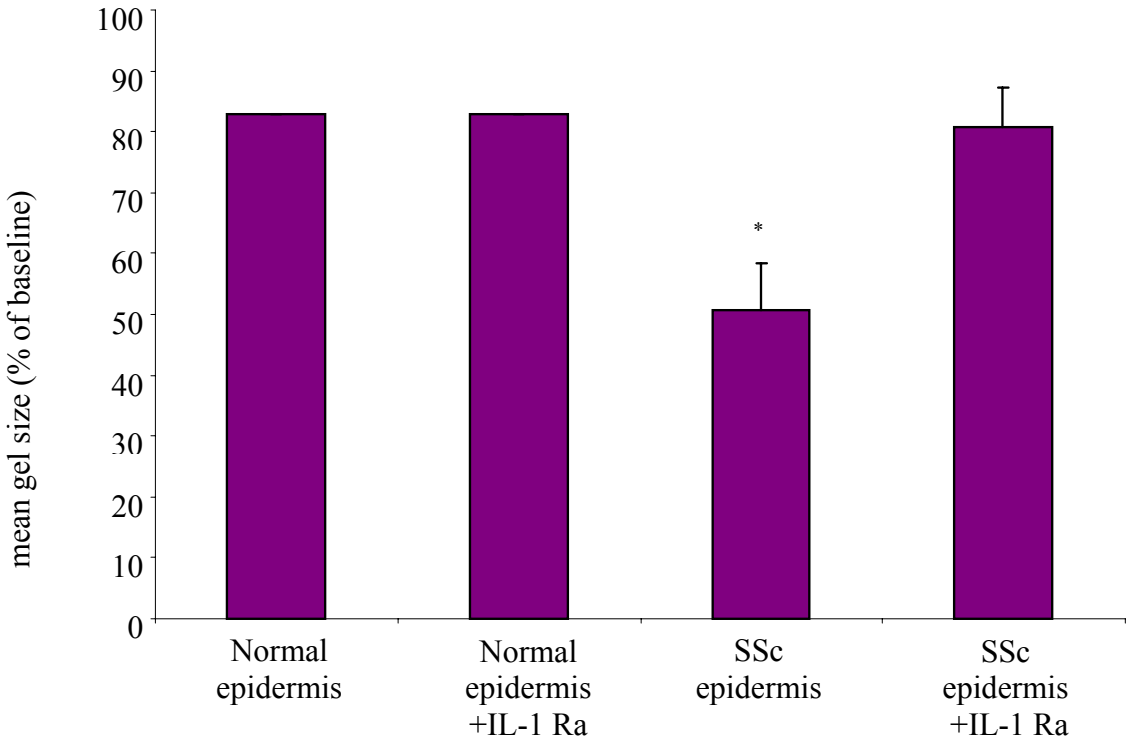


Figure 4.13: Histogram of SSc epidermis driven FPCL contraction after treatment with IL-1R antagonist. Mean FPCL sizes are shown as percentages (%) of baseline size after 48 hours of treatment with SSc and normal epidermal discs with and without the presence of IL-1 Receptor antagonist. *$p<0.001$

Paired biopsies were taken from SSc patients (n=4) and control subjects (n=2). One was embedded in the standard fibroblast collagen gel, and the other was embedded in a gel containing a recombinant human IL-1R antagonist (100 nM). SSc epidermis caused contraction of standard FPCLs as previously described (Figure 4.2). Normal epidermis produced minimal contraction of the lattices. IL-1 Receptor antagonist inhibited contraction of SSc-treated FPCLs ($p<0.001$) (Figures 4.12, 4.13).

These results mean that fibroblast activation to contractile state by SSc epidermis is IL-1, TGF-β, and ET-1 dependant. I went on to try to determine whether these factors were derived from the epidermis or were induced in fibroblasts under epidermal influence.

4.3 SSc epidermis leads to induction of ET-1 and TGF-β in normal human fibroblasts.

Since I had found the SSc epidermis activating normal human fibroblasts into a contractile state, I hypothsised that SSc epidermis was inducing the expression of pro-fibrotic growth factors in these fibroblasts. Therefore I measured the induction of TGF-β, ET-1 and IL-1 in protein extracts obtained by lysis of FPCLs after exposure to SSc epidermis, normal epidermis and TGF-β.

4.3.1 Western blot of FPCLs for ET-1:

I began by measuring the induction of ET-1 following treatment of standard and bosentan impregnated FPCLs by SSc epidermis and TGF-β (Figure 4.14).

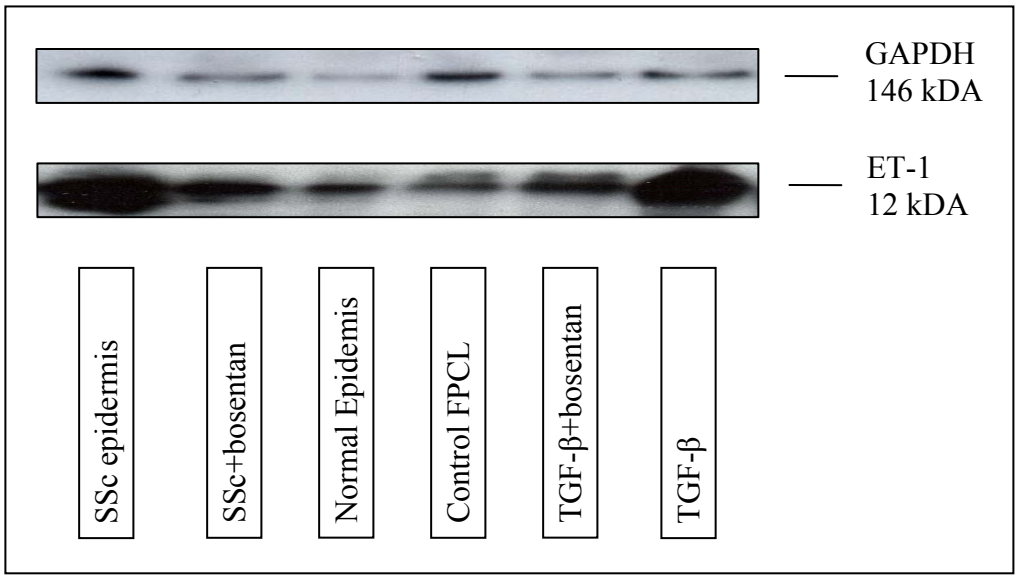


Figure 4.14: Western blot assay of ET-1 expression in FPCLs (with and without bosentan) following exposure to SSc epidermis, TGF-β (4 ng/ml), normal epidermis and control medium. GAPDH was used as protein loading control.

Treatment with SSc epidermis and TGF-β lead to induction of fibroblast ET-1 expression, which was antagonised by the ET $_A$ and $_B$ receptor antagonist bosentan (10 μM). Untreated FPCLs and normal epidermis treated ones had similar low levels of ET-1 expression (Figure 4.14, 4.17).

4.3.2 Western blot of FPCLs for TGF-β:

TGF-β expression in the FPCLs treated with SSc epidermis was also measured (Figure 4.15).

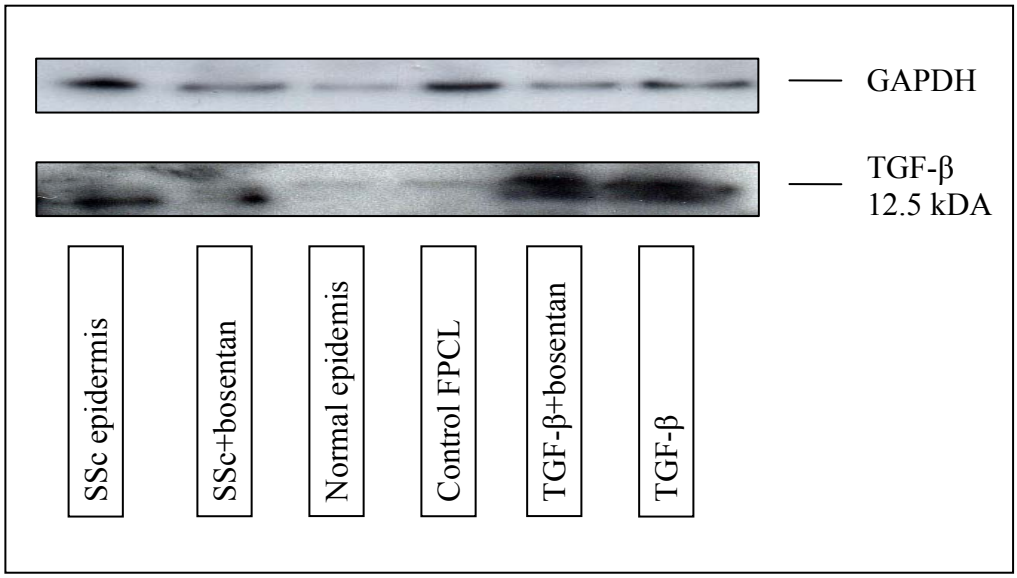


Figure 4.15: Western blot assay of TGF-β expression in FPCLs (with and without bosentan) following exposure to SSc epidermis, TGF-β (4 ng/ml), normal epidermis and control medium. GAPDH was used as protein loading control.

TGF-β caused increased expression of TGF-β in the FPCL, this expression was not inhibited by bosentan. SSc epidermis treatment also caused increased TGF-β production in the lattice, but to a lesser extent than TGF-β treatment. Bosentan partially inhibited this production. Control untreated lattices and lattices treated with normal epidermis had negligible TGF-β expression.

4.3.3 Western blot of FPCLs for IL-1:

I went on to test whether IL-1 was being induced in FPCLs. I suspected that IL-1 was in fact being released by the epidermal cells to cause fibroblast activation as shown in some previous co-culture experiments (P. Shephard *et al.* 2004h).

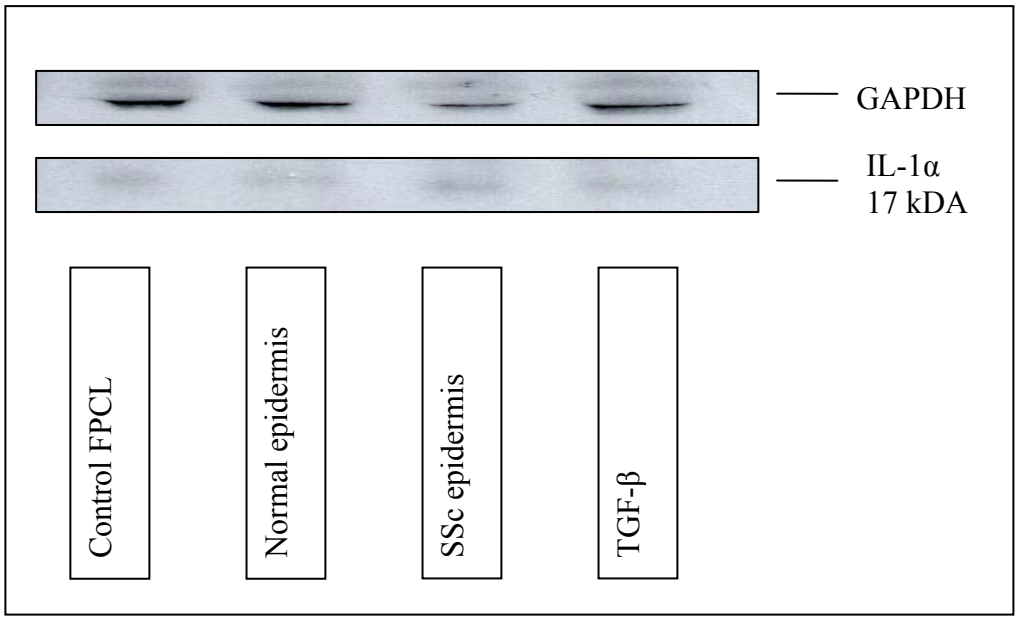


Figure 4.16: Western blot assay of IL-1 expression in FPCLs following exposure to SSc epidermis, TGF-β (4 ng/ml), normal epidermis and control medium. GAPDH was used as protein loading control.

Consistent with previous experimental data I did not observe IL-1 induction in FPCLs treated with SSc epidermis or TGF-β, only low level basal expression was seen (Figure 4.16).

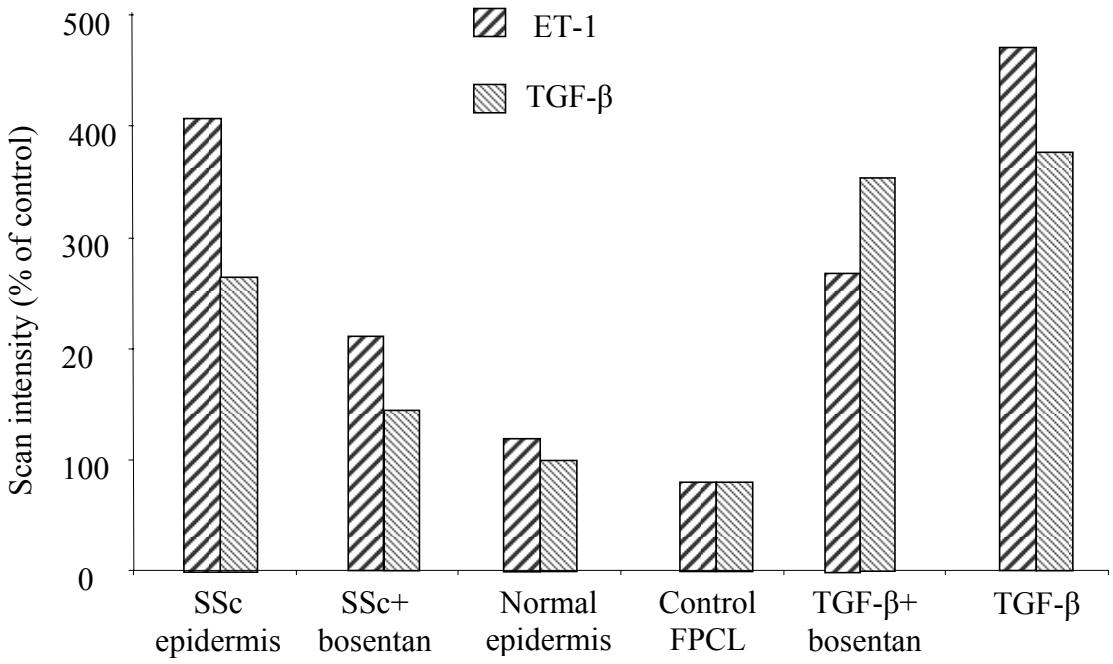


Figure 4.17: Gel intensity scan. Results for Western blots performed above for ET-1 and TGF- β content of standard and bosentan impregnated FPCLs treated with SSc or normal epidermis, TGF-β, or medium only (control) expressed as percentage (%) of control values and adjusted for GAPDH loading variability.

Therefore, based on these studies I concluded that fibroblast activation by SSc epidermis was being driven by IL-1 derived from KCs, which in turn lead to TGF-β and ET-1 induction in fibroblasts.

Discussion

SSc epidermis has a wound healing phenotype both structurally and functionally. It is known that in the setting of normal skin healing KCs are capable of secreting profibrotic mediators that can activate dermal fibroblasts and cause fibrosis (P. Shephard *et al.* 2004c;P. Shephard *et al.* 2004i;W. Xia *et al.* 2004c).

Experimental work presented in this chapter was designed to test whether the SSc epidermis in its capacity as a wound healing-like structure had fibroblast activating properties in vitro. It was hypothesised that persistent activation of the SSc epidermis contributes to disease pathogenesis, maintaining a wound healing, matrix overproducing phenotype in the disease.

The normal human FPCL contraction studies provided a convenient model for studying the in vitro epidermal-dermal interaction. Pre-stressed FPCLs mimic the tension of dermal matrix which causes fibroblast contraction after tension is released and growth factor stimulation is applied (F. Grinnell 2003a).

