Intracellular Sphingosine Kinase Activity as a Regulator of Endothelial Cell Inflammatory and Angiogenic Potential.

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ABSTRACT

The normal endothelium is in a non-activated state evidenced by its inability to support leukocyte adhesion and its lack of angiogenesis. I present evidence in this thesis that the intracellular levels and activity of Sphingosine Kinase (SK) regulate the inflammatory potential of the endothelium and are also an important determinant of the ability of the endothelium to undergo angiogenesis. Moderate (three to fivefold) elevations in SK activity in human umbilical vein endothelial cells resulted in endothelial activation, as indicated by the heightened expression of adhesion molecules, and further sensitized the cells to subliminal doses of inflammatory cytokines. This corresponded with enhanced neutrophil binding in the un-stimulated and stimulated states. Over-expression of a dominant-negative SK (G82D, containing the substitution glycine to aspartate and which blocks agonist-induced activation of SK) inhibited the adhesion molecule response to inflammatory cytokine and inhibited leukocyte adhesion. Over-expression of SK increased cell survival under the stressful conditions of serum deprivation and loss of attachment with extracellular matrix, which was associated with suppression of apoptotic mechanisms. Raised SK activity also stimulated cell migration and cellular remodeling, additional measures of angiogenesis. Over-expression of SK enabled activation of the phosphatidyl inositol-3kinase (PI-3K/Akt) pathway in response to serum deprivation, and this pathway was obligatory in mediating SK-induced cell survival. Activation of the PI-3K/Akt pathway in cells with raised SK activity was mediated by the cell junctional molecule platelet endothelial cell adhesion molecule-1 (PECAM-1), which was upregulated and dephosphorylated and critical in SK-induced cell survival. Thus raised intracellular SK activity enables a PECAM-1-dependent activation of the PI-3K/Akt pathway to augment cell survival, thus exposing a hitherto unexplored pathway of endothelial cell survival which may be manipulated therapeutically. The findings suggest a possible role for SK levels in the regulation of angiogenic phenomena as well as in the capacity

of endothelial cells to survive in suspension (circulating endothelial cells). Thus SK could be considered as a novel target for therapeutic manipulation in diseases of aberrant inflammation and angiogenesis.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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ABSTRACTS

1. Limaye V, Xia P, Moretti P, Pitson S, Parsons M, Vadas M, Gamble J
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ABBREVIATIONS

7-AAD	7 amino-actinomycin D
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
°C	degrees celcius
cAMP	cyclic adenosine monophosphate
cDNA	complimentary DNA
CI	confidence interval
CsCl	cesium chloride
CMV	cytomegalovirus
DAPI	4'6-Diamidine-2'-phenylindole dihydrochloride
DMS	N'N'-dimethylsphingosine
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E Selectin	endothelial selectin
E Coli	*Escherisciae coli*
ECGS	endothelial cell growth supplement
EDG	endothelial differentiation gene
EDTA	ethylenediaminetetra acetic acid
ELISA	enzyme linked immunosorbent assay
ERK	extracellular-signal-regulated kinase
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FGF	fibroblast growth factor
g	grams
G	gauge
GFP	green fluorescent protein
GCSF	granulocyte colony stimulating factor
GMCSF	granulocyte-macrophage colony stimulating factor
HA	haemaglutinin
HBSS	Hank's buffered salt solution
HCl	hydrochloric acid
HLA	human leukocyte antigen
HM	HUVE medium
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobulin
IFN	interferon
IL	interleukin
KDa	Kilodalton
LB	Luria Bertani
µg	micrograms
µl	microlitres
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinases
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulphophenyl)-2H-tetrazolium
ng	nanograms

O.D.	optical density
^{32}P	radiolabeled phosphorous
PBS	phosphate buffered saline
PECAM-1	platelet/endothelial cell adhesion molecule-1
PE	phycoerythrin
PKC	protein kinase C
PI-3K	phosphatidyl inositol 3-kinase
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SK	sphingosine kinase
S1P	sphingosine-1-phosphate
Sph	sphingosine
TE	Tris/EDTA
TIMP	tissue inhibitor of metalloproteinases
TLC	thin layer chromatography
TNFα	tumour necrosis factor alpha
Tween 20	polyoxyethylenesorbitan monolaurate (Sigma Chemical Co, MO, USA)
V	volts
v/v	volume/volume
VCAM-1	vascular cell adhesion molecule-1
VE Cadherin	vascular-endothelial cadherin
VEGF	vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder targeting the diarthrodial synovial joints. It has a worldwide distribution and affects 1 % of the world's adult population (1). The clinical course varies greatly, from self- limited disease to progressive destructive deforming disease with extra-articular involvement.

1.1 Synovial pathology in Rheumatoid Arthritis

The primary inflammatory joint lesion involves the synovium, with the earliest changes documented in the synovial microvasculature. The microvascular changes include angiogenesis, occlusion of the lumen, and alteration of the endothelium to an activated "high endothelial venule" phenotype, with swelling of endothelial cells (EC) and gaps between cells. The synovial lining comprises two cell types; Type A synoviocytes, of bone marrow origin that are like macrophages, and Type B synoviocytes of mesenchymal origin. In early RA there is mild proliferation on the superficial lining layer with oedema and fibrin exudation.

1.1.1 The role of T lymphocytes in Rheumatoid Arthritis

Cellular infiltration is an early feature of RA. T lymphocytes gather in the synovium utilizing specific adhesion molecules, and form nodular aggregates within a diffuse infiltrate. CD4+ T cells expressing cell surface antigens characteristic of memory or activated cells are prominent in the nodular aggregates, while CD8+ T cells dominate in the diffuse infiltrates. Activated T cells within RA synovial tissue express a characteristic pattern of cell surface molecules which allow binding to the endothelial cells and subsequent transmigration. In

response to cytokines, EC in turn express specific adhesion molecules to facilitate the egress of leukocytes from blood into synovial tissue.

Although CD4+ T cells are the major infiltrating cell and oligoclonal expansion is frequent, there is a paucity of T-cell-derived cytokines in synovial inflammation. However it needs to be remembered that T cells are engaged extensively in heterotypic adhesive reactions through which signalling can occur, including communication through release of low abundance lymphokines at privileged sites of intercellular engagement. Factors within the local milieu in the rheumatoid joint appear to condition the functioning of T cells. Prostaglandins, which suppress interferon γ secretion, and tumour necrosis factor-α (TNF-α), a T cell suppressive cytokine, are abundant in synovial inflammation, suggesting the local environment modulates T cell functioning. Cytokines consistent with a Th1 response (IFNγ, interleukin-2 and lymphotoxin) have however been demonstrated in inflamed synovium by immunohistochemistry (2),(3).

1.1.2 Rheumatoid Factor

While many RA patients have IgM autoantibodies (rheumatoid factor, RF) which recognize the Fc region of IgG, these are not disease specific. Although associated with a more aggressive disease course, it is not understood how RF contributes to the disease process. Other autoantibodies are also seen, including antifilaggrin antibodies, implicating at least a minor role for B cells (4).

1.1.3 Chronic changes in the synovium

With ongoing inflammation, follicular aggregates of lymphocytes develop, and plasma cells, mast cells, and multinucleated giant cells can be seen. The synovium eventually becomes hypertrophic from neovascularization and proliferation of synovial fibroblasts. This hypertrophic tissue can organize into areas known as pannus, which spread over and damage articular cartilage and invade supporting structures including periarticular bone.

The consequence of ongoing synovial inflammation is destruction of articular cartilage, bone, ligaments and tendons. Metalloproteinases (collagenases, gelatinases, and stromolysins) that degrade the extracellular matrix are produced by synovial fibroblasts under the control of the macrophage-derived cytokines interleukin-1 (IL-1), TNFα, and transforming growth factor-β (TGFβ).