I showed that SSc epidermis activated normal fibroblasts in vitro, equivalent to the result seen with exogenous TGF-β application (Figures 4.2, 4.3).

The ability of the SSc epidermis to cause contraction of normal human fibroblasts is a new finding. It was therefore important to determine which mediator had a role in this interaction. Well known fibrotic mediators TGF-β and ET-1, as well as an important wound healing mediator IL-1 were studied.

1. Contraction of fibroblast collagen gels by SSc epidermis is TGF-β dependent:

The attention was first turned to TGF-β. TGF-β is a well recognized stimulus of fibroblast contraction (T. Mori *et al.* 1999b;S. A. Cotton *et al.* 1998). The pro-contractile effect of the SSc epidermis was reversed by the neutralising antibody to

TGF-β (1D11) added to the lattice at baseline (Figures 4.6, 4.7). This result suggests that TGF-β is important in SSc epidermis driven FPCL contraction. I went on to consider whether TGF-β was involved at the level of the epidermis or downstream of epidermal stimulation.

Immunoblotting of the FPCLs treated with SSc and normal epidermal discs and TGF-β was conducted. TGF-β was found to be stimulated in the lattice fibroblasts by exposure to TGF-β itself (a positive control) as well as the SSc epidermis (Figure 4.17). Untreated and normal epidermis treated fibroblasts did not produce TGF-β. CTGF expression was also assessed in the fibroblasts as a marker of TGF-β pathway activation in the lattice, and found to be significantly increased after both TGF-β and SSc epidermis treatment, but not after control or normal epidermal exposure. This was confirmed by immunostaining the FPCLs for CTGF (Figure 4.5).

Results from the previous chapter have shown that the SSc epidermis has increased expression of LAP-TGF-β compared to normal control (Figure 3.4, 3.7).

Since the difference in SSc versus normal epidermal LAP-TGF-β content is slight, its release from the epidermis might not explain the large and consistent difference in the ability of SSc epidermis to activate and contract human fibroblasts.

One explanation for the contraction inducing properties of SSc epidermis could be that the absolute value of LAP-TGF-β is not important for the epidermal-dermal interaction. Since TGF-β is normally secreted in its latent form, the biological function of this growth factor is thought to be largely determined by its activation from the latent state. LAP-TGF-β is known to be activated by epithelium-specific integrins such as αvβ6 and αvβ8 via the integrin-binding sequence (RGD) of the LAP that allows LAP-TGF-β sequestration to the cell surface where its activation

via a conformational change or proteolytic cleavage is locally controlled (J. S. Munger *et al.* 1998;D. Mu *et al.* 2002). These integrins are expressed during epithelial wound healing to allow KC migration over the wound defect. As part of the wound-healing phenotype SSc KCs would be expected to express these integrins, which could result in the release of the free TGF-β form. Sine TGF-β has the ability to stimulate the expression of αvβ6 integrins in epithelial cells, a positive feedback could ensue resulting in unchecked TGF-β activation (D. Sheppard *et al.* 1992).

A similar integrin - TGF-β interaction is thought to be important in epithelial-mesenchymal interplay of lung development. Since αvβ8 is over-expressed in adult lung epithelium exposed to an injury or inflammatory stimuli, and epithelial injury is known to promote lung fibrotic reactions, this provides a potential link between epithelial injury, LAP-TGF-β activation and fibrosis (J. Araya *et al.* 2006).

In addition, the same levels of TGF-β in the epidermis could have very different effects in a dermis pre-sensitised to its stimulation. In fact, several studies have found raised TGF-β receptor levels in SSc fibroblasts (H. Ihn *et al.* 2001a;T. Kawakami *et al.* 1998). In the disease, active TGF-β released from the latent form, or free TGF-β could be immediately sequestered by the disease fibroblasts as part of SSc pathogenesis.

Another reason absolute amounts of TGF-β may not be important, is if it is the temporal pattern of factor release that determines the fibroblast response. A snap shot concentration may not represent the fact that these factors would be constantly produced by the disease epidermis. Once bound to their receptors TGF-β molecules would have their effect and get immediately degraded to be replaced by newly released ones. In healthy skin the pro-fibrotic mediators would be there transiently in

response to a specific stimulus, and not be continuously produced.

Having considered the possibility of epidermal TGF-β release causing dermal fibrosis in SSc, one has to consider the fact that TGF-β actions may be restricted to dermal fibroblasts only. It is well known that SSc fibroblasts have increased levels of TGF-β expression (J. Peltonen *et al.* 1990f;J. Peltonen *et al.* 1990d;L. Rudnicka *et al.* 1994d). Findings in this chapter show increased levels of TGF-β in normal human fibroblasts after exposure to SSc epidermis (Figure 4.17). TGF-β could be responsible for maintaining dermal fibrosis after the initial stimulation from the epidermis is applied to the area.

2. Contraction of fibroblast collagen gels by SSc epidermis is ET-1 dependent: ET-1 is known to be important in skin fibrosis (Y Hamada 1999;M. B. Kahaleh 1991c;S. Xu *et al.* 1998). Previous experimental evidence supports the role of ET-1 in fibroblast activation downstream of TGF-β (X. Shi-Wen *et al.* 2004b;P. Shephard *et al.* 2004d). ET-1 antagonist bosentan was found to reverse the FPCL contraction caused by SSc epidermis (p=0.01) (Figures 4.8, 4.9). Bosentan also inhibited TGF-β-driven FPCL contraction (Figures 4.12, 4.13), confirming the role of ET-1 downstream of TGF-β.

The next step is to determine whether the involvement of ET-1 in FPCL contraction is at the level of the epidermis or the fibroblasts.

Results from the previous chapter show increased ET $_{A}$ and $_{B}$ receptor content in SSc epidermis, but no difference was found in terms of ET-1 itself (Figures 3.5, 3.11, 3.12). This result does not exclude epidermal ET-1 secretion since even if the expression of ET-1 is only slightly increased in the disease, one can speculate that when secreted from the epidermis, ET-1 works on the fibroblasts that are pre-

sensitised to its effects. We know that SSc fibroblasts have reduced ET_A receptor expression (X. Shi-Wen *et al.* 2001b). This could mean that either the receptor is more sensitive to ET-1 stimulation, or that the already activated downstream mechanisms in SSc fibroblasts/myofibroblasts are hyper-responsive to its effects in vivo.

Just as discussed above for the role of TGF-β, absolute amounts of epidermal ET-1 may not be important if it is the temporal pattern of factor release that determines the fibroblast response.

Having considered the possible epidermal ET-1 contribution to SSc pathogenesis, one should consider its role at the level of the dermis. ET-1 is well known to activate fibroblasts in SSc (S. Xu *et al.* 1998;S. W. Xu *et al.* 2004b). I found increased levels of ET-1 in normal human fibroblasts after their stimulation by SSc epidermis for 48 hours (Figure 4.14). The levels were similar to those after TGF-β stimulation, and both were inhibited by bosentan. This would strongly support the role of ET-1 in maintaining fibrosis at the level of the dermis, and implies that ET-1 induction is required for fibroblast activation by SSc epidermis.

3. Contraction of fibroblast collagen gels by SSc epidermis is IL-1 dependent:

IL-1 is an important pleiotropic cytokine involved in KC and fibroblast activation after injury. IL-1 is vital in KC response to epidermal disruption, but can also be found in fibroblasts (I. M. Freedberg *et al.* 2001e). Cultured dermal fibroblasts from SSc patients have been reported to have higher levels of IL-1α than normal controls (Y. Kawaguchi 1994). IL-1 is also known to prolong SSc myofibroblast survival time and cause epidermis driven fibroblast activation (I. M. Freedberg *et al.* 2001d;P. Shephard *et al.* 2004j;T. Z. Kirk, M. D. Mayes 1999).

I found that IL-1 Receptor inhibitor reversed SSc epidermis-driven FPCL contraction, a result consistently repeatable over three experiments (Figures 4.12, 4.13). I needed to determine whether IL-1 was driving FPCL contraction from the SSc epidermis or whether it was being induced at the level of the fibroblasts. ELISA immunoassay and immunostaining demonstrated greatly increased IL-1 and its receptor content in SSc epidermis (Figures 3.6, 3.13), with no increase in IL-1 levels in SSc epidermis treated fibroblasts from the lattice (Figure 4.16). It is notable that previous experimental evidence suggests that IL-1α and β are both capable of IL-1β gene induction in normal skin fibroblasts (N. Maas-Szabowski *et al.* 1999c). It is possible that the epidermal IL-1 does not induce IL-1α in dermal fibroblasts, or requires a different time scale to achieve this induction (FPCL contraction was achieved within 7 hours of SSc epidermal exposure, whereas IL-1 gene induction required 24 hours of treatment).

Previous experimental evidence confirms the ability of IL-1 to induce ET-1 production in human cells and in KC-fibroblast co-culture (X. Shi-Wen *et al.* 2000a;Y. Mori *et al.* 2003c). This ET-1 induction seems to be important in early fibroblast contraction before TGF-β (inhibited by NFkB activation) or αSMA expression come into play.

In summary, the experimental data presented above demonstrates that TGF-β and ET-1 are induced in normal human fibroblasts by the SSc epidermis. This implies that these mediators are important at the level of the dermis, and may play a significant role in maintaining fibroblasts in an activated state. IL-1, on the other hand, is not found to be increased in SSc epidermis treated fibroblasts, which leaves it as a potential candidate for an epidermis-derived pro-fibrotic agent.

IL-1 is likely to be the trigger that sets up a loop causing local autocrine KC stimulation as well as the paracrine activation of dermal fibroblasts. The latter respond by secreting TGF-β and ET-1 and setting up their own autocrine cycling to produce pathological ECM secretion and ultimately fibrosis.

4. Mechanism of SSc epidermis-driven activation of dermal fibroblasts:

I suggest that the most likely mechanism involved in SSc epidermis-driven activation of dermal fibroblasts is that of an epidermal-dermal paracrine loop, which, once initiated, then goes on to self-potentiate and maintain the pro-fibrotic cycle (Figure 6.1).

This type of loop has previously been demonstrated for IL-1/KGF production in normal cell epithelial-mesenchymal interactions examined in the setting of healing and repair (Y. Asano *et al.* 2006b;Y. Asano *et al.* 2006a). In this environment, the absolute quantities of the secreted factors are not important as positive feedback mechanisms are set in motion. On the other hand, any interruption to such a loop, as achieved with ET-1, TGF-β or IL-1 inhibitors in the above experiments, would have a major impact on continuation of the fibrotic processes.

The concept of epidermal-dermal stimulation implies either a direct cellular interaction between KCs and dermal fibroblasts, and/or factor diffusion across the basement membrane. Normally, an intact basement membrane would prevent the interaction. This may change, however, if the skin layers were to be disturbed as in the situation of wounding or after the onset of inflammation. We know that SSc epidermis has a wound healing phenotype. This may allow the epidermal-dermal interplay in SSc the way it does in wound healing.

In addition, it is important to bear in mind that at some stage activated fibroblasts can become autonomous. In this case they stop requiring stimulation from the

epithelium and continue secreting ECM which eventually leads to the skin sclerosis seen in SSc in an entirely unchecked manner (Y. Mori *et al.* 2003b;H. Gardner *et al.* 2006;Y. Mori *et al.* 2003a;F. K. Tan *et al.* 2005).

In line with the autonomy theory, recent studies demonstrated that αvβ5 integrin found to be over-expressed in SSc fibroblasts, promoted the formation of the TGF-β/ TGF-β receptor complex and subsequent establishment of autocrine TGF-β signalling in these cells leading to constitutive myofibroblast phenotype of SSc fibroblasts (L. Chung, P. J. Utz 2004;P. Q. Hu *et al.* 2003). In addition, ligand-independent SMAD signalling and gene activation has been demonstrated in SSc fibroblasts (S. S. Baroni *et al.* 2006).

The question of what sets the positive feedback in motion remains, however. An attractive idea is that of serum immunoglobulins causing the initial KC and/or fibroblast activation. Several autoantibodies are associated with SSc and implicated in disease pathogenesis. These are anti-endothelial, anti-fibroblast, anti matrix metalloproteinase, anti-DNA topoisomerase, and anti-fibrillin-1 antibodies (S. Narumiya 1995).

A recent paper finds PDGFR antibodies in serum of patients affected by SSc, and links them to fibroblast oxidative damage via Ras/ERK signalling. This cascade is thought to be responsible for fibroblast activation in the disease (E. J. Goetzl *et al.* 1995b).

Recent unpublished data from this laboratory demonstrates increased binding of serum antibodies from SSc patients to normal human KCs. KCs exposed to SSc patient serum release greater levels of IL-1 than those exposed to serum from normal controls. This supports the hypothesis of antibody-mediated skin activation in SSc,

with KCs as a primary target.

Other factors initiating KC/fibroblast activation could be epithelial injury or chemical exposure, both of which have been linked to SSc (P. J. Nietert, R. M. Silver 2000b).

Conclusions

- The data presented above demonstrates for the first time that SSc epidermis is capable of activating normal human fibroblasts in an in vitro model of epidermal-dermal cross-talk.
- SSc epidermis induces contractile and CTGF-expressing phenotype in normal human fibroblasts in vitro.
- Activation of fibroblasts by SSc epidermis is TGF-β, ET-1 and IL-1 dependent.
- SSc epidermis leads to the induction of TGF-β and ET-1, but not IL-1 in human fibroblasts.
- IL-1 levels are significantly raised in SSc epidermis.
- It is reasonable to suggest that the SSc epidermis is an active secretory organ where wound healing-like epidermis driven mechanisms cause persistent downstream fibroblast activation.
- I put forward a model where epidermis-derived IL-1 causes TGF-β and ET-1 dependant fibroblast activation.

CHAPTER 5

The Role of PGE_2 in TGF-β Driven KC Function

Introduction

Previous chapters demonstrate that the SSc epidermis has a wound healing phenotype in terms of its structure, signalling capacity, raised IL-1 content and the ability to activate underlying matrix. I also showed that the SSc epidermis caused human fibroblast activation suggesting that epithelial processes need to be tightly controlled if the correct skin structure is to be maintained. A similar epidermal-fibroblast interplay is known in the setting of wound healing (P. Shephard *et al.* 2004k).

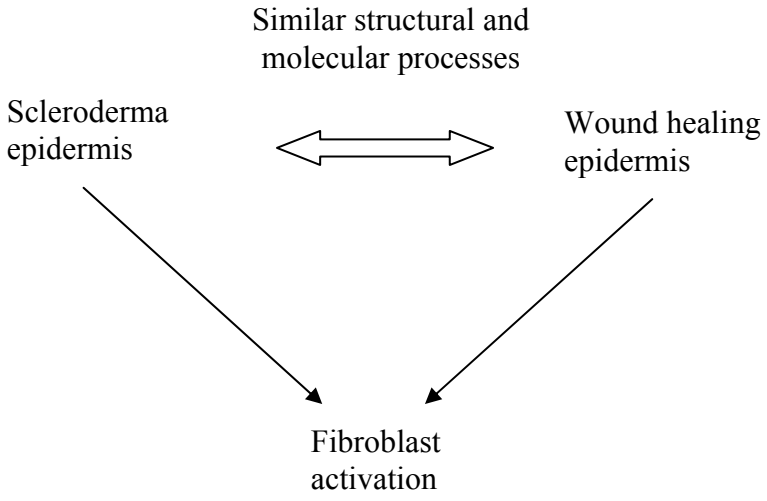


Figure 5.1: Comparison is made between SSc epidermis and a wound healing epidermis based on experiments in Chapter 3. The similarities lead to the interest in KC wound healing biology with potential implications for dermal fibrosis.

Because of this I became interested in the regulation of KCs during wound repair. I went on to study epithelial cell biology in the setting of wound healing in order to explore the idea of altering epithelial processes to control dermal fibrosis.