1.2 The Pathogenesis of RA: Genetic factors

Although the precise aetiology is unknown, it is conceivable that RA disease expression results from the interaction of a genetically predisposed individual with an as yet undefined environmental trigger. Substantial evidence is available for genetic factors in RA and both HLA (human lymphocyte antigen) and non-HLA genes have been implicated. Allelic polymorphisms occur in the HLA DRB region and these alleles in RA patients share the sequence motif QK/RRAA in the third hypervariable region of the β chain, from amino acids at positions 70-74. Disease-associated alleles include DRB1*0401, *0404, *0408 alleles, and DRB1* 0101/2 in the Caucasian population. The specific sequence motif forms a pocket in the antigen binding site of the DR molecule. The pocket is large, shallow and accommodates the side chains of the bound peptide. The RA-associated sequence favors presentation of peptides with a net negative charge and thereby influences which peptides may be bound by the pocket, in order to engage in T cell receptor – HLA interactions.

1.3 Pathogenesis of RA: Altered Regulation of Angiogenesis

Neovascularization in RA is an early and critical feature which sustains the developing pannus. Angiogenesis involves the proliferation and migration of endothelial cells, accompanied by tube formation, establishment of a basement membrane, migration of smooth muscle cells and pericytes, along with secretion of matrix metalloproteinases by endothelial cells.

In inflamed rheumatoid synovium, endothelial cells express c-myc and c-fos, indicating activation (5), along with Ki-67, a marker of cell proliferation (6). The endothelium in rheumatoid synovium has an immature phenotype, with increased expression of matrix metalloproteinases, E selectin, and the vitronectin ligand $\alpha v\beta 3$ (7-9). Although there is increased endothelial proliferation in inflamed synovium, there is also increased endothelial cell death, as determined by terminal uridyldeoxynucleotide nick-end labeling (TUNEL), indicating increased vascular regression (9). Thus, there is increased vascular turnover in RA synovium, reflecting a complex interplay of angiogenic and anti-angiogenic factors.

1.3.1 The Balance of Pro-angiogenic and Anti-angiogenic Factors

Factors known to promote or inhibit angiogenesis, relevant to rheumatoid synovium are indicated in Table 1. In essence in RA there is a relative abundance of pro-angiogenic factors and a relative deficiency of factors which suppress angiogenesis.

1.3.1.1 Vascular Endothelial Growth Factor (VEGF)

Of particular importance to angiogenesis in RA is the role of VEGF. VEGF exerts its effects by binding the tyrosine kinase linked cell surface receptors flt-1 (VEGF-R1), and flk-1 (VEGF-R2), which are expressed exclusively on endothelial cells. VEGF levels are increased in synovial fluid from patients with active RA and message can be detected in synovial macrophages (10). The protein is detectable in the microvasculature in the pannus and within synovial macrophages (11). VEGF exerts mitogenic effects on endothelial cells, increases vascular permeability (12), stimulates the secretion of matrix metalloproteinase-1 (MMP-1) (an interstitial collagenase) (13), and the expression of tissue factor (14).

The use of angiogenesis inhibitors in rats with collagen-induced arthritis (CIA) lead to clinical improvement which was accompanied by a fall in serum levels of VEGF (15). Moreover, murine CIA was improved by the use of soluble flt-1, providing further evidence for a role of this important angiogenic mediator in RA (16). Induction of VEGF by the hypoxic conditions within the inflamed joint is well documented (17), and is presumably an attempt at compensatory neovascularization to allow greater nutrient delivery to the growing pannus. However VEGF also facilitates further egress of inflammatory cells into the joint. VEGF may thus provide a link between angiogenesis and inflammation, two processes which are frequently but not invariably codependent.

1.3.1.2 Integrins and angiogenesis in RA

The role of cell adhesion receptors, integrins, in angiogenesis is also established, and in particular, $\alpha v\beta 3$ integrin is recognized as a key player. Normal endothelium expresses very low levels of $\alpha v\beta 3$, which is markedly upregulated in rheumatoid synovial microvasculature (18). The various angiogenic cytokines signal through distinct integrins to mediate their biological effects. Basic FGF signals through $\alpha v\beta 3$ (19), while VEGF signals through $\alpha v\beta 5$

in a protein kinase C (PKC)-dependent manner. Antagonists of αvβ3 administered to rats with adjuvant-induced arthritis reduced disease severity (20), implying a role for this integrin in the disease process.

1.3.1.3 Matrix metalloproteinases

Endothelial cell secretion of matrix metalloproteinases (MMPs) is an important feature of angiogenesis. Normally, the activity of MMPs is regulated by specific tissue inhibitors of metalloproteinases (TIMPs), which bind the MMPs to inhibit their effects. In RA secretion of TIMP-1 by endothelial cells is reduced, thus further favouring the progress of angiogenesis (21).

1.4 Pathogenesis of RA: The role of Cytokines in RA

In studying the pathogenesis of RA, in recent years, the attention has been diverted from the resident and invading cells within the synovium, to the cytokines produced by these cells. Cytokines are proteins which mediate communication between cells and are involved in normal processes such as cell growth, differentiation, and regulation of the immune response. Cytokines act either directly, or indirectly through the induction of other cytokines and effector proteins. Not surprisingly, some cytokines have more influence than others, and cytokine release is highly regulated, as is the expression of their cell surface receptors.

In RA synovial tissue, there is a particular abundance of the pro-inflammatory cytokines TNFα, IL-1, and IL-6 produce by macrophages, fibroblasts, and synovial endothelial cells.

IL-1 stimulates the release of metalloproteinases from chondrocytes and collagenase from synovial cells, thereby promoting tissue damage. IL-1 can activate T cells, stimulate bone resorption, and up-regulates adhesion molecule expression.

IL-17 is a recently described T cell cytokine which is abundant in RA synovium and which appears to have important effects on infiltrating monocytes and cells resident within periarticular tissues, especially when acting in synergy with IL-1 and TNFα (22).

1.4.1 TNFα: Its role in RA

There is now substantial evidence that TNFα is a key player in the pathogenesis of inflammation in RA. TNFα concentration is increased in synovial fluid from patients with active disease(23),(1;24) (1) and plasma levels have been correlated with joint pain (25). Immunohistochemistry has revealed predominant localization within macrophages (26;27). Not only is the expression of TNFα increased in RA, but there is evidence from animal models that it is directly involved in disease pathogenesis. In murine CIA, the elevation of TNFα expression in diseased synovium coincided temporally with disease onset (28-30). Disease is aggravated by exogenous administration of TNFα, and prevented/ attenuated by the use of inhibitors (antibodies to TNFα and soluble receptor constructs). An erosive arthritis with cartilage destruction, bone erosion, synovial hyperplasia, and leukocyte infiltration, not dissimilar to RA has been reported in transgenic mice overexpressing a human TNFα transgene (31). Furthermore, mice without functional TNF receptor 1 (TNF-R1) are relatively resistant to CIA, and if they develop disease, arthritis is mild (32).

TNFα directly, and by promoting the release of other proinflammatory cytokines, stimulates bone resorption (33;34), inhibits proteoglycan synthesis in cartilage (34), and stimulates collagenase production by synovial cells (35).

IL-1 and TNFα share many biological functions, although it has been proposed that TNFα may be more critically involved in propagating inflammation, while IL-1 may be more relevant in initiating cartilage destruction (36). The importance of TNFα in RA is underlined by the therapeutic success of TNFα blockers (chimeric and humanized antibodies to TNFα (37)) and etanercept, a recombinant human TNF-RII-Fc fusion protein (38).

1.4.2 TNFα: synthesis/ production

TNFα is a member of a family of peptide mediators comprising at least nineteen cytokines including TNFβ (lymphotoxin), Fas-ligand, nerve growth factor and CD40 ligand. Members of the TNF superfamily, with the exception of lymphotoxin, are homotrimeric proteins which exist primarily in a membrane-associated form (39). A variety of immune cells (B and T lymphocytes, basophils, eosinophils, monocytes, macrophages, NK cells and dendritic cells) and non-immune cells (smooth muscle cells, glial cells, osteoblasts, neurons) may produce TNFα, as can various tumour cells (39). However tissue macrophages are principally responsible for TNFα production at sites of inflammation. Macrophage production of TNFα is stimulated by ischaemia, trauma, cytokines (IL-1, IL-2, IFNγ, GMCSF, M-CSF, and TNFα itself), activated complement components, and microbial products.