One of the most fundamental properties of skin healing is epithelial re-surfacing. When skin is injured wound healing consists of simultaneous repair of dermal extracellular matrix and proliferation and differentiation of epidermal KCs (T. Sato *et al.* 1997d). A balance of KC migration and proliferation establishes wound coverage, these cells also stimulating the underlying dermal fibroblasts to complete skin closure (S. Werner, H. Smola 2001c;S. Werner, R. Grose 2003b;S. Werner *et al.* 2007c). Cell-cell interactions between KCs and dermal fibroblasts are known to contribute to the correct organisation of the epidermis (T. Sato *et al.* 1997c). PGE_2 has been shown to be involved in KC proliferation and differentiation and is a crucial mediator of epidermal repair (G. Furstenberger, F. Marks 1978b;A. P. Pentland, P. Needleman 1986b). It has been found that in KC-fibroblast co-culture experiments fibroblast derived PGE_2 production is dependent on IL-1 release from epidermal KCs , suggesting a feedback mechanism between theses cells (T. Sato *et al.* 1997b).

I found increased IL-1 and decreased COX II expression in SSc epidermis (Chapter3). KCs are known to secrete prostaglandins, but the contribution of PGE_2 and COX II to wound repair is not clear.

One interesting aspect of wound healing is that KCs proliferate and the epidermis expands in a TGF-β dominated environment which is anti-proliferative and pro-migratory in nature (C. Amendt *et al.* 2002c;P. A. Hebda 1988e;C. J. Kane *et al.*

1991c;S. Werner, H. Smola 2001b;B. Bandyopadhyay *et al.* 2006e). Results from this laboratory have previously demonstrated that prostanoids are able to antagonise TGF-β dependent events in human cells (R. Stratton *et al.* 2001b;R. Stratton *et al.* 2002e). I hypothesised, therefore, that the induction of prostanoids during wound healing frees KCs from the anti-proliferative effects of TGF-β. I went on to measure the induction of PGE_2 and COX II during wound repair, and studied the effect of PGE_2 on TGF-β-driven KC functions.

Results

5.1 COX II and PGE_2 induction in excisional mouse wounds:

Mice were anaesthetised and underwent excisional wounding (4x4 mm biopsies of dorsal skin) (see Methods). Mice were then euthanised at various time points (0, 1, 3, 7, 11, and 14 days), wound sites were excised and stored in liquid nitrogen for PGE_2 ELISA or fixed in formalin for histochemistry.

5.1.2 Staining for COX II in mouse skin:

Immunohistochemistry was used to localise COX II expression in sections obtained from mouse wounds during wound healing. COX II was induced in the epidermis on days 1-3 post wounding, the induction resolved by day 9 (Figure 5.2).

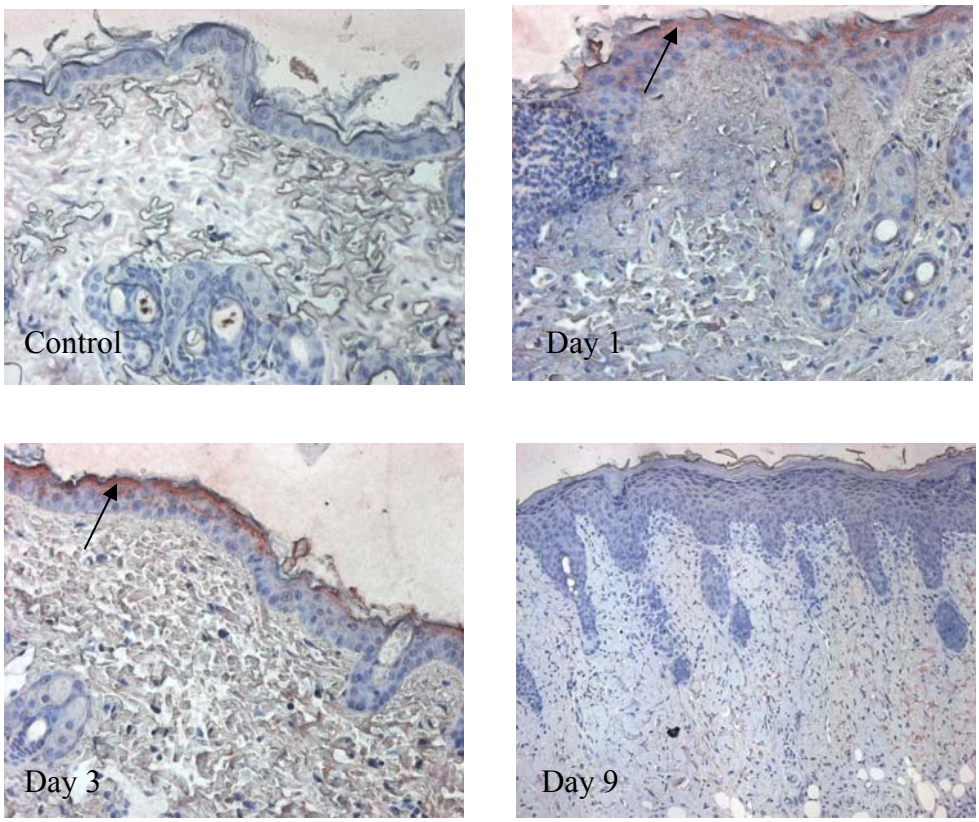


Figure 5.2: Induction of COX II (red staining) in the mouse epidermis during excisional wound healing. Cox II is visible on Day 1 post wounding (red staining, arrows) and is absent by Day 9.

5.1.2 Timecourse for PGE_2 induction in mouse wound:

Total wounds were then extracted and PGE_2 measured by ELISA of wound homogenates. Rapid induction of PGE_2 (up to 3 μg/mg of tissue) was seen (Figure 5.3). This induction peaked on day 1, diminishing thereafter.

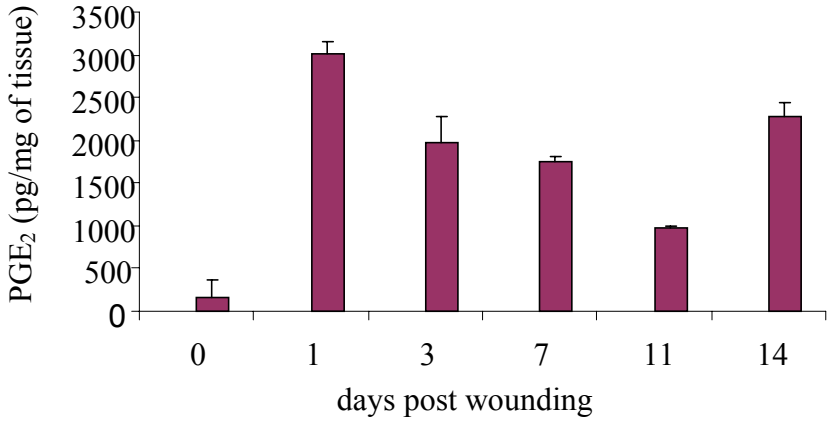


Figure 5.3: PGE_2 levels during excisional wound healing in the mouse. n=3

I concluded that following tissue injury COX II is rapidly induced in the epidermis and PGE_2 synthesis increases in parallel.

5.2 PGE2 partially blocks the anti-proliferative effects of TGF-β in human KCs:

Because of the above findings of increased COX II and PGE_2 production in early wounds and because we had found that prostaglandins antagonised TGF-β in human cells, I hypothesised that PGE_2 was freeing KCs at the wound edge from the anti-proliferative effects of TGF-β. I began by measuring the effect of PGE_2 at various concentrations on TGF-β inhibition of rapidly proliferating normal human KCs. Normal human KCs were cultured in 6 well plates in K-SFM with or without the addition of TGF-β (10 ng/ml). After 48 hours KC number was determined by counting of the cells in a haemocytometer.

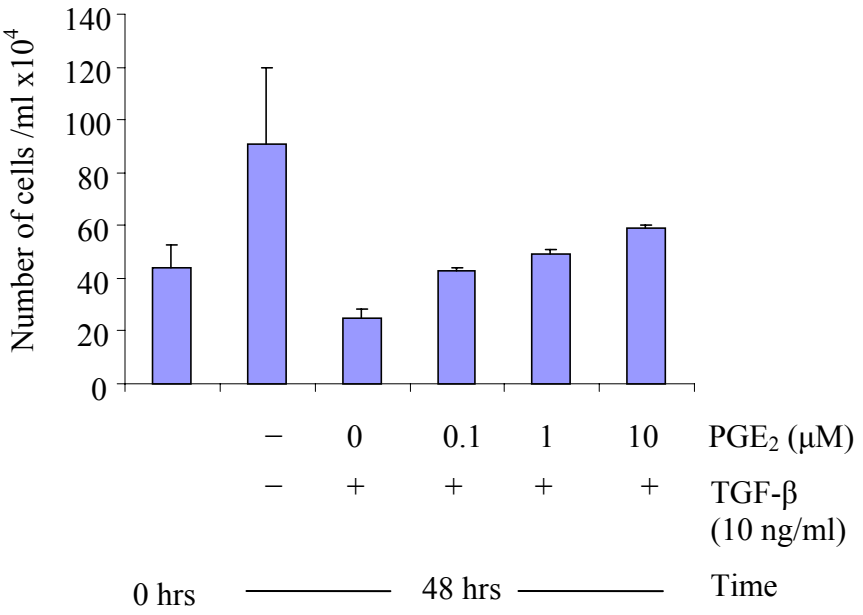


Figure 5.4: The effect of PGE_2 on TGF-β suppression of proliferation in human KCs. Normal human KCs were allowed to proliferate for 48 hours in the presence or absence of TGF-β, with or without PGE_2. TGF-β suppressed KC proliferation, and this effect was antagonised by PGE_2 (n=3).

TGF-β antagonised KC proliferation (Figure 5.4). Antagonism of KC proliferation by TGF-β was partially blocked in a dose dependent manner by PGE_2.

I then went on to explore whether TGF-β driven KC motility was also affected by PGE_2.

5.3 PGE_2 blocks TGF-β induced motility in human KCs:

KCs were grown on 6 well plates in K-SFM to 90% confluency. A scratch was induced with a pipette tip. TGF-β was added to the medium with or without co-addition of PGE_2 (0.1-10 μM).

I demonstrated that TGF-β (4 ng/ml) enhanced KC motility using a scratch model of KC migration. I then went on to test whether PGE_2 (0.1-10 μM) was able to antagonise TGF-β stimulated KC migration (Figure 5.5).

Photo time / **Well treatment**	**Baseline**	**12 hours**	**24 hours**	**36 hours**
Control	100	75.75	75.75	69.7
TGF-β only (4 ng/ml)	100	58.3	50	8.3
PGE_2 only (10 μM)	100	89.5	89.5	84.2
PGE_2 (0.1μM) +TGF-β (4	100	66.66	35.3	5.9
PGE_2 (1 μM) +TGF- β	100	72	64	48
PGE_2 (10 μM) +TGF- β(4	100	79.4	76.47	61.76

Table 5.1: The effect of PGE_2 and TGF-β on human KC migration. Scratch defect widths measured as percentages of day 1 width after treatment as indicated over 36 hours total. Photographs taken at 12 hour intervals (n=3).

Figure 5.5(a)

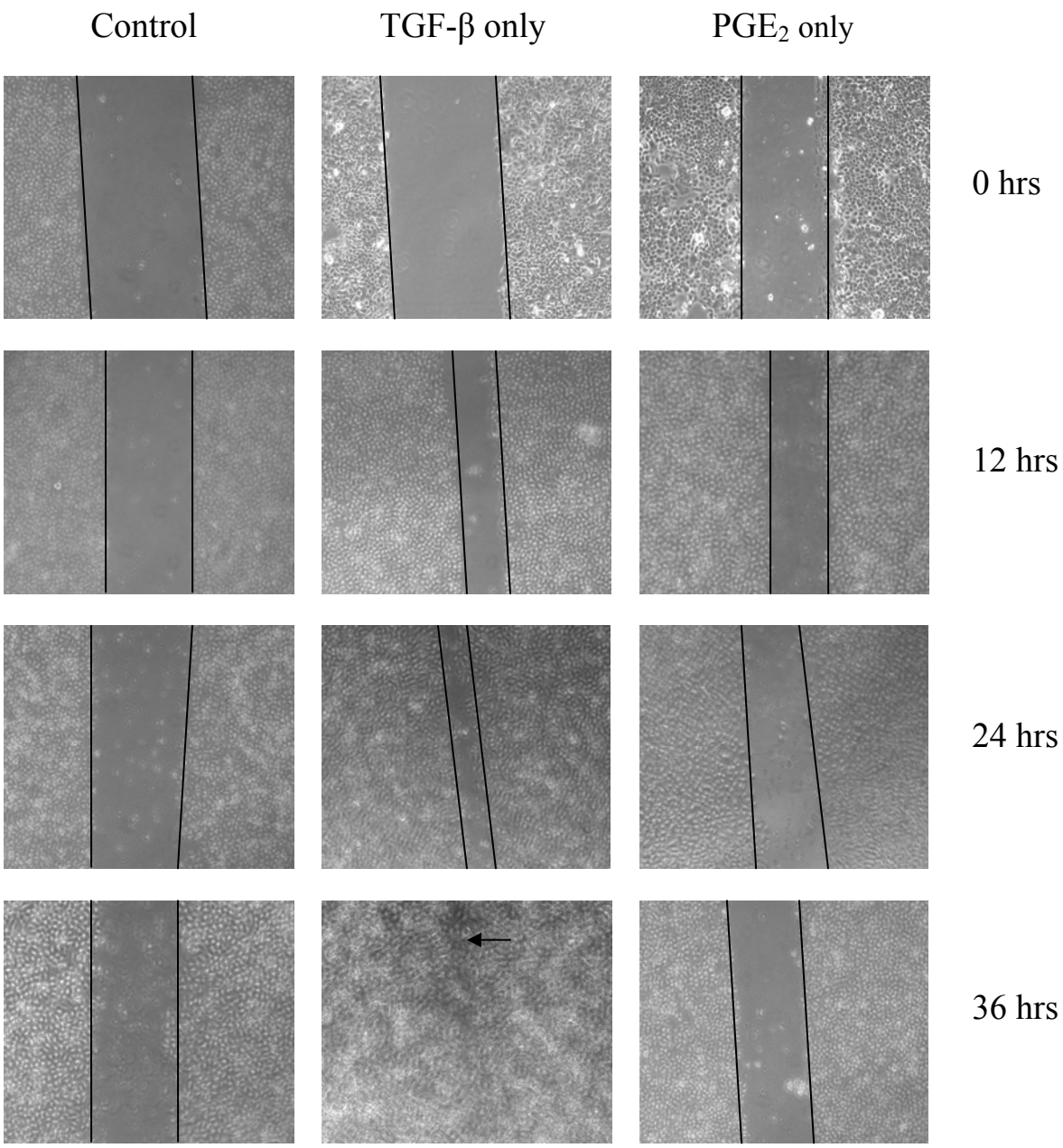


Figure 5.5(b)

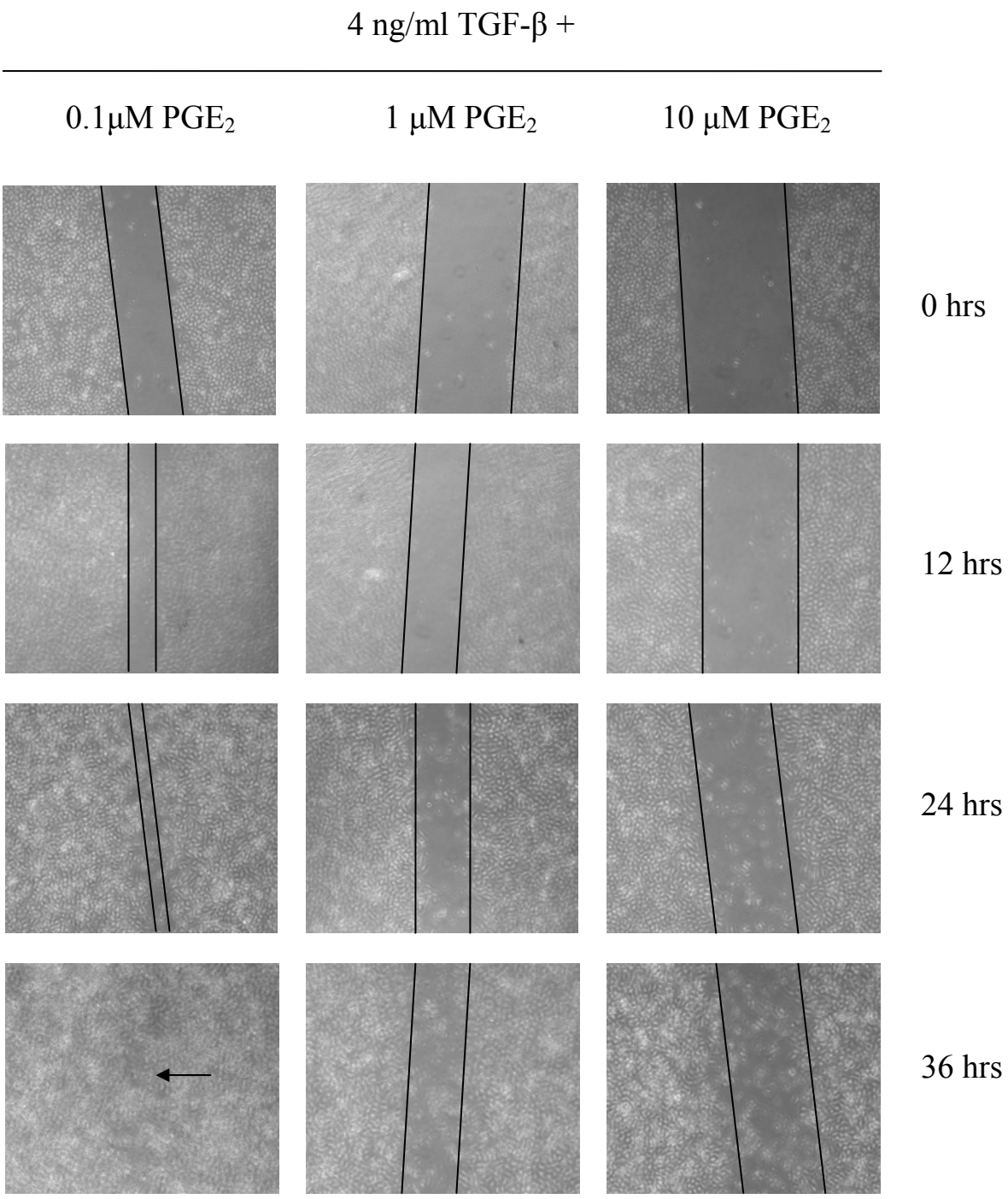


Figure 5.5 (a,b): Summary figure comparing scratch defects after treatment with TGF-β and PGE_2 as indicated at 12 hour time points. Lines and arrows indicate areas of interest.

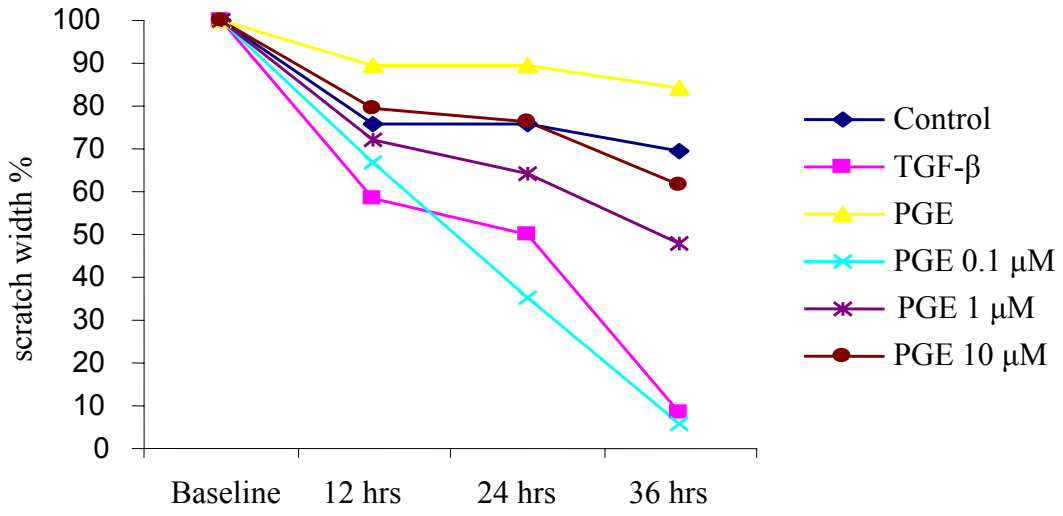


Figure 5.6: Scratch widths as percentages of baseline. Line graph to show scratch widths measured as percentages of baseline at 12 hour intervals for each well after treatment with 4 ng/ml TGF-β, 4 ng/ml TGF-β plus PGE_2 (0.1 μM, 1 μM, 10 μM), or 10 μM PGE2 only.

The pro-migratory action of TGF-β on cultured human KCs was confirmed (Figure 5.5). PGE_2 inhibited the pro-migratory action of TGF-β on KCs in a dose-dependent manner. Figure 5.5 delineates scratch closure patterns after treatment with TGF-β with or without PGE_2 and compares them to each other at 12 hour time points over a total of 36 hours. The control untreated KCs displayed some migration at 48 hours. At 48 hours the well containing TGF-β only had an almost completely closed scratch defect as expected, thus demonstrating a strong pro-migratory influence of TGF-β on KCs. At 0.1 μM concentration PGE_2 had no effect on the pro-migratory action of TGF-β. However, PGE_2 at 1 μM had an inhibitory effect on TGF-β induced KC migration, and at 10 μM PGE_2 almost completely inhibited scratch defect closure despite the presence of TGF-β.

Interestingly, the PGE_2 only wells showed less scratch defect closure than the control wells. This suggests that either there is background TGF-β activity in the control KCs, or that PGE_2 can act on a different pathway to inhibit scratch closure.

5.4 Effect of ERK signalling inhibition on TGF-β driven KC migration:

Because we had found previously that ras/MEK/ERK signalling was required for TGF-β dependent events in fibroblasts, I investigated whether this pathway was involved in TGF-β stimulated KC motility (R. Stratton *et al.* 2002). I used the small molecule inhibitor of MEK 1/2 U0126 to inhibit ras/MEK/ERK signalling (Y. Imamichi *et al.* 2005;J. V. Duncia *et al.* 1998) (Figure 5.7). I found that U0126 had a modest inhibitory effect on TGF-β dependent KC motility, which was not as great as the inhibitory effect of PGE_2 on KC motility observed in Figures 5.6, 5.7.

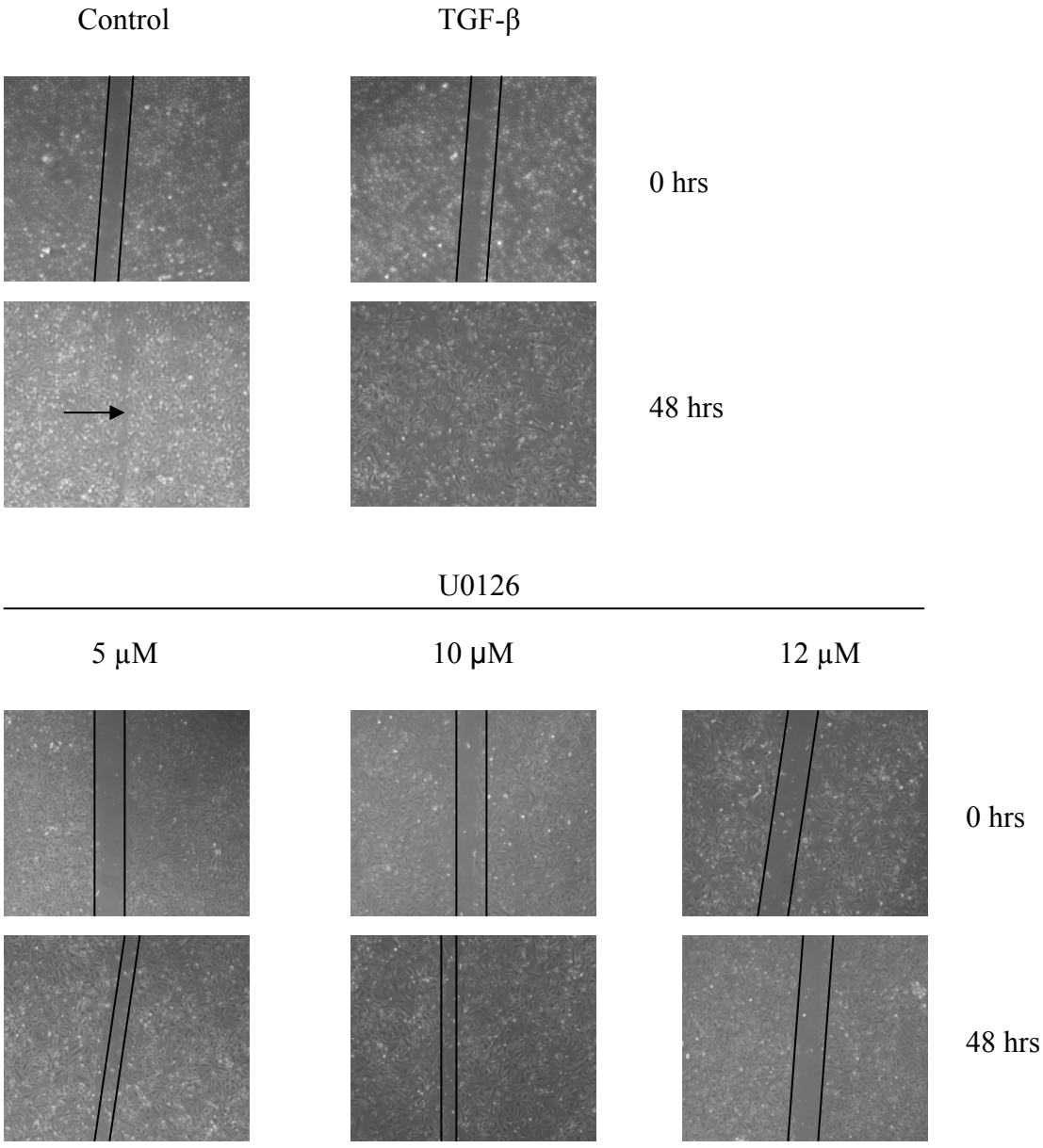


Figure 5.7: Summary figure comparing scratch defects after treatment with TGF-β and U0126. The baseline and 48 hour results are shown. Lines and arrows indicate areas of interest.

Photo time Well treatment	Baseline	12 hours	24 hours	36 hours	48 hours
Control	100	88.9	66.7	66.7	55.5
TGF-β only (4 ng/ml)	100	70	70	55	0
U0126 (5 μM)+ TGF-β (4 ng/ml)	100	77.8	72.2	69.4	61.5
U0126 (10 μM)+ TGF-β (4 ng/ml)	100	100	100	66.7	55.55
U0126 (12 μM)+ TGF-β (4 ng/ml)	100	89.5	78.9	73.7	63.16

Table 5.2: The effect of U0126 on human cultured KC migration. Scratch defect widths measured as percentages of day 1 width after treatment as indicated over 48 hours total. Photographs taken at 12 hour intervals (n=3).

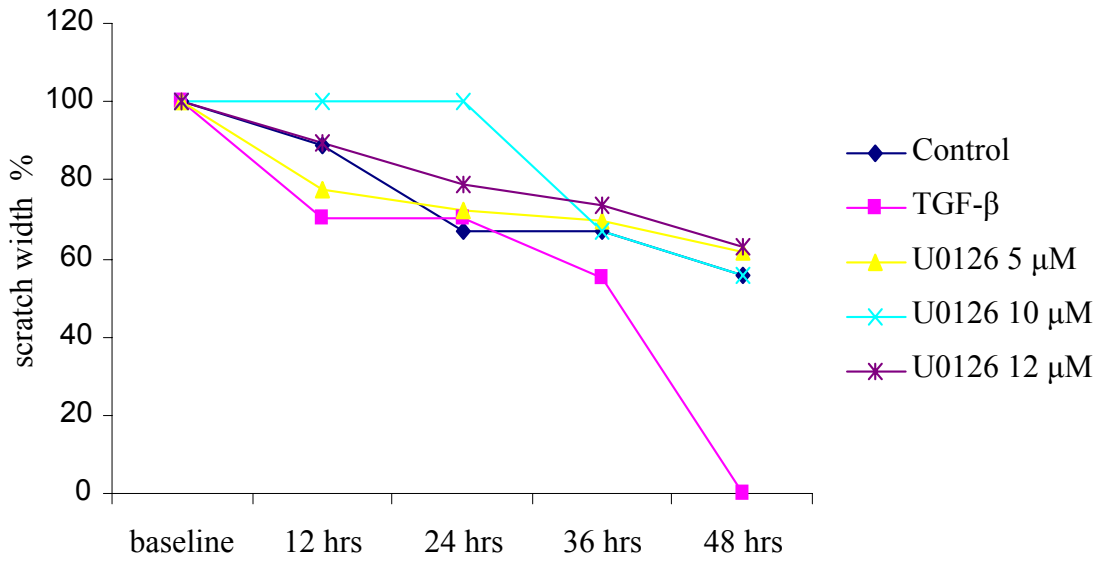


Figure 5.8: Scratch widths as percentage of baseline. Graph to show scratch widths measured as percentages of day 1 width at 12 hour intervals for each well after treatment with 4 ng/ml TGF-β only, or 4 ng/ml TGF-β plus U0126 (5μM, 10 μM, 12 μM).

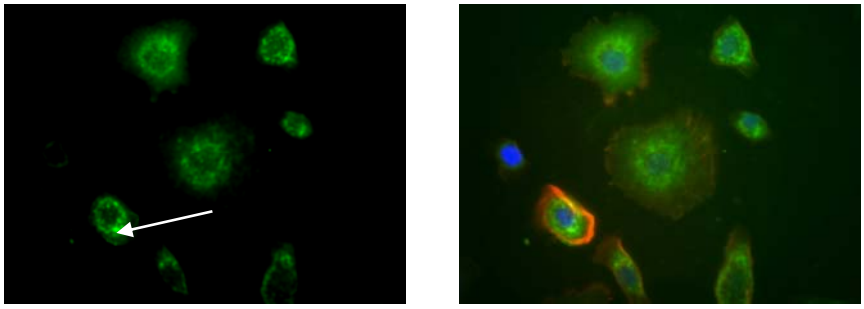
The MEK 2/3 inhibitor U0126 reversed the promigratory effect of TGF-β on human KCs in a scratch assay (Figures 5.7, 5.8, Table 5.2). The effect of U0126 was dose-independent at the 48 hour end-point with the 5 μM, 10 μM, and 12 μM solutions allowing scratch defect closure to 61.5%, 55.5%, and 63.16% of baseline respectively. TGF-β, however, caused a complete closure of the scratch defect after 48 hours.

5.5 Effect of PGE_2 on TGF-β driven timecourse of ERK phosphorylation in human KCs:

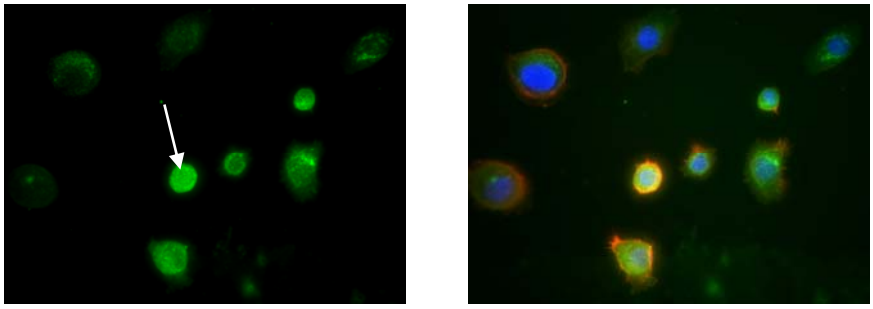
Previously we found that prostanoids prevent TGF-β signalling in fibroblasts via inhibition of ras/MEK/ERK pathways. Because of this I went on to determine whether these effects extend to KCs.