Human TNFα is initially displayed on the cell membrane as pro-TNFα, a 233 amino acid, 26 kDa proprotein which lacks a classic signal peptide (40). Membrane-associated TNFα exerts both cytotoxic and inflammatory effects by cell-cell contact (41). TNFα converting enzyme (TACE), a matrix metalloproteinase cleaves pro-TNFα in the extracellular domain to release the 157 amino acid, 17.3 kDa monomeric form. The secreted protein homotrimerizes. The N-terminus is not involved in trimeric interactions, and is trivial in terms of the biological activities of TNFα.

1.4.3 Regulation of TNFα

Homeostatic control of protective immunity and inflammation demands that cytokines with potent effects, such as TNFα, be intricately regulated and indeed, synthesis of TNFα is closely regulated at transcriptional and post-transcriptional levels. Under normal conditions, the TNFα gene is silenced and hence production is minimal in quiescent cells. When activated by any of the aforementioned stimuli, macrophages are capable of producing substantial amounts of TNFα, and message levels rise promptly without requirement for de novo protein synthesis.

TNFα mRNA is unstable by virtue of a series of AUAU sequences (common to mRNA of inflammatory cytokines) which render it susceptible to degradation by cytosolic RNases, once the inciting stimulus is abolished (42-44). The message thus has a short half-life once transcription is interrupted.

Compensatory mechanisms exist to provide negative feedback for TNFα production. Corticosteroids, and prostaglandin E2 (PGE2) induced by TNFα, transcriptionally down-regulate TNFα expression (45;46). IL-10, induced by TNFα is an anti-inflammatory cytokine which similarly provides negative feedback for TNFα production (47).

1.4.4 TNFα: biological effects relevant to RA

1.4.4.1 The role of TNFα in immunity

TNFα (like IL-1) plays a central role in innate (antigen independent) immunity which is central to the body's defence against microbial invasion. Effects are mediated both directly

and via induction of other proinflammatory cytokines including IL-1, 2, 4, 6, 10, 12, 18, IFNγ, and TGFβ (48;49).

TNFα also plays a role in antigen-specific immunity (acquired immune response) through its modulation of the Th1 immune response. By inducing IL-12 and IL-18, it augments IFNγ production. This increases activation of CD4+ T cells and amplifies the Th1 response, which in turn, stimulates TNFα production by macrophages.

1.4.4.2 Effects of TNFα on the vascular endothelium

(i) Inflammation

The effects of TNFα on the vasculature are critical to its role in RA pathogenesis. The mechanisms whereby circulating leukocytes leave the intravascular space and accumulate at sites of inflammation has been a subject of much interest. It is noteworthy that the normal quiescent endothelium does not bind leukocytes, and it is only the activated endothelium, (in response to cytokines such as TNFα), which may bind leukocytes. The activated endothelium expresses adhesion molecules, and the selectin family of adhesion molecules plays an important role in initial engagement of leukocytes by the endothelium, while firm adhesion is mediated by adhesion molecules of the immunoglobulin superfamily (ICAM-1 and VCAM-1) and the integrins to which they bind (Fig 1.1). Transmigration of the leukocyte between the endothelial cells is coordinated by both ICAM-1 and the junctional molecule PECAM-1, and the leukocyte then moves by chemotaxis towards an increasing gradient of chemoattractant.

TNFα up-regulates the expression of endothelial adhesion molecules including E Selectin (CD62E), intercellular adhesion molecule (ICAM-1) (CD54), and vascular cell adhesion

molecule VCAM-1 (CD106) (50). It also causes sulfation of CD44, another adhesion receptor, thus increasing its binding affinity (51). These adhesion molecules facilitate leukocyte transmigration to extravascular sites of inflammation (52).

(ii) Other effects upon the endothelium

TNFα confers upon the endothelium a procoagulant phenotype, by enhancing production of tissue factor, and downregulating thrombomodulin. It stimulates angiogenesis, a characteristic feature of rheumatoid synovium. It increases vascular permeability, inhibits endothelial cell proliferation and enhances production of prostacyclin by endothelial cells. In vascular endothelial cells, TNFα stimulates the production of IL-1, IL-3, GCSF, and GMCSF and induces nitric oxide synthase, all of which contribute to inflammatory reactions.

(iii) Effects on other cell types

TNFα stimulates the release of MMPs from neutrophils, fibroblasts, and chondrocytes (53-55). In polymorphonuclear leukocytes, it enhances adherence to extracellular matrix and stimulates production of superoxide. In lymphocytes, TNFα activates the invasiveness of cytotoxic T cells, and induces apoptosis in mature T cells. It induces synthesis of reactive oxygen species in B cells and by inducing cyclooxygenase 2, it increases the production of prostaglandins.

1.4.5 TNFα receptors

One of the strategies to inhibit TNFα is to block its interaction with its receptor and the challenge has been to identify the TNFα receptors and to elucidate the downstream signalling

pathways they influence. Indeed, TNF receptor expression is up-regulated in actively inflamed synovium. In both the serum and synovial fluid of patients with RA, there are increased levels of soluble forms of TNF receptor (56) and the concentration of the shed receptor correlates with disease activity (57-59). The precise role of these endogenous soluble receptors remains elusive (60), but they may reflect an attempt to sequester excess TNFα. In RA this adaptive mechanism may be insufficient to overcome the over-abundance of TNFα, thereby allowing the inflammatory state to persist.

1.4.5.1 TNF receptor I and II

There are two cell surface receptors for TNFα: TNF-RI, a 55 kDa protein (p55), and TNF-RII (p75), a 75 kDa protein (61;62). Both are transmembrane glycoproteins and are expressed by all cell types except erythrocytes. Endothelial cells preferentially express TNF-RII. TNF-RI has a more widespread distribution, and is constitutively expressed, in contrast to TNF-RII which is inducible.

Proteolytic cleavage by MMPs releases truncated soluble forms of the receptor, comprising the extracellular ligand-binding domain (33;63). Binding of TNFα to its receptor in fact stimulates receptor cleavage and the release of the shed receptor. Thus in inflammatory states, these soluble truncated receptors may serve as an adaptive mechanism to modulate the biological effects of TNFα (64;65). The concentration of shed receptors is elevated in serum and synovial fluid in RA (56;66-68), and they have been identified in synovial tissue, and at the cartilage- pannus junction (69;70). Eventually the shed receptors are excreted by the kidney (71-73).

TNFα binds both receptors with high affinity (74), however the kinetics of release from the two receptors differs. Binding to TNF-RI is almost irreversible, in contrast to the rapid dissociation from TNF-RII. It is TNF-RI rather than TNF-RII which mediates the inflammatory responses to soluble TNFα(75), and it has been proposed that TNF-RII may function as a ligand passer, passing trimeric soluble TNFα at low concentrations to TNF-RI (76).

1.4.5.2 TNF receptor signalling

The extracellular domains of both TNFα receptors have four cysteine-rich regions which are conserved between the two receptors. The intracellular domains are devoid of enzymatic activity, and differ in structure, consistent with distinct downstream signalling pathways. The intracellular domain of TNF-RI is marked by a "death domain" (DD) at its carboxy terminus, and this region is involved in TNF-induced apoptosis, antiviral activity, and induction of nitric oxide synthase (77-79).

Within each TNFα trimer are three potential sites for interaction with the receptor (80). Receptor- ligand binding causes trimerization of the receptor, and the recruitment of a number of downstream signalling molecules. In the case of TNF-RI, the intracellular death domain recruits adaptor proteins containing cognate death domains. One such protein, TNF-receptor-associated death domain (TRADD), a 34 kDa adaptor protein, interacts with TNF-RI via its C-terminal death domain. TRADD in turn, when activated, recruits Fas-associated death domain protein (FADD), and RIP (receptor interacting protein) which lead to activation of caspases and apoptosis as shown in Fig 1.2 (81;82).