5.5.1 Timecourse KC pERK1/2 induction in the presence of TGF-β:

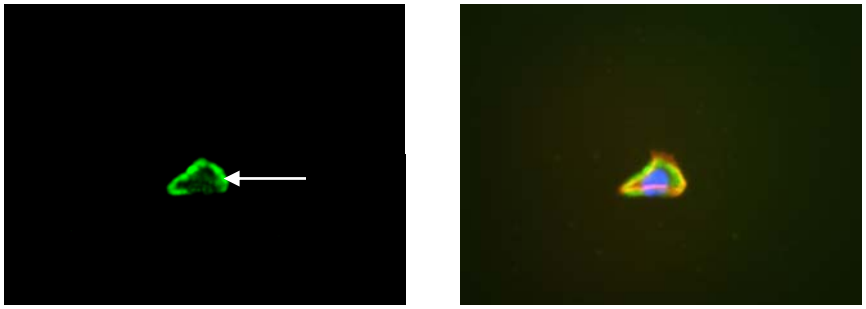
To begin with the time course of ERK phosphorylation following exposure of KCs to TGF-β was shown using immunostaining with and without DAPI nuclear staining (Figure 5.9).



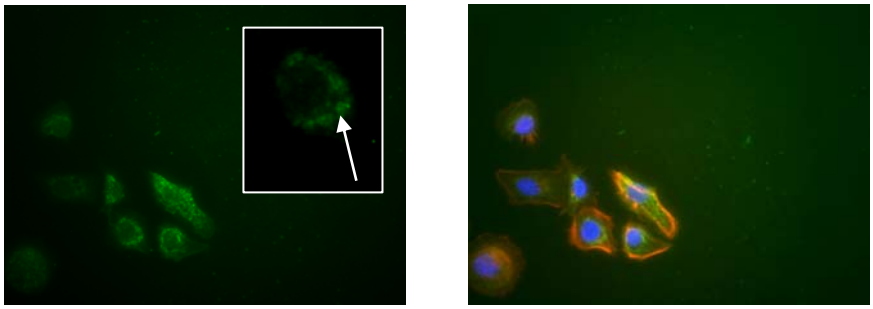
Control pERK



TGF-β pERK (15 min)



TGF-β pERK (30 min)



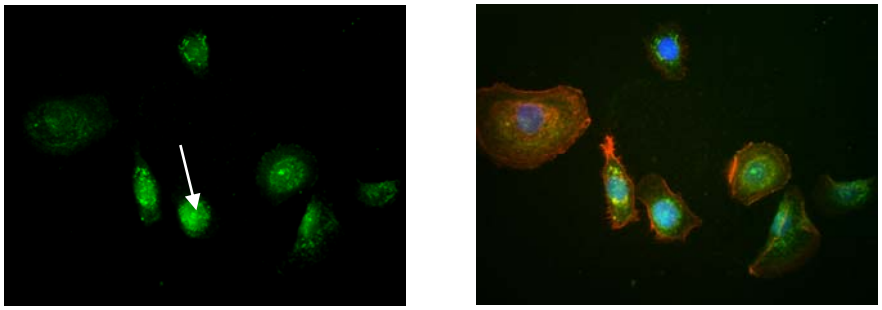
TGF-β pERK (60 min)

Figure 5.9 Timecourse of TGF-β driven KC pERK1/2 induction. KCs were grown on chamber slides following exposure to TGF-β (4ng/ml) for 15, 30, and 60 minutes. Immunohistochemical staining (arrow) for pERK1/2 with and without DAPI nuclear staining (blue) was performed.

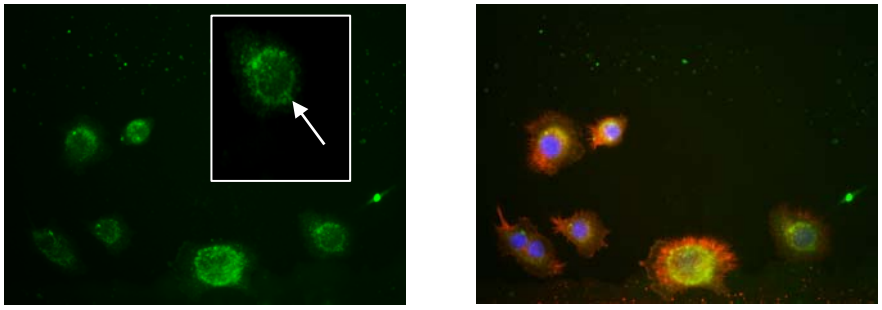
I found that under basal conditions phosphorylated ERK was present within the KC cytoplasm and that after 15 minutes of exposure to TGF-β (4 ng/ml) the pERK migrated to the nucleus. This effect was transient, however, and pERK appeared cytoplasmic again after 30 minutes of TGF-β treatment. pERK was still detected in the cytoplasm after 60 minutes.

5.5.2 Timecourse of KC pERK1/2 induction in the presence of TGF-β and PGE_2:

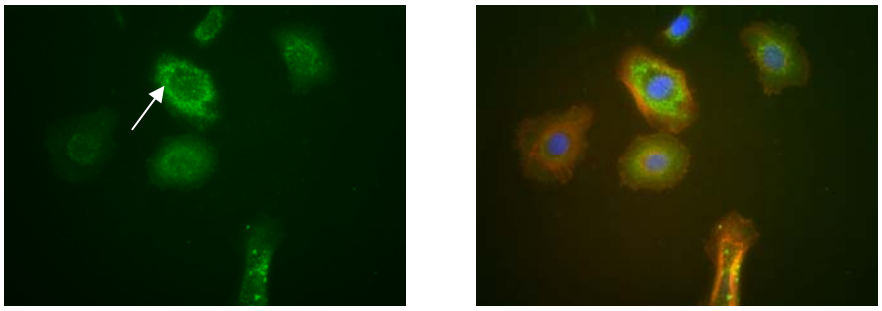
The effect of PGE_2 on TGF-β driven timecourse of ERK phosphorylation was studied by adding the two factors to the KC culture wells and staining for pERK at 15, 30 and 60 minutes post exposure (Figure 5.10) with and without DAPI nuclear staining.



TGF-β+PGE_2 pERK (15 min)



TGF-β+PGE_2 pERK (30 min)



TGF-β+PGE_2 pERK (60 min)

Figure 5.10: Timecourse of KC pERK1/2 induction in the presence of TGF-β and PGE_2. KCs were grown on chamber slides following exposure to TGF-β (4ng/ml) with and without PGE_2 (10 μM) for 15, 30, and 60 minutes. Immunohistochemical staining (arrow) with and without DAPI nuclear staining (blue) for pERK1/2 was performed. For control staining see Figure 5.9.

Addition of PGE_2 (10 μM) did not affect pERK migration to the nucleus.

I concluded that ERK was phosphorylated under basal conditions in KCs grown in serum-free conditions, and that following exposure to TGF-β pERK rapidly translocated to the nuclear compartment. PGE_2 had no effect on this process.

5.6 Effect of TGF-β and PGE2 on total cellular ERK phosphorylation in KCs:

In these experiments KCs were cultured on 6 well plates and treated with TGF-β with and without PGE_2 and lysed at various time points in Western lysis buffer containing protease inhibitors. Western blot analysis confirmed the presence of pERK1/2 under basal conditions.

Exposure to TGF-β did not alter the total pERK1/2 concentration, in addition PGE_2 did not affect the total cellular pERK content (Figure 5.11).

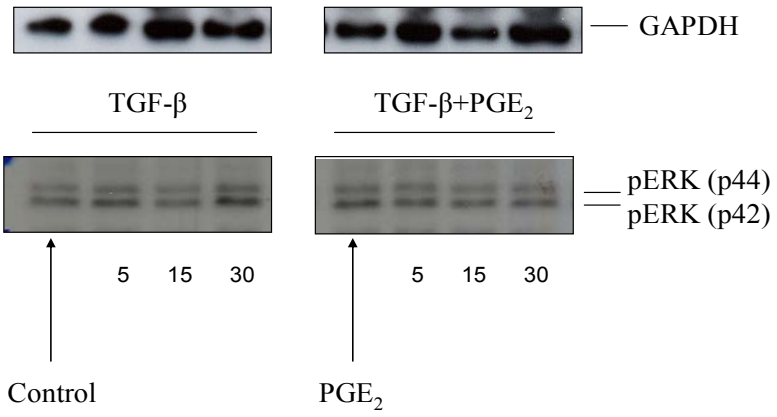


Figure 5.11: Western blot assay for pERK1/2 content of TGF-β and/or PGE_2 treated KCs. Western blot of KCs for pERK1/2 after treatment with 4 ng/ml TGF-β alone, 10 μg/ml PGE_2 alone, and 4 ng/ml TGF-β + 10 μg/ml PGE_2 over 1 hour. pERK1/2 expression after TGF-β and TGF-β + PGE_2 treatment assessed at 5, 15, and 30 minutes (n=3). Representative figure with GAPDH loading control shown.

From these studies I concluded that pERK was constitutively expressed in the cytoplasm of normal human KCs, and that TGF-β treatment did not enhance total pERK expression. PGE_2 did not affect pERK content or timecourse in these KCs.

Discussion

Wound healing is one of the most fundamental functions of the epidermis largely controlled by mediators secreted by epidermal KCs themselves. When the highly regulated process of wound healing becomes disordered, the consequences can range from chronic ulceration to fibrosis and even malignancy. It is therefore important to study the cellular events involved in wound healing and the soluble mediators that orchestrate these events. Two important secreted factors involved in wound healing regulation are PGE_2 and TGF-β.

Human KCs are known to produce prostaglandins and to express all 4 types of PGE_2 receptor (E-series PG receptors: EP1, EP2, EP3 and EP4) (S. Narumiya 1995). PGs are involved in wound healing, particularly in the inflammatory and proliferative phases. This explains the erythema, increased blood flow, vasodilation and white cell influx in the wounded area (E. J. Goetzl *et al.* 1995a). One of the mechanisms responsible for the PGE_2 induction post tissue injury is the stabilisation of COX II mRNA by IL-1α through p38 and NF-kappaB expression (J. F. Di Mari *et al.* 2007;J. F. Di Mari *et al.* 2003;M. Lasa *et al.* 2000). However, the exact role of PGs in KC function is not clear. Studies have suggested that they are pro-proliferative autocoids for the epidermis. For instance, intradermal injection of PGE_1 and PGE_2 increases epidermal proliferation (C. B. Bentley-Phillips *et al.* 1977b). NSAIDs (pharmacological PG inhibitors) reduce the proliferation rate of KCs. Topical addition of PGI_2 to indomethacin-treated non-confluent KC cell cultures restores their normal proliferation rate (A. P. Pentland, P. Needleman 1986c).

It is thought that overall PGE_2 improves wound healing kinetics (T. Brzozowski *et al.* 1993b;B. Gonul *et al.* 1993b;L. E. LeDuc *et al.* 1993a).

TGF-β is known to affect nearly all aspects of wound repair, and this growth factor is consistently found in the wound healing environment (S. Werner, R. Grose 2003a). It has been shown that TGF-β itself is present in excisional wounds from day 1 post injury (L. I. Gold *et al.* 1997). The overall function of TGF-β is to enhance wound repair in vivo (C. Amendt *et al.* 2002b;I. M. Freedberg *et al.* 2001c;P. A. Hebda 1988d;C. J. Kane *et al.* 1991b;S. Werner, H. Smola 2001a;B. Bandyopadhyay *et al.* 2006d;P. A. Hebda 1988c).

One interesting aspect of wound healing is that KCs proliferate and the epidermis expands in a TGF-β dominated environment which is pro-migratory but anti-proliferative in nature (C. Amendt *et al.* 2002a;B. Bandyopadhyay *et al.* 2006c;I. M. Freedberg *et al.* 2001b;P. A. Hebda 1988b;C. J. Kane *et al.* 1991a;H. Smola *et al.* 1993a).

We have previously found that prostanoids are able to antagonise TGF-β signalling in human cells(R. Stratton *et al.* 2001c;R. Stratton *et al.* 2002b). In addition I found SSc epidermal KCs to have reduced COX II expression while LAP- TGF-β levels were raised (Chapter 3). I therefore hypothesised that one of the roles of prostanoids was to regulate the responses of cells to TGF-β in the epidermis, and began by studying the effect of PGE_2 on TGF-β in normal cultured human KCs.

In this chapter I show that COX II and PGE_2 are induced in the epidermis of healing excisional mouse wounds (Figures 5.2, 5.3). The KC proliferation study shows that PGE_2 is capable of reversing the anti-proliferative effect of TGF-β on human KCs in a concentration-dependent manner (Figure 5.4). I suggest that the antagonistic effect of PGE_2 on KC functioning could have evolved to release KCs from the anti-proliferative effect of TGF-β during wound healing and thus allow effective epidermal defect closure.

Previous studies show that TGF-β enhances KC motility(B. Bandyopadhyay *et al.* 2006b;P. A. Hebda 1988a;E. B. Tredget *et al.* 2005a). I hypothesised that PGE_2 antagonised this TGF-β driven function also. Using a scratch assay the pro-migratory influence of TGF-β on KCs was confirmed (Figure 5.5(b)), and this effect was antagonised by PGE_2. This goes against the idea of PGE_2 accelerating wound healing. However, PGE_2 production in the epidermis is transitory, the reduction in its level from day 1 onwards could allow the pro-migratory function of TGF-β to come through.

It went on to delineate the mechanism through which PGE_2 may be inhibiting TGF-β functioning in KCs. Normal TGF-β signalling involves TβR dependent phosphorylation of SMAD2 and 3 effector proteins, which combine with each other and SMAD4 to form a complex. This complex either directly binds DNA prmoters, or binds with various other transcription factors such as AP-1 and CREB (cAMP response element binding protein), which modify its function (R. Derynck *et al.* 1998b). It has been shown that PGE_2 interfered with TGF-β-induced SMAD-specific gene transactivation via a PKA-dependent mechanism which disrupted the interaction of SMAD3 with transcription co-activators CREB-binding protein (CBP) and p300 (Figure 5.12) (M. Schiller *et al.* 2003b;R. Derynck *et al.* 1998a).

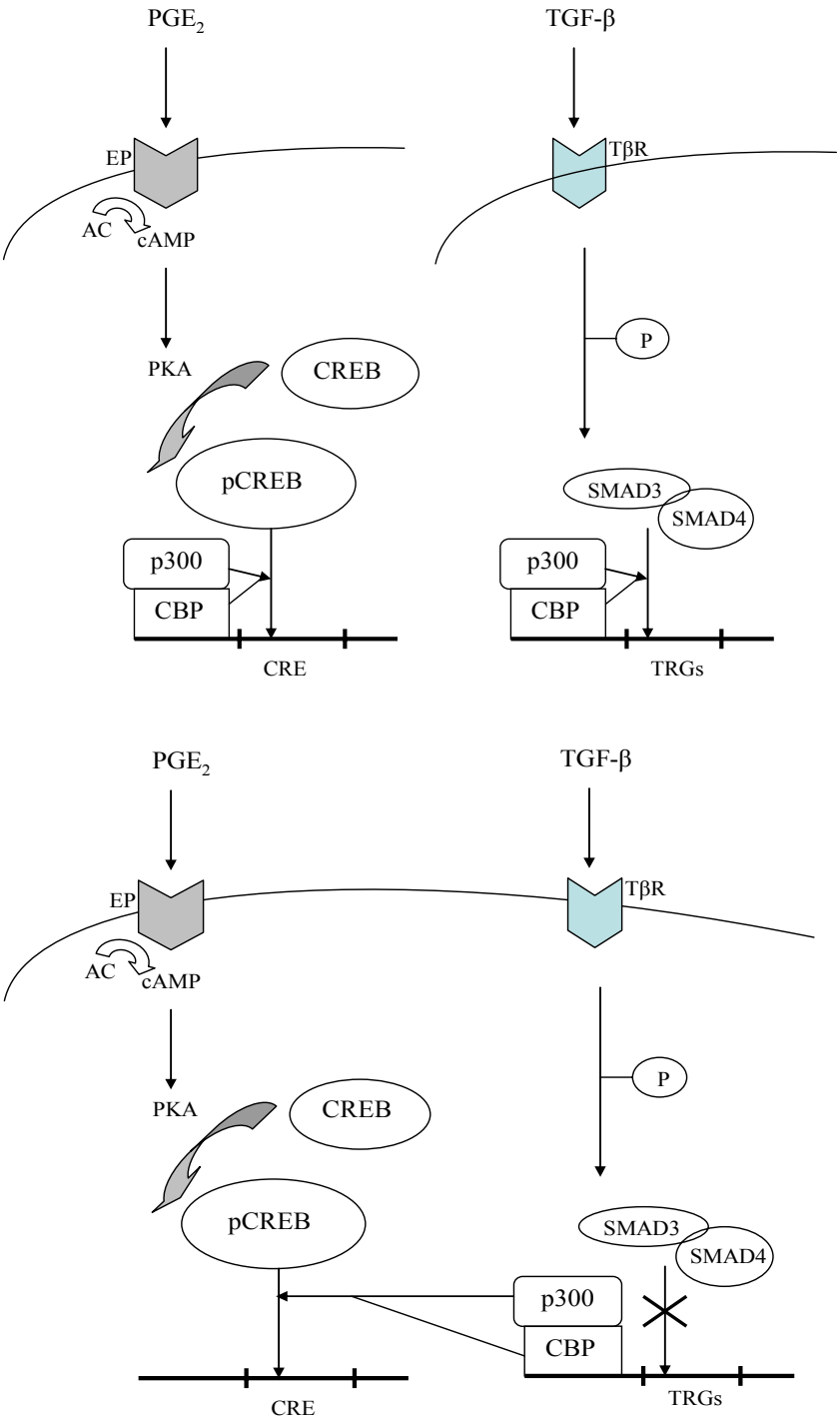


Figure 5.12: Schematic diagram of interactions between PGE_2 and TGF-β signalling pathways. PG activates EP receptor on the cell surface resulting in adenylate cyclase (AC) activation. AC catalyses activation of cAMP, which in turn activates cAMP-dependent protein kinase A (PKA). PKA phosphorylates cAMP response element binding protein (CREB) allowing its association with transcriptional coactivators CREB-binding protein (CBP) and p300. The complex then binds cAMP-responsive element (CRE). TGF-β acts through activation of the SMAD complex which also requires the binding to transcriptional coactivators CBP and p300. When the cAMP pathway is activated it sequesteres these coactivators, thus preventing effective binding of the SMAD complex to to the TGF-β responsive genes (TRGs) (R. Derynck *et al.* 1998d;M. Schiller *et al.* 2003c).

Ras/MEK/ERK signalling is normally required for KC housekeeping functions including cell cycling (J. D. Weber *et al.* 1997). It is known that ERK signalling is essential for KC motility (M. E. Zeigler *et al.* 1999). It was not surprising, therefore to find pERK expression in control cultured KCs under basal conditions (Figure 5.10). A small molecule inhibitor of MEK1/2 U0126 inhibited KC migration, confirming that this pathway is important in TGF-β driven cell motility (Figures 5.7, 5.8).

However, I showed that TGF-β did not enhance pERK expression in KCs and PGE_2 did not appear to block ras/MEK/ERK signalling in these cells.

I suggest that the overall effect of PGE_2 is to allow wound edge KCs to proliferate in a TGF-β rich environment and partially arrest their early migration across the wound. As PGE_2 levels decline from days 5-7 post wounding, TGF-β responses are restored and KCs re-acquire their migratory non-proliferative phenotype.

Conclusions

- PGE_2 inhibits TGF-β driven KC migration.
- PGE_2 inhibits the anti-proliferative effect of TGF-β on human KCs.
- PGE_2 antagonism of TGF-β driven KC function may have evolved to enable effective wound closure in the presence of TGF-β.
- PGE_2 antagonism of TGF-β driven anti-proliferative effect on KCs may be partially responsible for the proliferative and carcinogenic effects of PGE_2 on epidermal KCs.

CHAPTER 6

Summary Discussion and Conclusions

Studies into the pathogenesis of SSc skin sclerosis to date have predominantly given emphasis to the fibroblast activation in the dermis as key to causing fibrosis. TGF-β, CTGF and ET-1 have been identified as factors capable of causing fibroblast activation and transformation to the highly contractile myofibroblast form (A. Desmouliere *et al.* 2005a).

Relatively little attention has been paid to the epidermis in SSc. We do know from clinical practice that SSc often involves areas of the skin which have had repetitive superficial external stimulation such as rubbing with a belt on the abdomen or around the neck by a heavy pendant. We also know that the SSc epidermis is often hyper-pigmented, a phenomenon specific to the epidermis and its melanocytes. ET-1, a growth factor implicated in SSc skin fibrosis, is increased in the epidermal cell layers of SSc lesional skin and correlates with pigmentary changes in the disease (H. Tabata *et al.* 2000h;X. Shi-Wen *et al.* 2004c;S. W. Xu *et al.* 2004a). TGF-β (L. Rudnicka *et al.* 1994e), monocyte chemo-attractant protein-1 (MCP-1) (O. Distler *et al.* 2001b), VEGF (C. A. Davies *et al.* 2006a) and IL-21 receptor (IL-21R) (J. H. Distler *et al.* 2005d) have all been shown to be up-regulated in the epidermis in SSc.

Epidermal maturation is a complex process and KCs play a major role in its regulation. KCs synthesise structural components of the epidermal barrier through a programmed process of differentiation (K. S. Rao *et al.* 1996b). In the normal epidermis, proliferative cells are located in the basal layer where they strongly express cytokeratins 5 and 14. As they withdraw from the cell cycle and migrate

upwards, KCs differentiate and start expressing keratin 1 and keratin 10 (E. Fuchs 1993). I found that in SSc forearm skin lesions, the pattern of keratin expression is altered. Cytokeratin 14 is seen to persist into subrabasal layers and this is accompanied by a delay in the expression of terminal differentiation marker keratins 1 and 10. This change represents a disruption in terminal differentiation which results in a delay in epidermal maturation (Figure 3.2). I also found that in SSc the epidermis is expanded consistent with persistence of active proliferative KCs. These changes in SSc epidermis are similar to those described in wound healing during which the epidermis becomes expanded and shows persistence of the basal phenotype into superficial layers (M. L. Usui *et al.* 2005b;G. K. Patel *et al.* 2006b;M. D. Hertle *et al.* 1992a).

Integrins are transmembrane receptors, which play a crucial role in forming bonds between cells of the basal layer of the epidermis with the basement membrane. They stabilise cell-to-cell interactions and cell matrix adhesion in the epidermis (A. Sonnenberg *et al.* 1991). The hemidesmosome-associated integrin-α6, which forms a complex with the β4-subunit, is sharply localised at the basal surface of basal KCs in normal skin (M. D. Hertle *et al.* 1992e). However, I found that in the disease, the expression of the α6-integrin was no longer confined to the basal membrane zone and this was accompanied by loss of basal intensity (Figure 3.2). This change also represents a shift towards a proliferative undifferentiated poorly stratified epidermis seen in the disease. Persistent suprabasal integrin-α6 expression is a feature of a hyperproliferative wound healing epidermis (M. D. Hertle *et al.* 1992f).

Following epidermal injury or in hyperproliferative disorders such as psoriasis or cancer, KCs become activated and express activation makers, such as keratin 6 and 16 (K. S. Rao *et al.* 1996a). The positive immunostaining for the presence of keratin

6 and 16 which I found in SSc epidermis, provides evidence of activation of KCs in the disease, further supporting the view that the SSc epidermis exhibits changes described during wound healing (Figure 3.3) (N. Aden *et al.* 2008c).

Overall, I find that the SSc epidermis is expanded, lacks terminal differentiation and exhibits a cytokeratin marker pattern typical for a wound healing epidermis (N. Aden *et al.* 2008d). The fact that the SSc epidermis is altered and has a wound healing phenotype is an important new finding based on the results of my thesis.

Activated KCs are known regulators of wound healing in general and extracellular matrix production in particular (J. N. Barker *et al.* 1991a;I. M. Freedberg *et al.* 2001a;F. Grinnell 1992b;S. Werner *et al.* 2007b). KCs are known to be active secretory cells capable of producing profibrotic mediators that via diffusion into the dermis cause fibroblast activation and transformation to the myofibroblasts phenotype (N. Maas-Szabowski *et al.* 1999b). Co-culture of normal human KCs and fibroblasts induces fibroblast transition to the myofibroblast form in a TGF-β and ET-1 dependent manner (B. S. Bauer *et al.* 2002). Evidence for KC-derived fibroblast activation also comes from studies of keloid KC and fibroblast co-cultures (W. Xia *et al.* 2004d).

In wound healing, the KC-fibroblast activation loop is thought to be that of double paracrine signalling, with KCs instructing fibroblasts to produce and secrete growth factors which are in turn capable of KC activation (N. Maas-Szabowski *et al.* 1999a).

In this thesis I proposed that SSc skin fibrosis is at least partially caused by persistence of the wound healing phenotype of the epidermis causing production of pro-fibrotic mediators by KCs. This in turn would lead to continuous and uncontrolled activation of dermal fibroblasts.

First I began with a simple ELISA measurement of homogenised normal and SSc epidermal samples, and assayed KC-derived factors known to be involved in fibroblast induction (C. P. Denton, D. J. Abraham 2001;X. Shi-Wen *et al.* 2001a). Specifically, I measured levels of IL-1, ET-1, and TGF-β (free and LAP-associated). I found that IL-1 levels were greately elevated in SSc epidermis, but ET-1 and TGF-β levels were similar in disease and control specimens. In SSc there was a slight elevation of the LAP-TGF-β form and a trend towards a higher ET-1 receptor level. Also, my studies of SMAD 2/3 phosphorylation are consistent with more active TGF-β signalling in SSc epidermis (Figures 3.5, 3.5, 3.10, 3.12, 3.13).

These findings are important because IL-1, which is an inflammatory molecule stored within intact KCs and released upon cell stress or mechanical injury, has previously been shown to be involved in the KC-fibroblast activation loop (P. Shephard *et al.* 2004e;P. Shephard *et al.* 2004l). In fact, IL-1 is known to be the initiator of fibroblast activation in this setting and induces a large number of genes important for epidermal-fibroblast cross-talk (L. Florin *et al.* 2004a;S. Werner *et al.* 2007a)

There is some conflict in the literature because some fibroblast responses to IL-1 act so as to delay the induction of extracellular matrix production (D. Nowinski *et al.* 2002). However, it is thought that there is a difference in the pattern of signalling molecule activation between KCs and fibroblasts depending on the time scale of their co-culture. Specifically, in the early stages IL-1 activates NF-κB - a transcription factor that inhibits TGF-β effects and collagen production (P. Shephard *et al.* 2004m). We have shown, however, that KCs in SSc resemble active day 7 wound healing cells (N. Aden *et al.* 2008e), at which point the NF-κB effects are likely to become redundant and TGF-β would act to transform fibroblasts into the

myofibroblast form (P. Shephard *et al.* 2004n). Since most of the previous co-culture experiments have been done in the context of a wound healing environment, i.e. the time scale of less than 5 days, and with cultured KCs rather than intact tissue, the SSc skin biopsy disc - FPCL co-culture set-up used in my experiments represents a more life-like environment in terms of modelling SSc events. The signalling milieu in my experiments is likely to be different from cell co-cultures used by other groups. It is known, for instance, that IL-1 activates IL-6, IL-8, TNF-α, and GM-CSF in the epidermis (H. Uchi *et al.* 2000b). GM-CSF is known to activate SSc ECM production, and its receptors are over-expressed on SSc dermal fibroblasts (L. Postiglione *et al.* 2002;L. Postiglione *et al.* 2005). IL-6 is also known to be pro-fibrotic, and SSc patients homozygous for a specific allele of this interleukin are known to have higher disease activity scores (R. Sfrent-Cornateanu *et al.* 2006). Both IL-6 and IL-8 have been found to be inducible in SSc fibroblasts by IL-1 (T. Kadono *et al.* 1998). Activated SSc epidermal KCs are likely to be causing fibroblast activation indirectly via stimulation of the above mediators, the study of which is beyond the scope of this thesis. Overall, IL-1 most likely initiates complex KC-fibroblast interactions but delays ECM production until the mid phase of wound healing when ECM genes such as Type I collagen are induced.

Overall, the structural and signalling changes described above represent persistent SSc epidermal activation.

In addition, I went on to study signalling events in SSc and normal epidermal biopsy samples using phosphoprotein microarray (Kinexus). These studies demonstrated a significant increase in phosphoproteins associated with wound healing and cell cycling (including receptor kinases, transcription proteins and signalling molecules), and a reduction in apoptosis related phosphoproteins in SSc (Tables 3.1, 3.2). This

supports the notion of a chronic injury phenotype and activation in SSc epidermis. Most significantly, c-Jun, p38, HGF and STAT3 phosphorylated kinases have been found to be significantly elevated in SSc epidermis. All of these factors are known to be involved in epithelial-mesenchymal cross-talk. c-Jun and p38 are both downstream of IL-1 in its signalling cascade (Figure 1.3) (Y. Kida *et al.* 2005), which confirms a tissue injury-like process in SSc epidermis and indicates the presence of injured or stressed cells. I also found that HGF receptor tyrosine kinase has an elevated phosphoylation state in the disease. This is important because in injured tissues mesenchymal cells release HGF which causes epidermal cell activation via the HGF c-MET receptor (R. Zarnegar 1995). Also previous studies indicate that SSc fibroblasts do indeed hypersecrete HGF and this is IL-1 dependent (Y. Kawaguchi *et al.* 2002).

I went on to study whether these changes in SSc epidermis translated into the ability to cause dermal fibroblast activation. In order to mimick the natural tissue architecture and cell interactions in vitro, three-dimentional co-cultures of a biopsied epidermis with normal human fibroblast populated collagen matrices were used. I demonstrated that the SSc epidermis, and not normal epidermis, caused fibroblast activation in a TGF-β, ET-1 and IL-1 dependent manner (Figures 4.2-4.13).

SSc epidermis was shown to cause ET-1, TGF-β, and CTGF, but not IL-1 overproduction by the stimulated collagen lattice fibroblasts (Figures 4.14 - 4.17).

I therefore propose that IL-1 is the epidermis-derived cytokine triggering fibroblast activation, whereas ET-1 and TGF-β are involved at the level of the fibroblast maintaining the activated phenotype of these cells.

I also propose a mechanism whereby the SSc epidermis is activated by a stimulus/insult (such as an auto-antibody or a chemical irritant), causing IL-1 release

from KCs that in turn causes fibroblast activation. The fibroblasts then enter an autocrine loop involving ET-1 and TGF-β which maintains the fibrotic matrix-overproducing phenotype of the cells. The dermal factors are then likely to feedback to the epidermis setting up a paracrine activation loop similar to that seen in wound healing (Figure 6.1). While initially maintained by an external insult, the loop is likely to become autonomous in the long run. The initial inflammatory stage of the disease that correlates with the clinical picture of red, pruritic skin (the IL-1 phase), eventually burns out leaving the patient with permanent sclerosis.