Alternatively, TRADD may signal through a distinct pathway, bypassing the route to apoptosis, and leading to inflammation (Fig 1.2). TNF-receptor- associated factors (TRAFs), are a family of proteins involved in signal transduction with six identified members. TRADD may bind either TRAFI or TRAF2, and binding to TRAF2 initiates activation of the transcription factor NFκB with subsequent induction of inflammatory proteins (83;84). Signal transduction molecules downstream of TRADD thus determine whether TNF-RI binding leads to apoptosis or an inflammatory state.

Although TNF-RII does not have a TRADD/FADD binding region, it may also mediate apoptosis when bound by agonist (85;86). The induction of TRAF2 is thought responsible for this effect (Fig1.2) (86). TNF-RII may also interact with TRAF2 to activate NFκB and lead to inflammation.

1.4.6 TRAFs

TRAFs are a family of six adaptor proteins, of which TRAF2 is the prototype. All six identified TRAFs are characterized by a RING finger and a zinc finger motif located at the N terminus (87). The TRAF domain allows for self association and interaction with other signalling proteins and is located in the C-terminal region (88;89). TRAF1 and TRAF5 are found in spleen, thymus, and lungs, while TRAF4 is found in breast cancer cells. There is ubiquitous expression of TRAF2, TRAF3, and TRAF6.

TRAF2 may interact with both TNF receptors, either directly with TNF-RII or indirectly with TNF-RI, via TRADD (Fig 1.2) (84). TRAF2 interacts with various downstream signalling molecules including RIP, NIK, and subsequently activates NFκB (83). An alternative signalling pathway downstream of TRAF2 exists, causing activation of JNK, and there is

evidence supporting a role for apoptosis-signal-regulating kinase 1 (ASK1), a MAPK kinase kinase in mediating TRAF2-induced JNK activation (90). TRAF2 thus lies at the bifurcation of signal transduction pathways to activate either NFκB or JNK (Fig 1.2). Although not absolutely essential for TNF-induced NFκB activation, TRAF2 is obligatory in TNF-induced JNK activation (91;92).

The mechanisms involved in regulating the distinct biological responses elicited by TRAF2 signalling have been unclear. Recent work however, implicates a role for sphingosine kinase (SK), a lipid kinase, in signal transduction by TRAF2, to specifically and preferentially activate NFκB with a subsequent anti-apoptosis and pro-inflammatory effect (93).

1.5. Sphingolipid metabolism

The concept of sphingolipids as inert structures within cell membranes has been challenged by evidence supporting a role for sphingolipid metabolites as second messengers mediating diverse cellular functions including proliferation, migration and survival.

Of particular interest to the biomedical research field is the sphingomyelin (SM) pathway (Fig 1.3). As shown, TNFα activates sphingomyelinase (SMase) which catalyzes the hydrolysis of SM to ceramide, a pro-apoptotic lipid second messenger (94;95). Ceramide may be metabolized by ceramidase to form sphingosine (Sph). Sphingosine kinase (SK) then phosphorylates Sph to generate sphingosine-1-phosphate (S1P) (96). TNFα is a potent inducer of SK activity, independent of activation of SMase, and the product, S1P, antagonizes TNFα-induced apoptosis (97). Thus preferential formation of ceramide or S1P, intermediates within a single metabolic pathway, has opposing biological effects, leading to either cell death, or cell survival respectively. Differential activation of the enzymes responsible for

these products, by specific stimuli thus determines the fate of cells – activation of SMase by cytokines, versus growth factor activation of SK (98).

1.6. Sphingosine Kinase

1.6.1 Enzymatic properties

Sphingosine kinase catalyzes the phosphorylation of the 1-hydroxy group of Sph to generate bioactive S1P. This reaction is the rate-limiting step in the sphingomyelin pathway and is dependent on adenosine triphosphate (ATP) and magnesium. SK activity has been found in a variety of cells/ tissues including bovine brain (99) and kidney (100), rat liver (101), and human and porcine platelets (102). Rat SK has been purified (103), and the enzyme from mouse and yeast has been cloned (104;105)

Only recently has human SK been purified and cloned from placenta (106). The active purified enzyme was expressed in HEK293T cells and *Escherichia coli,* and subsequently, the purified placental enzyme was characterized along with *E Coli*-derived recombinant SK. In terms of substrate specificity and kinetics, and catalytic properties, the recombinant enzyme was virtually identical to native SK, suggesting that SK is a constitutively active enzyme which does not rely on eukaryotic post-translational modifications for its activity (106).

Constitutively active SK may serve a house-keeping function, to keep cellular levels of Sph and ceramide in check. SK may however assume a role in cell signalling, when activated by agonist, by catalyzing the formation of S1P.

Sequence analysis of purified human SK reveals it shares amino acid sequence similarity with two murine SK sequences (104) and with two Saccharomyces cerevisiae SKs (105). There are five blocks of highly conserved regions towards the N terminus, and three putative calcium/ calmodulin binding sites. Amplification of human SK to identify signalling domains demonstrated similar amino acid sequences in residues 16-153 with the putative diacylglycerol kinase catalytic domain. There was however very little similarity to the ATP binding site of the catalytic domain of diacylglycerol kinase (106). The purification process using anion exchange chromatography revealed two peaks of SK activity from human placenta, suggesting the presence of two isoforms of the enzyme, SK1 and SK2. Similarly in human platelets, the majority (72%) of SK activity is associated with the membrane fraction. The cytoplasmic and membrane-associated enzymes in human platelets differ in their response to detergents and SK inhibitors, again suggesting the existence of several enzymatic forms (107). Human umbilical vein endothelial cells (HUVEC) in contrast have a single enzymatic isoform of SK, which elutes at the same point as human placental SK1 on anion exchange chromatography. No other latent isoforms were revealed upon TNFα treatment of HUVEC, rather, SK1 activity is stimulated (106).

1.6.2 Sphingosine kinase 2

1.6.2.1 Tissue distribution and enzymatic properties

A second type of mammalian SK, SK2 has been cloned and characterized (108). SK2 is a larger protein than SK1 with an additional 236 amino acids. Its tissue distribution/ expression are distinct, with abundant expression in brain, heart, kidney, testis and liver. Histochemical analysis of SK1 in human tissues has revealed its expression in white matter in the cerebrum and cerebellum, red nucleus, midbrain and kidney, platelets and in the vascular endothelium

(109). The temporal pattern of expression of the SK1 and SK2 in murine development differs. The two enzymes differ in terms of substrate specificities; the naturally occurring D erythrosphingosine isomer is the preferred substrate for SK1, but D-erythro dihydrosphingosine is a better substrate for SK2.

1.6.2.2 Sphingosine kinase 2: a role in apoptosis

In light of the above differences in tissue distribution, kinetic properties, and substrate specificities, it is conceivable that SK2 may have a unique role, distinct from SK1. However the physiological role of SK2 has not been addressed until recently. A recent investigation into the role of SK2 indicates that SK2 enhances apoptosis and inhibits cellular proliferation, despite enhancing the production of S1P(110).

The same study addressed the mechanism underlying the pro-apoptotic effects of SK2, revealing that this was explained by the presence of a critical sequence of nine amino acids which resembled a sequence seen in Bcl-2 homology 3 (BH3)-only proteins(110). The BH3-only proteins are a subfamily of the Bcl-2 group of proteins, which by virtue of this critical amino acid sequence, interact with the pro-survival Bcl-2 proteins, resulting in apoptosis(111). That it was the BH3 domain present at the N-terminal of the protein which is responsible for apoptotic effects was confirmed by enforced expression of truncated forms of SK2 in NIH3T3 fibroblasts and PC12 cells. Expression of the N-terminal fragment, containing the BH3 domain, but not the C-terminal fragment, induced apoptosis in these cell types(110).