These findings have therapeutic implications because epithelial-fibroblast interactions could be blocked by biologic therapies. Specifically IL-1 receptor antagonists such as Anakinra (licensed for use in inflammatory conditions) could be tried in early inflammatory disease stages. In addition, blocking HGF and its receptor c-MET could be used to antagonise KC-fibroblast interaction in the disease.

Expansion of the epidermis, delayed maturation, and altered α6 integrin and cytokeratin 1, 10, 14, 6, and 16 expression are all features of the epidermis during wound healing. Because of this I became interested in KC regulation during wound repair. During wound repair and in SSc the epidermal layer becomes thickened and expanded despite a TGF-β-dominated environment, which is anti-proliferative in nature. I reasoned that some component of epidermal cell activation during wound repair releases KCs from the anti-proliferative effect of TGF-β. In the past, prostanoids have been shown to inhibit TGF-β functioning in human cells (R. Stratton *et al.* 2002k). Kinexus phosphoprotein studies have highlighted reduced phosphorylation of COX II in SSc epidermis. This prompted the studies of COX II and its product PGE_2 in wounding. I showed increased COX II and PGE_2 production in early excisional mouse wounds (Figure 5.2).

I then showed that PGE_2 inhibits anti-proliferative affects of TGF-β. I also found that PGE_2 blocked the pro-migratory affects of TGF-β. I suggest that PGE_2 induced at the wound edge on days 1-7 transiently blocks TGF-β events in KCs and enables their proliferation at wound margin. The mechanism of this inhibition does not seem to involve erk phosphorylation. Published data shows that the two pathways interact at the level of transcription co-factors CREB and p300 (Figure 5.12).

Regeneration in rapidly renewing epidermal tissue requires proper functioning of control mechanisms of growth and differentiation, which are mainly regulated by epithelial-mesenchymal interactions (N. Maas-Szabowski *et al.* 2000). PGE_2 is gradually emerging as an important mediator that influences the progression of fibrotic processes. In the lung, for instance, PGE_2 is known to be protective against interstitial fibrosis conditions such as IPF (V. Lama *et al.* 2002;B. B. Moore *et al.* 2000;B. B. Moore *et al.* 2005). Outside the lung environment, PGE_2 has been shown to be anti-fibrogenic in the liver (L. Treffkorn *et al.* 2004). PGE_2 has also been shown to inhibit keloid scar dermal fibroblast contraction and TGF-β-induced collagen production (V. C. Sandulache *et al.* 2007). Results from this laboratory have also previously established that prostanoids are able to antagonise TGF-β signalling in human dermal fibroblasts (R. Stratton *et al.* 2002l).

Experimental evidence has established that at least some of the antifibrotic actions of PGE_2 are produced through inhibition of TGF-β. For example, there is definitive evidence to show that PGE_2 reduces fibroblast activation and collagen production in human lung fibroblasts via inhibition of TGF-β activity (A. Diaz *et al.* 1989b). It has also been confirmed more recently that PGE_2 inhibits TGF-β-induced fibroblast to myofibroblast transformation and collagen I expression (J. E. Kolodsick *et al.* 2003).

In fact, it has been suggested that normally, through stimulation of endogenous PGE_2 production, TGF-β exhibits autoregulatory control to limit connective tissue synthesis by human lung fibroblasts (A. Diaz *et al.* 1989a).

In human skin fibroblasts the relationship between PGE_2 and TGF-β is also well recognised. There is evidence that PGE_2 inhibits collagen and fibronectin production by these cells (J. Varga *et al.* 1987).

In clinical practice, iloprost (a synthetic analogue of prostacyclin, a prostanoid related to PGE_2) is known to improve skin fibrosis, and has previously been shown to inhibit fibroblast activation (R. Stratton *et al.* 2002m).

Epithelial-mesenchymal interaction involving PGE_2 has also been noted. It has been shown that inflammatory mediators TNF-α and IL-1, known to be released from normal human KCs after wounding, have antifibrotic properties by inhibiting transcription of α1(I)procollagen gene partly via PGE_2 release from human fibroblasts (A. Diaz *et al.* 1993).

Alveolar epithelial cell (AEC) dysregulation has been directly implicated in lung fibroblast activation and subsequent fibrosis (M. Selman *et al.* 2001). More recently, epithelium-driven lung fibrosis has been attributed to a reduction in PGE_2 production by damaged AECs (B. B. Moore *et al.* 2003a). It is known that in SSc lung disease AEC damage is partly responsible for the underlying parenchymal fibrosis via a TGF-β mechanism (R. K. Hoyles *et al.* 2008). By drawing analogy between SSc lung and skin pathology I propose that the reduction in PGE_2 availability is partly responsible for abnormalities in SSc KC proliferation and migration (via inhibition of TGF-β driven KC function), which could in turn lead to the structural abnormalities seen in the disease epidermis, with fibrosis as a pathophysiological result of this epidermal disfunction.

Increasing epithelial PGE_2 production has previously been put forward as a potential candidate for therapeutic interventions in fibrotic lung disease (B. B. Moore *et al.* 2003b). Since the above experimental evidence confirms the role of PGE_2 in inhibition of epithelial TGF-β function, PGE_2 is a candidate therapeutic agent for the inhibition of epithelium-driven fibroblast activation also. One has to bear in mind, however, that PGE_2 has been shown to induce IL-1β production in normal human fibroblasts. IL-1β is known to be profibrotic in the lung. Its close isoform IL-1α (which has identical biological properties and binds to the same receptor) has been shown to be an important epithelium-derived fibrotic mediator in chapter 3. This may mean that PGE_2 on its own is not enough to suppress fibrosis, and may require an addition of an IL-1 inhibitor.

Excess PGE_2 is strongly linked to increased incidence of skin and gastrointestinal malignancy. We must keep in mind that the anti-proliferative action of TGF-β is known to be important in protection against epidermal carcinogenesis (B. Pasche 2001). Early skin tumour development is suppressed by TGF-β through its effect on cell cycling (W. Cui *et al.* 1996;A. B. Glick *et al.* 1993). Transgenic SMAD 3+/- heterozygous knockout mice have a greater resistance to DMBA-induced skin carcinogenesis than the wild-type or SMAD 2+/- counterparts thus implicating this TGF-β pathway in tumour protection (S. H. Tannehill-Gregg *et al.* 2004).

In the recent years COX II and prostaglandins including PGE_2 have emerged as important mediators of epithelial carcinogenesis (K. Muller-Decker *et al.* 1995;Y. M. Sung *et al.* 2005). COX II over-expression has been found in basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) (G. Gasparini *et al.* 2003), and both SCC initiation and progression have recently been shown to be enhanced by PGE_2 (S. Brouxhon *et al.* 2007). PGE_2 and COX II have been found to be over-

expressed in human KCs following UVB irradiation confirming the important role of PGE_2 in UV-induced carcinogenesis (S. Y. Buckman *et al.* 1998). COX II inhibitors inomethacin and colecoxib have been found to be protective in this setting (S. M. Fischer *et al.* 1999b).

PGE_2 has also been implicated in the development of several gastrointestinal malignancies including gastric and colorectal (C. E. Eberhart *et al.* 1994). COX II inhibitors are associated with an up to 50% reduction in the morbidity and mortality from colorectal cancer (L. J. Marnett, R. N. DuBois 2002). Antagonism of TGF-β could explain in part the mechanism of pro-proliferative and tumourgenic action of PGE_2 in epithelial structures and the protective effect of NSAIDs (M. Schiller *et al.* 2003a).

The set aims of this thesis were:

1. To determine whether the SSc epidermis has an altered structural and signalling phenotype.

2. To determine whether the SSc epidermis activates normal human fibroblast in vitro.

3. To determine whether PGE_2 modifies the responses of KCs to TGF-β.

I have found that:

1. The SSc epidermis has an altered structural and signalling phenotype. The phenotype resembles a wound healing epidermis and has pro-fibrotic potential.

2. The SSc epidermis activates normal human fibroblasts in vitro.

3. PGE_2 inhibits the TGF-β driven anti-proliferative and pro-migratory KC functions. This may explain the ability of KCs to proliferate at the wound margin in a TGF-β dominated environment.

Figure 5.13 summarises the findings of this thesis and ties them in with known mechanisms of epithelial-mesenchymal intreaction.

I therefore demonstrate that the hypothesis of the thesis was correct and SSc epidermis does contribute to activation of fibrotic processes in the disease.

It is important to appreciate that the success of future therapeutic agents for SSc treatment will depend on changing our target from the dermal/interstitial structures to epidermal/epithelial ones.

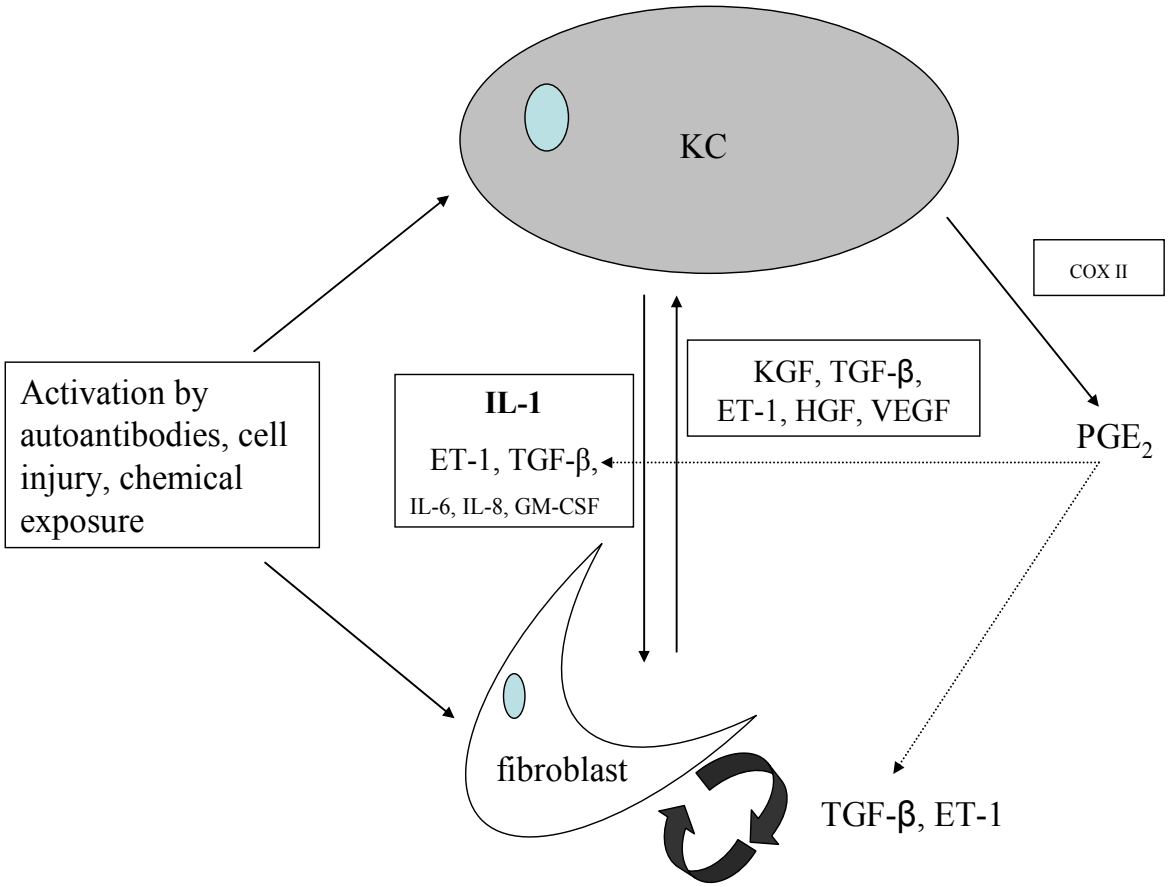


Figure 6.1: Schematic representation of epidermal-fibroblast interaction in SSc. Summary of the mechanism proposed to account for epidermis-driven dermal fibroblast activation in SSc. Initial external stimuli (autoantibody, cell injury, or chemical exposure) activate KCs as well as fibroblasts. Activated KCs assume a wound healing phenotype with an altered structure and function resulting in the production of profibrotic mediators such as IL-1. These factors subsequently act on dermal fibroblasts setting up a positive feedback paracrine loop (maintained by TGF-β and ET-1) to cause pathological fibrosis seen in the disease. Feedback mechanisms operate from dermis to epidermis involving KGF and HGF. COX II is also activated post epidermal insult and causes PGE_2 production. PGE_2 inhibits TGF-β-driven KC function which has anti-fibrotic potential.

CHAPTER 7

Future Work

The work presented in this thesis shows that the interaction between SSc epidermis and dermal fibroblasts is important in pathogenesis of fibrosis in the disease. However several areas were beyond the scope of the thesis. Additional experimental work will provide a clearer picture of the disease pathways.

Further studies of SSc epidermis to include:

1. Staining of SSc and normal epidermal biopsy material for most significantly altered factors highlighted with Kinexus phosphoprotein assay, eg. HGF, members of MAPK cascade, markers of apoptosis and cell proliferation.
2. SSc and normal KC culture and staining for intracellular presence of IL-1.
3. IL-1 release from SSc KCs determined using ELISA and Western blotting of media.
4. mRNA PCR of SSc and normal epidermal KCs for IL-1 expression.

Further studies of SSc epidermis-fibroblast co-culture to include:

1. Specific ET-1 A and B receptor inhibitors used in co-culture experiments.
2. Staining of epidermal-dermal junction from co-culture experiment for HGF receptor c-Met as a marker of fibroblast feedback.

Further studies into PGE_2/TGF-β interactions in human KCs to include:

1. Measurement of the effect of topical PGE_2 on epithelial coverage of excisional wounds in the mouse.
2. Measurement of the effect of topical PGE_2 on KC proliferation at the wound edge.
3. Repeating these experiments in a COX II knock-out mouse.