At present any other biological effects of SK2 remain unknown, despite its ubiquitous expression.

In this thesis, SK refers to SK1, unless otherwise specified.

1.6.3 Activation of SK

Although constitutively active, sphingsoine kinase may be further activated by a number of agonists including PDGF (112), phorbol esters (98), serum (113), nerve growth factor (114;115), activation of PKC (116;117), and cross linking of FcεR1 and FcγR1 (118). Importantly, TNFα can activate SK in HUVEC independently of its activation of SMase (97). TNFα treatment of HUVEC results in a prompt but transient rise in SK activity, peaking within ten minutes.

Until recently, the enzymatic alterations which lead to activation of SK in response to agonist binding have not been addressed. The critical role of TRAF2 in the activation of SK in response to TNFα has been established (93), however the mechanisms underlying this had not been elucidated. In an attempt to elucidate the mechanism of catalysis of SK, in our laboratory, the substrate-binding sites of SK have been examined. By using site-directed mutagenesis and affinity labeling with the ATP analogue FSBA, the role of Gly82, and Lys103 in nucleotide binding by SK have been established (119). More recently in our laboratory, it has been shown that activation of SK is mediated by phosphorylation of SK at Ser225, and that this is mediated by the ERK1/2 kinases (120). Furthermore, it was shown that this phosphorylation leads to translocation of the activated enzyme from the cytosol to the plasma membrane. The structural changes consequent upon phosphorylation of SK leading to enhanced catalytic activity remain unknown, in the absence of a defined crystal structure for the enzyme to date.

Activation of SK by TNFα is paralleled by an increase in S1P, and resistance to TNFα-induced apoptosis. In a spontaneously transformed endothelial line (C11), SK may be activated by PMA but not by TNFα. This cell line is susceptible to TNFα-induced apoptosis, but is protected by the addition of exogenous S1P or stimulation of SK by PMA. Normal HUVEC are rendered sensitive to TNFα mediated apoptosis when treated with N,N,dimethylsphingosine (DMS), a competitive inhibitor of SK. TNFα thus activates SK, an obligatory enzyme in protecting the cell from its own apoptotic effects (97). Over-expression of SK1 in rat phaeochromocytoma PC12 cells (accompanied by increased intracellular S1P and kinase activity) provided resistance to apoptosis induced by withdrawal of trophic factors or ceramide. This was accompanied by suppression of stress-activated protein kinase c-Jun N-terminal kinase (SAPK/JNK), and inhibition of caspase 2,3, and 7 activation. Unlike NGF induced survival, SK over-expression did not influence ERK1/2 or Akt (121).

1.6.4 Role of SK in mediating signalling downstream from TNFα

1.6.4.1 Adhesion molecules

Of particular relevance to the pathogenesis of a chronic inflammatory disease such as RA, is the up-regulation of adhesion molecules (E Selectin, VCAM-1, ICAM-1) on vascular endothelium, which facilitates egress of leukocytes from the intravascular space to the site of inflammation. A critical study, investigating the mechanisms underlying these effects of TNFα in HUVEC has demonstrated convincingly a role for SK in mediating this effect (122). S1P, the product of SK activity, was shown to stimulate VCAM-1 and E Selectin expression in a dose-dependent manner, with prompt increase in mRNA levels in HUVEC. The effect was specific to S1P; sphingosine or ceramide had no effect. N'N'-dimethylsphingosine

(DMS), a competetive inhibitor of SK, reduced adhesion protein and message levels (50-70%), implying a dominant role of SK in mediating the TNFα action. Addition of exogenous S1P reversed the effects of DMS, and moreover, DMS had no effect on S1P-induced adhesion molecule expression. This establishes SK as an important enzyme in mediating the adhesion protein expression induced by TNFα. Evidence that high density lipoprotein (HDL) inhibits TNFα-induced SK activation in HUVEC with concomitant decrease in S1P production and adhesion molecule expression (123) provides further strength to these data.

1.6.4.2 ERK activation

TNFα and S1P can both activate extracellular signal related kinase (ERK), a member of the MAPK family, which may lead to endothelial activation. SK has been shown to mediate this effect of TNFα (122).

1.6.4.3 NFκB

Adhesion protein gene transcription stimulated by TNFα is dependent on NFκB (124), and a role for SK in mediating TNFα-induced NFkB activation has been established by the use of competitive inhibitors which specifically prevented NFκB activation, but spared other transcription factors (122). NFκB activation by SK is dependent on TRAF2 (93). TRAF2 binding depends on the presence of a consensus motif (P/S/T/A)X(Q/E)E present on TRAF2 binding receptors including TNF-RII and CD40 (125;126). SK has recently been demonstrated to contain two TRAF2 binding motifs at positions 240-243 (PLEE) and 379-382 (PPEE) (93).

1.6.4.4 Interaction with TRAF2

Over-expression of SK (in HEK293 cells) activated NFκB and potentiated TRAF2- induced NFkB activation. The use of dominant negative SK (a catalytically inactive SK containing the substitution glycine to aspartate at position 82) (127) completely abolished TNFα and/ or TRAF2-induced NFκB activation (93). Thus the interaction of TRAF2 and SK activates SK which subsequently mediates TRAF2-induced NFκB activation. The alternative signalling pathway downstream of TRAF2, leading to JNK activation, was SK independent. NFκB protects against apoptosis by the production of a family of anti-apoptotic proteins, the inhibitors of apoptosis proteins (128), however it is not an obligatory factor in TRAF2 mediated anti-apoptosis (91;92). The activation of SK by its interaction with TRAF2 mechanistically defines an anti-apoptotic pathway independent of NFκB (93).

1.6.5 An oncogenic role for SK

Over-expression of SK (in NIH3T3 fibroblasts) can confer upon the cells a transformed phenotype, with an escape from contact inhibition and serum dependence. This is accompanied by clustering (focus formation) by cells transfected with SK, and the ability to form tumours in NOD/SCID mice (129). SK thus has an oncogenic role in its own right, while in addition, it is essential in Ras-mediated transformation (129). This is in accordance with the documented anti-apoptotic role of SK and S1P (130-132). Furthermore, SK inhibits DEVDase and caspase-3 activation, again in keeping with the above findings (130;131).

1.7 Sphingosine-1-phosphate

S1P, a bioactive polar sphingolipid metabolite, is generated by the phosphorylation of sphingosine by SK. S1P is either cleaved at the C2-C3 bond by S1P lyase to produce ethanolamine phosphate and fatty aldehyde (133;134), or is dephosphorylated by S1P phosphatase. S1P lyase is absent in platelets but is otherwise ubiquitously expressed in cell membranes within mammalian cells.

S1P influences diverse biological processes such as cell proliferation, survival, migration, calcium mobilization and differentiation (135). Whether these effects are mediated intracellularly or via cell surface receptors has been the subject of much controversy, but there is now evidence to support a role for both intracellular and extracellular actions.

1.7.1 Extracellular effects of S1P

1.7.1.1 EDG receptors

S1P is a ligand for EDG-1 (endothelial differentiation gene 1), a member of a growing family of G-protein-coupled receptors (GPCR) including EDG1,3,5,6,8 (131;136). EDG-1 is a 380 amino acid protein with high affinity for S1P which signals via Gi/Go (137-139) to mediate downstream effects. EDG-3,5,6,8 all bind S1P with high affinity and specificity, although less is known about these.

EDG-1 is abundantly expressed by HUVEC (EDG-3 to a lesser degree) and may be induced by PMA. When bound by S1P, it induces cell migration (140), a process critical for wound healing, tumour metastasis, and inflammation. Binding of S1P to EDG-1 inhibits cyclic AMP formation, and activates ERK-2 (136) (Fig 1.4). ERK activation by S1P shows variable sensitivity to pertussis toxin (141;142). Calcium mobilization by S1P has also been linked to EDG-1 receptor binding (138).