CHAPER 8
Appendix

Appendix 1: List of Antibodies

Antibody	Company	ID number	Host Species
αSMA	DAKO	MO851	mouse
αv integrin	Abcam	ab6133	mouse
β-Actin	Sigma	A 5441	mouse
COX II	Santa Cruz Biotechnology	sc-1746	goat
CTGF	Santa Cruz Biotechnology	sc-14939	goat
Cytokeratin 14	Vector Laboratories	VP-C410	mouse
Cytokeratin 16	Labvision	LLO25	mouse
Cytokeratin 7	R&D	AF 1339	goat
Cytokeratin 6	Labvision	LHK6B	mouse
Cytokeratins 1 and 10	Cymbus Biotech	CBL266	mouse
ERK1/2 (p44/42)	Cell Signalling Technology	9122	rabbit
ET-1	Bachem	T-4049	rabbit
ET-A Receptor	Abcam	ab 12977	rabbit
ET-B Receptor	Abcam	ab 12980	rabbit
GAPDH	Abcam	ab8245-100	mouse
IL-1α	Santa Cruz Biotechnology	sc-9983	mouse
IL-1α Receptor	Santa Cruz Biotechnology	sc-25775	rabbit
LAP- TGF-β	R&D Systems	AB-246-NA	goat
TGF-β	Santa Cruz Biotechnology	sc-146	rabbit
pERK1/2 (Phospho-P44/42)	Cell Signalling Technology	9101	rabbit
pSMAD2/3	Santa Cruz Biotechnology	sc-11769	rabbit

Appendix 2: Patient Biopsy Cohort

68% Female, Age range 35-67, Mean age 51

Antibody profile: ScL 70 31%; RNA polymerase 18%; u3RNP 9%, Nucleolar 4.5%;

Centromere 22.7%; ANA positive, ENA negative 13.6%

Appendix 3: Full list of Kinexus Phosphoprotein Data

Fold Increase	Phosphoprotein Site (Human)	Full Target Protein Name
5.55	S70	Tyrosine hydroxylase isoform a
2.89	Y1003	Hepatocyte growth factor (HGF) receptor-tyrosine kinase
2.63	Pan-specific	Protein-serine phosphatase 4 - regulatory subunit (PPX/A'2)
2.62	S605	Synapsin 1 isoform Ia
2.57	S674	Protein-serine kinase C eta
2.43	Pan-specific	Male germ cell-associated protein-serine kinase
2.38	Pan-specific	Wee1 protein-tyrosine kinase
2.37	S63	Jun proto-oncogene-encoded AP1 transcription factor
2.29	Pan-specific	NIMA (never-in-mitosis)-related protein-serine kinase 7
2.23	T514	Protein-serine kinase C gamma
2.18	Y705	Signal transducer and activator of transcription 3
2.18	Pan-specific	Dual specificity protein kinase
2.18	T674	Protein-serine kinase C gamma
2.17	Pan-specific	Integrin-linked protein-serine kinase 1
2.15	Y1189/Y1190	Insulin receptor/Insulin-like growth factor 1 receptor
2.13	T180+Y182	Mitogen-activated protein-serine kinase p38 alpha
2.10	Pan-specific	PCTAIRE-1 protein-serine kinase
2.09	Pan-specific	NIMA (never-in-mitosis)-related protein-serine kinase 4
2.07	Pan-specific	Polo-like protein kinase 3 (cytokine- inducible kinase (CNK))
1.96	Pan-specific	Protein kinase C-related protein-serine kinase 1
1.94	S18	Tyrosine hydroxylase isoform a
1.92	Pan-specific	Nucleotide diphosphate kinase 7 (nm23-H7)
1.91	S716	Microtubule-associated protein tau

1.86	T514	Protein-serine kinase C gamma
1.84	Pan-specific	BNDF/NT3/4/5 receptor- tyrosine kinase
1.84	Pan-specific	RYK tyrosine-protein kinase
1.83	S576	Protein-tyrosine phosphatase 1D (SHP2, SHPTP2, Syp, PTP2C)
1.81	Pan-specific	ZIP kinase (death associated protein-serine kinase 3 (DAPK3))
1.81	Pan-specific	MAPK/ERK protein-serine kinase 6 (MKK6)
1.80	Y40	Bone marrow X protein-tyrosine kinase
1.79	Y279/ Y216	Glycogen synthase-serine kinase 3 alpha
1.79	Pan-specific	TGF-beta-activated protein-serine kinase 1
1.77	Pan-specific	Proliferating cell nuclear antigen
1.77	Pan-specific	Phosphatidylinositol 4-phosphatase 5-kinase type 2 alpha
1.76	Pan-specific	Aurora Kinase A (serine/threonine protein kinase 6)
1.76	Pan-specific	p53-induced protein PIGPC1
1.76	Pan-specific	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and protein phosphatase and tensin homolog deleted on chromosome 10
1.76	Y576	Focal adhesion protein-tyrosine kinase
1.75	Y754	Platelet-derived growth factor receptor kinase alpha
1.73	Pan-specific	MAPK/ERK protein-serine kinase 2 (MKK2)
1.71	Pan-specific	Protein-serine phosphatase 1 - catalytic subunit - beta isoform
1.71	Pan-specific	Intestinal cell protein-serine kinase (MAK-related kinase (MRK))
1.70	Pan-specific	Extracellular regulated protein-serine kinase 4
1.70	Pan-specific	TGF-beta-activated protein-serine kinase 1
1.66	Pan-specific	Protein-serine phosphatase 2A - A regulatory subunit - alpha and beta isoforms
1.65	Pan-specific	MAPK/ERK kinase kinase 2
1.65	Pan-specific	p70 ribosomal protein-serine S6 kinase beta
1.64	S465+S467	SMA- and mothers against decapentaplegic homolog 2
1.64	Pan-specific	cAMP-dependent protein-serine kinase regulatory type 2 subunit alpha
1.63	S738+S742	Protein-serine kinase C mu (Protein kinase D)
1.63	T334	Mitogen-activated protein kinase-activated protein kinase 2 alpha/beta
1.63	Y572+Y574/Y579+Y581	Platelet-derived growth factor receptor kinase alpha/beta
1.62	S370	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and protein phosphatase and tensin homolog deleted on chromosome 10
1.62	Pan-specific	Phosphoinositide-3-kinase, regulatory subunit

		4
1.62	Pan-specific	Signal transducer and activator of transcription 2
1.59	Pan-specific	Tousled-like protein-serine kinase 1
1.58	S116	Phosphoprotein-enriched in diabetes/astrocytes 15
1.58	Pan-specific	Axl proto-oncogene-encoded protein-tyrosine kinase
1.57	Pan-specific	Vaccinia related protein-serine kinase 1
1.56	Pan-specific	Nucleotide diphosphate kinase 6 (nm23-H6)
1.56	Pan-specific	Protein-serine kinase C zeta
1.56	Pan-specific	Inhibitor of NF-kappa-B protein-serine kinase beta
1.56	Pan-specific	Src proto-oncogene-encoded protein-tyrosine kinase
1.55	Pan-specific	Tank-binding protein 1
1.54	S218+S222, S4	MAPK/ERK protein-serine kinase 1 (MKK1) + B23 (nucleophosmin, numatrin, nucleolar protein NO38)
1.54	Pan-specific	Suppressor of cytokine signalling 4 (SOCS7)
1.53	S645	Protein-serine kinase C delta
1.53	T210	Polo-like protein-serine kinase 1
1.53	S910	Focal adhesion protein-tyrosine kinase
1.53	Pan-specific	Mitogen-activated protein-serine kinase p38 alpha
1.53	Pan-specific	Eukaryotic translation initiation factor 2 alpha
1.52	S82	Heat shock 27 kDa protein beta 1 (HspB1)
1.52	Pan-specific	Mammalian target of rapamycin (FRAP)
1.52	T229	p70/p85 ribosomal protein-serine S6 kinase alpha
1.50	T232	Fos-c FBJ murine osteosarcoma oncoprotein-related transcription factor
1.50	S114	cAMP-dependent protein-serine kinase regulatory type 2 subunit beta
1.50	Pan-specific	Kinase homologous to SPS1/STE20 (MAP kinase kinase kinase protein-serine kinase 5 (MEKKK5)
1.50	S8	Glial fibrillary acidic protein

Fold Decrease	**Phosphoprotein Site (Human)**	**Full Target Protein Name**
0.31	Pan-specific	Diacylglycerol kinase zeta
0.33	Pan-specific	Bcl2-like protein 1
0.34	Pan-specific	Pro-caspase 3
0.36	Pan-specific	caspase 5

0.39	Pan-specific	Janus protein-tyrosine kinase 2
0.39	Pan-specific	Cyclin-dependent protein-serine kinase 4
0.40	Pan-specific	Cyclo-oxygenase 2 (prostaglandin G/H synthase 2 precursor)
0.40	Pan-specific	Cell division cycle 25B phosphatase
0.40	Pan-specific	Heme oxygenase 2
0.40	T286	Calcium/calmodulin-dependent protein-serine kinase 2 alpha
0.40	Y612	Insulin receptor substrate 1
0.41	Pan-specific	Cell division cycle 2-like protein-serine kinase 5
0.41	S129+S133	cAMP response element binding protein 1
0.42	Pan-specific	Cyclin-dependent protein-serine kinase 5
0.43	Pan-specific	Calcium/calmodulin-dependent protein-serine kinase 2 beta
0.43	Pan-specific	p21-activated serine kinase 5 (Serine/threonine-protein kinase PAK 7)
0.44	Pan-specific	Leukocyte common antigen CD45 receptor-tyrosine phosphatase (LCA, T200)
0.44	S36	Caveolin 2
0.45	Pan-specific	Calcium/calmodulin-dependent protein-serine kinase 1 delta
0.46	Pan-specific	Heat shock 70 kDa protein 1
0.47	Pan-specific	Large tumor suppressor 1 protein-serine kinase (WARTS)
0.48	S789	Caldesmon
0.50	Pan-specific	Calcium/calmodulin-dependent protein-serine kinase 2 gamma
0.51	S45	Catenin (cadherin-associated protein) beta 1
0.52	Pan-specific	Cyclin-dependent protein-serine kinase 1
0.52	Pan-specific	Calmodulin-dependent protein-serine kinase kinase
0.52	S1107	Eukaryotic translation initiation factor 4 gamma 1
0.53	S80	Acetyl coenzyme A carboxylase
0.53	S530	Microtubule-associated protein tau
0.53	T183+Y185	Jun N-terminus protein-serine kinases (stress-activated protein kinase (SAPK)) 1/2/3
0.54	Pan-specific	Cyclin-dependent protein-serine kinase 6
0.54	Y412	Abelson proto-oncogene-encoded protein-tyrosine kinase
0.55	Pan-specific	Cyclin-dependent protein-serine kinase 8
0.55	Pan-specific	Anaplastic lymphoma kinase
0.55	Pan-specific	Pro-caspase 4 (ICH2 protease, ICE(rel)-II)
0.56	Pan-specific	Pro-caspase 8 (ICE-like apoptotic protease 5 (ICE-LAP5), Mch5, FLICE, CAP4)
0.56	S676	Protein-serine kinase C theta
0.57	Pan-specific	Jun N-terminus protein-serine kinases (stress-activated protein kinase (SAPK)) 1/2/3
0.57	Y15	Cyclin-dependent protein-serine kinase 1/2

0.57	Y999	Insulin receptor
0.57	S722	Focal adhesion protein-tyrosine kinase
0.57	Pan-specific	Heat shock transcription factor 4
0.57	Pan-specific	Calcium/calmodulin-dependent protein-serine kinase 2 delta
0.58	T218+Y220	Extracellular regulated protein-serine kinase 5 (Big MAP kinase 1 (BMK1))
0.58	Pan-specific	Extracellular regulated protein-serine kinase 2 (p42 MAP kinase)
0.59	Pan-specific	Pro-caspase 12 (mouse)
0.59	T320	Protein-serine phosphatase 1 - catalytic subunit - alpha isoform
0.59	Pan-specific	Aurora Kinase A (serine/threonine protein kinase 6)
0.60	Pan-specific	Putative protein-serine kinase WNK4
0.61	T199	B23 (nucleophosmin, numatrin, nucleolar protein NO38)
0.62	Pan-specific	STE20-like protein-serine kinase
0.63	Pan-specific	Cyclin-dependent protein-serine kinase 7
0.63	S10	Histone H3.3
0.63	Pan-specific	ER protein 57 kDa (protein disulfide isomerase-associated 3; 58 kDa glucose regulated protein)
0.64	S15	Heat shock 27 kDa protein beta 1 (HspB1)
0.64	Y15	Cyclin-dependent protein-serine kinase 1/2
0.64	Pan-specific	Heme oxygenase 1
0.65	Pan-specific	C-terminus of Src tyrosine kinase
0.66	Pan-specific	Casein protein-serine kinase 1 epsilon
0.66	Pan-specific	Cyclin-dependent protein-serine kinase 7
0.66	Pan-specific	Hematopoetic progenitor protein-serine kinase 1

Appendix 3: Publications

'Proteomic analysis of scleroderma lesional skin reveals activated wound healing phenotype of epidermal cell layer' Aden,N.; Shiwen,X.; Black,C.; Nuttall,A.; Denton,C.P.; Leask,A.; Abraham,D.; Stratton,R.
Rheumatology (Oxford) 2008 Dec; 47(12):1754-60

Abstract: OBJECTIVE: To identify using proteomic analysis, proteins of altered abundance in the skin of patients with SSc. METHODS: 4 mm excision biopsies were obtained from the forearm involved skin of 12 diffuse SSc patients and 12 healthy controls. Two-dimensional gel electrophoresis was used to separate and define proteins in normal and SSc skin biopsy material. Proteins of altered abundance in the disease were formally identified by mass spectroscopy. Abnormalities of the epidermis were confirmed by immunohistochemistry. RESULTS: Proteomic analysis revealed altered abundance of proteins involved in extracellular matrix production, myofibroblast contractility, energy metabolism and response to oxidative stress. In addition, proteins specific to the epidermis and involved in epidermal cell differentiation were altered in abundance in the disease. SSc epidermis is thickened, has an expanded nucleated cell layer, and exhibits abnormal persistence of basal marker keratin 14, delayed expression of maturation markers keratin 1/10 and the induction of keratins 6 and 16, normally absent from interfollicular skin and induced following epidermal injury. These changes closely resemble the activated phenotype seen during wound healing. CONCLUSIONS: Consistent with previous models of SSc pathogenesis these data are showing increased contractility, increased extracellular matrix and response to oxidative stress in the involved skin of recent onset SSc patients. In addition, we show that SSc epidermis has an activated, wound healing phenotype. These findings are important because epidermal cells activated by injury induce and regulate local fibroblasts during wound repair.

‘Scleroderma Epidermis Promotes TGF-β and ET-1 Dependent Fibroblast Activation’

Nuttall,A.; Shiwen,X.; Aden,N.; Denton,C.P.; Abraham,D.; Stratton,R.

ACR Supplement, November 2007, F 220

BACKGROUND: Scleroderma (SSc) is a systemic disorder of unknown aetiology, characterised by uncontrolled scarring of skin and internal organs. We have found previously that SSc epidermis has an activated wound-healing phenotype. When injured, wound healing keratinocytes can promote fibroblast activation which depends on TGF-β and ET-1. Epithelial-mesenchymal interactions are a well recognised phenomenon in scarring conditions and wound environments. Since both TGF-β and ET-1 are important in SSc pathogenesis, we set out to determine whether epithelial stimulation of fibroblasts could be important in SSc skin fibrosis.
We have previously demonstrated the ability of SSc epidermal biopsies to cause contraction of normal human fibroblasts in vitro, and shown that TGF-β is involved in the process. Here we address the involvement of ET-1 in fibroblast activation by the epidermis and look at the characteristics of the SSc vs normal epidermis in terms of their TGF-β and ET-1 content.
METHODS: 2 skin punch biopsies (4 mm) were obtained from each of the forearms of 3 patients with recent onset active diffuse SSc (within 24 months), and 3 control subjects. The epidermis was separated with trypsin. Fibroblast-populated stress collagen lattices (FPCL) were set up in wells. Control wells were left untreated. The other wells were exposed to either SSc or normal epidermal discs for 48 hrs. Further FPCL containing ET inhibitor bosentan (10μM) were also exposed as above.
Further biopsies collected from 4 patients with SSc and 4 control subjects were snap frozen in liquid nitrogen and the epidermises surgically cut off. Lysates of epidermal discs were assayed for TGF-β and ET-1 content using ELISA (R&D Systems).
Further biopsies were stained using immunohistochemistry for TGF-β and LAP-TGF-β.
RESULTS: We found similar levels of ET-1 in SSc and normal epidermal lysates (18.4 pg/ml, vs 18.3 pg/ml, p=0.1117). Free TGF-β content was slightly reduced in the SSc epidermal lysates vs normal controls (135.3 pg/ml vs 168 pg/ml, p=0.129).

There was a significant difference between SSc and normal epidermal LAP-TGF-β content (1436 pg/ml vs 1222.7 pg/ml, p=0.04). This was confirmed by immunohistochemical staining of epidermal biopsies.
SSc epidermis caused FPCL contraction (gel size 56.26% of original). This contraction was significantly inhibited by ET inhibitor bosentan (gel size 90.7% of original), p=0.01.
CONCLUSIONS: SSc epidermis has fibroblast activating properties in vitro, which are dependent on ET-1 and TGF-β. We suggest that activated epithelial cells maintain fibrosis by interaction with dermal fibroblasts. Since ET-1 and free TGF-β content of the SSc epidermis is not significantly increased, we believe a double paracrine epithelial-mesenchymal loop potentiates dermal activation and could therefore be a target for theraputic intervention.

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