EDG receptors have subsequently been renamed S1P receptors, for example S1P1, S1P2, however in this thesis to avoid confusion with S1P, they have been referred to as EDG receptors.

1.7.1.2 EDG receptors and angiogenesis

In endothelial cells S1P functions as an angiogenic regulator by activating EDG-1 and 3 (143), as shown in Fig 1.4. Addition of exogenous S1P (but not intracellular microinjection) increased actin stress fibres and cortical actin structures, by signalling through Rac and Rho small GTPases, and independent of the Gi inhibitory pathway. Exogenous S1P induced the formation of adherens junctions, by translocation of VE-cadherin and catenin (α,β,γ) molecules to discontinuous structures at cell-cell junctions, an effect again dependent on S1P-induced Rho and Rac activation. The use of antisense oligonucleotides to specifically block EDG1 and 3 expression inhibited S1P-induced VE-cadherin accumulation at cell-cell junctions. Antisense EDG-1 inhibited Rac-dependent cortical actin structure assembly, whereas antisense EDG-3 specifically attenuated Rho-dependent stress fibre formation. EDG-1 thus signals through the Rac pathway, and EDG-3 through the Rho pathway, and both are critical for S1P-induced adherens junctions and endothelial cell morphogenesis. The EDG-1/Gi pathway was shown to activate ERK and thereby mediate endothelial cell survival. Cell-cell junction assembly, survival, and morphogenesis are thus under the dynamic control of S1P, implicating this lipid as an important modulator of angiogenesis.

1.7.1.3 S1P has synergistic effects with FGF to induce angiogenesis

S1P is known to cooperate with VEGF and FGF to induce angiogenesis (143;144). There is evidence that SK in endothelial cells is active constitutively and exported to the extracellular space where it generates S1P, in quantities sufficient to achieve receptor stimulation (145). Cells over-expressing SK1 had heightened release of the enzyme, and potentiated FGF-2 driven angiogenesis. These effects were attenuated by antisense EDG-1 and EDG-3 oligonucleotides (145). Dysregulated angiogenesis in rheumatoid synovium (and other pathological conditions) raises the possibility of inappropriate activity of S1P-EDG-1/3 pathways.

1.7.1.4 S1P and platelets

Platelets have persistently active SK under resting conditions and lack S1P lyase activity (146;147), and consequently have high intracellular levels of S1P in comparison with other cell types. S1P treatment of platelets induces changes in platelet shape and mobilizes intracellular calcium stores (148;149). S1P activated platelets; sphingosine did not despite being promptly converted to S1P by SK, suggesting S1P activates platelets from the exterior of the cell.

The S1P receptor on platelets is shared by lysophosphatidic acid (LPA) (150), a bioactive lipid structurally similar to S1P. LPA can itself induce platelet aggregation (151) and is released from activated platelets (152). Agonist activation of platelets through PKC stimulated the release of S1P, however platelet agonists which do not signal through PKC did not induce S1P release. Activation of PKC in platelets thus appears to be involved in S1P release (149).

1.7.1.5 S1P activation of non-receptor tyrosine kinases

S1P can activate various non-receptor tyrosine kinases to mediate biological effects. Activation of Crk stimulates mitogenesis (153), whereas phosphorylation of p125 FAK (focal adhesion kinase) causes cytoskeletal changes and stimulates the formation of focal adhesions (131;154). EDG-5 and Rho are thought to be involved in S1P-stimulated p125 FAK (131) (154) phosphorylation. S1P activates Syk, in a PKC dependent fashion to signal downstream to Ras and PI3 kinase (155). S1P also stimulates gene transcription of the AP-1 transcription factors c-Jun and c-Fos (156), and EDG-3 and EDG-5 have been shown to be responsible for these effects (157).

1.7.2 Intracellular effects of S1P

Agonist stimulation of SK, over-expression of SK in transfected cells, the use of SK inhibitors (DMS) and, more recently, the generation of a dominant negative SK (127) (which lacks kinase activity and is resistant to activation by agonists) provide evidence for an intracellular role of S1P as a second messenger. The precise intracellular targets of S1P remain elusive at present. As shown in Fig 1.4, intracellularly, S1P has been shown to mobilize calcium from internal stores independently of inositol triphosphate (96;158), stimulate FAK phosphorylation (96;158), and activate phospholipase D (159). In EDG-1 transfected HEK293 cells stimulated with exogenous S1P, S1P mobilized intracellular calcium, and activated phospholipase D independently of EDG-1 expression (131). Calcium mobilization and activation of phopholipase D are critical in cellular proliferation. The same study further revealed that the anti-apoptotic and mitogenic effects of S1P were related to intracellular uptake of S1P and independent of EDG-1 expression.

DMS treatment to block the formation of endogenous S1P, inhibited cellular proliferation driven by PDGF and fetal calf serum, but did not influence S1P receptor binding or receptor mediated reduction in cAMP levels (112). S1P-induced p125 FAK phosphorylation was not influenced by suramin, an inhibitor of receptor - ligand interactions, indicating that the mitogenic effects of S1P are not mediated by receptor binding (154).

S1P is thus a unique lipid mediator which may function both as an intracellular second messenger, and as a ligand for cell surface receptors.

1.8 AIMS

The important role of TNFα in RA, and the above evidence for sphingosine kinase in mediating many of the biological actions of TNFα, suggest a role for this enzyme in the pathogenesis of RA. This then justifies a systematic enquiry into the role of sphingosine kinase in mediating angiogenesis and inflammation in RA. It will be the intention here to conduct such an investigation.

The specific aims of this project are:

1. To establish a system for over-expressing SK in endothelial cells.
2. To determine the phenotypic consequences of raised intracellular levels/ activity of sphinsogine kinase in endothelial cells, with enquiry specifically directed to in vitro measures of angiogenesis and inflammation.
3. To define intracellular signalling pathways activated by raised intracellular SK activity.

4. To compare and/or contrast the effects of mild-moderate increases in intracellular SK activity in endothelial cells, to those effects seen when enzymatic activity is markedly raised.

1.9 Hypothesis

It is hypothesized that raised intracellular SK activity in endothelial cells will result in significant changes to the phenotype of these cells:

1. Endothelial cells over-expressing SK will show greater inflammatory potential. Specifically, over-expression of SK will result in increased expression of inflammatory proteins such as VCAM-1 and E Selectin. It is hypothesized that over-expression of SK will augment the normal endothelial response to inflammatory cytokines such as TNFα (that of induction of adhesion molecule expression). It is further hypothesized that any such alterations in adhesion molecule expression seen in cells over-expressing SK will result in functional alterations in the ability of the endothelium to bind leukocytes.
2. Over-expression of SK in endothelial cells will enhance the ability of the endothelium to undergo angiogenesis. Specifically, it is hypothesized that over-expression of SK will enhance cell proliferation, cell survival, confer resistance to apoptosis, enhance cell migration, and stimulate cellular remodeling.
3. Over-expression of SK will result in changes to cellular pathways involved in the regulation of cell proliferation, survival, apoptosis, and migration.

Pro-angiogenic factors	Anti-angiogenic factors
VEGF (10;11)	Thrombospondin (12)
bFGF (13;14)	TIMP-1 (15)
IL-1 (16)	TIMP-2 (15)
IL-8 (17)	IFNγ (18;19)
PDGF (20)	
hypoxia	

Table 1 shows the factors relevant to RA which act to promote angiogenesis (Pro-angiogenic factors) and those which act to suppress angiogenesis (anti-angiogenic factors). VEGF: vascular endothelial growth factor, bFGF: basic fibroblast growth factor, IL: interleukin, PDGF: platelet derived growth factor, TIMP: tissue inhibitors of metalloproteinase, IFN: interferon.

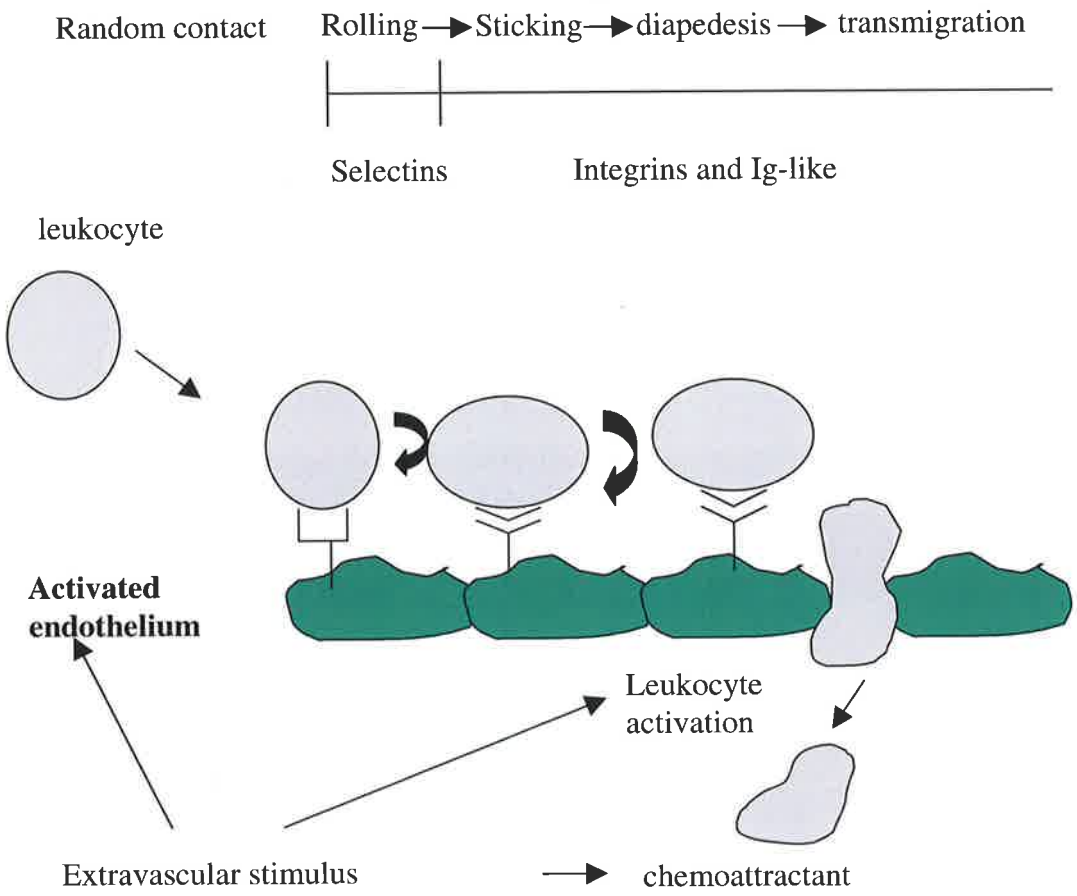


Fig 1.1 shows a schematic representation of the mechanisms whereby circulating leukocytes adhere to the activated endothelium. The initial weak adhesion is mediated by the selectins, and firm adhesion is mediated by the immunoglobulin superfamily. Transmigration and chemotaxis ensue.

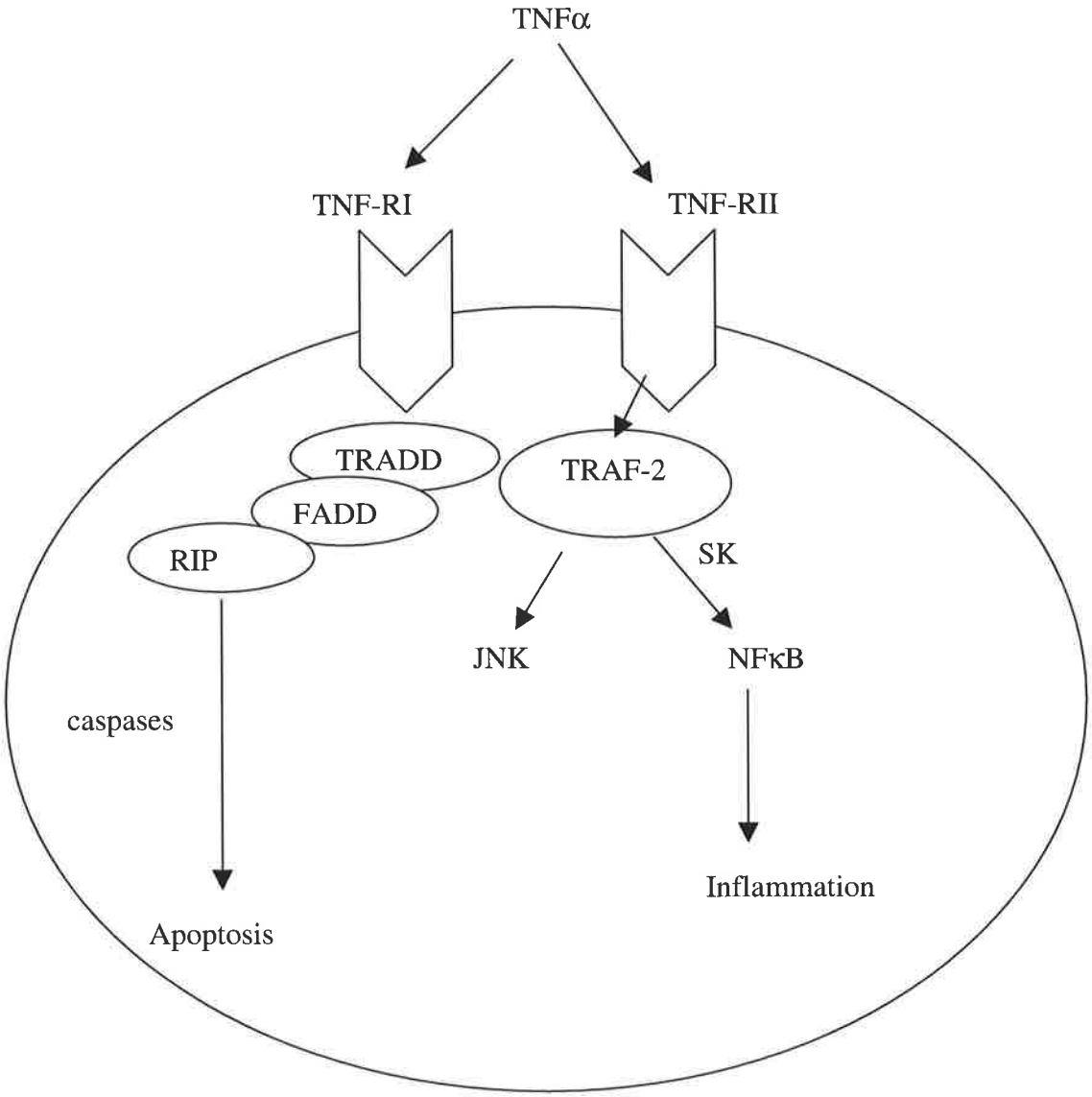


Fig 1.2 shows a schematic representation of the downstream signalling pathways resulting from interaction of TNFα with either of its receptors (TNF-RI or TNF-RII). TRAF2 thus lies at the bifurcation of pathways which may lead to inflammation or apoptosis. (TRADD= TNF receptor associated death domain, RIP = receptor interacting protein, FADD= Fas associated death domain protein, SK= sphingosine kinase).

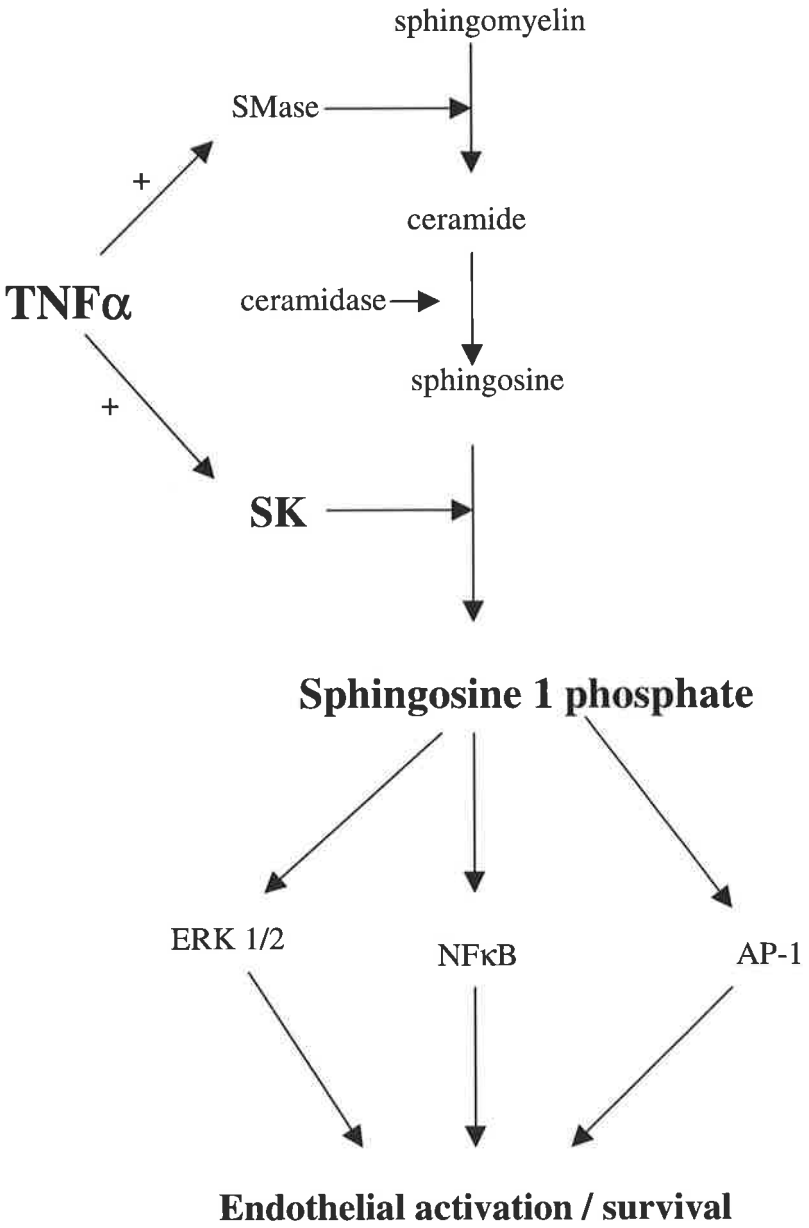


Fig 1.3 shows the sphingomyelin pathway. SMase: sphingomyelinase, SK: sphingosine kinase. TNFα: tumour necrosis factor α, NFκB: nuclear factor κB, ERK: extracellular signal related kinase, AP-1: AP-1 transcription factors, +: activation

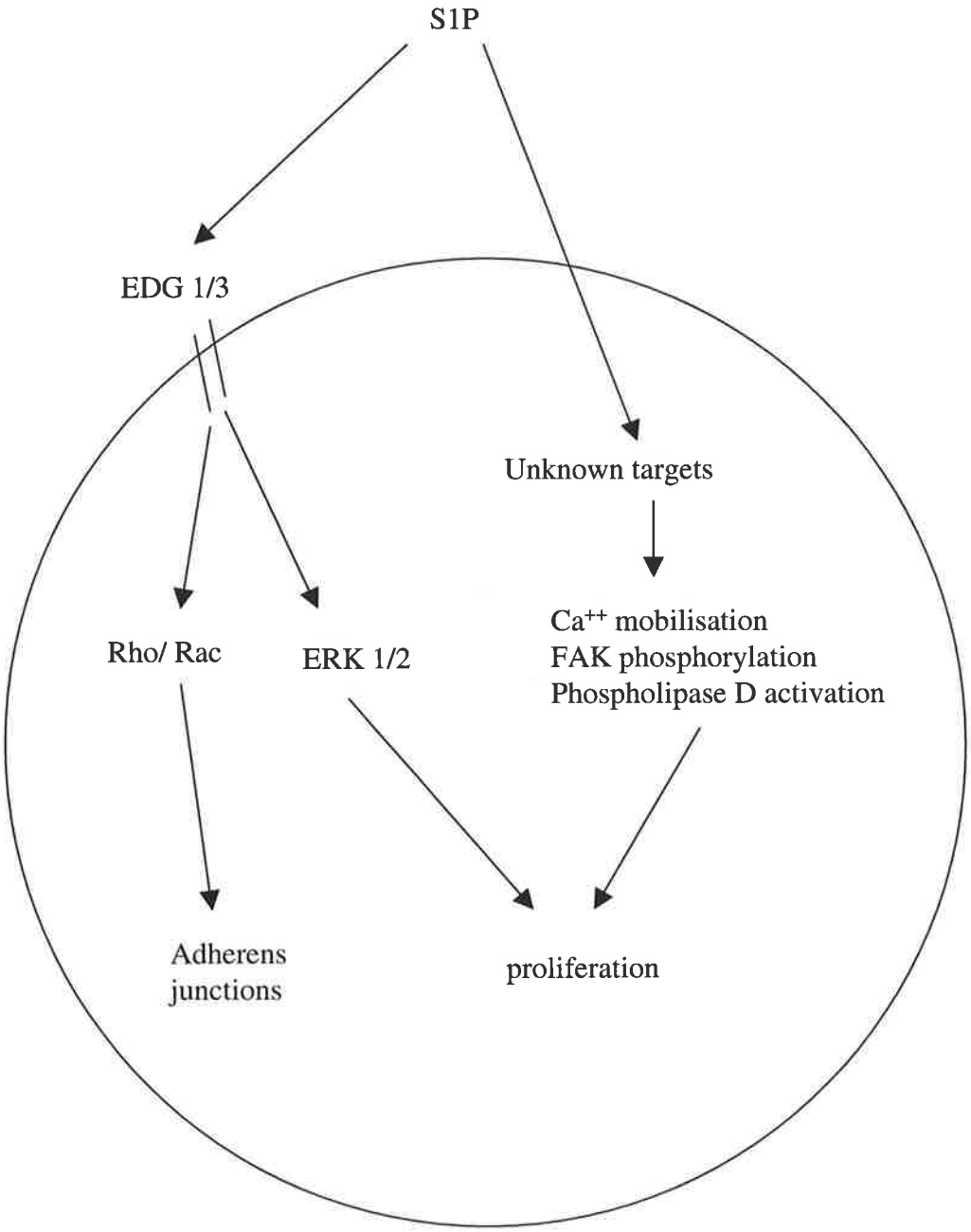


Fig 1.4 shows the action of S1P in endothelial cells. Binding of S1P to EDG receptors to activate the Rho/ Rac GTPases and ERK 1 /2 is shown, along with the intracellular actions on undefined targets to enhance cell proliferation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Culture media- Reagents

2.1.1 From JRH Biosciences

Medium 199 (199/EBSS) with Earle/s Balanced Salts + 0.68mM L-Glutamine

RPMI 1640 Medium +2.05mM L-Glutamine

DMEM (Dulbecco's Modified Eagle/s Medium)

HEPES Buffer solution 1M

L-Glutamine Solution 200mM in saline

Sodium bicarbonate solution (endotoxin free) 7.5%

MEM Nonessential amino acids solution (100x)

Sodium pyruvate 100mM

Trypsin (0.05% trypsin, 0.02% EDTA)

2.1.2 Growth supplements/ antibiotics

Penicillin G 5000U/ml, Streptomycin sulphate 5000μg/ml (Commonwealth Serum Laboratories, Victoria, Australia)

G418 sulfate (Promega, WI, USA)

Heparin (Sigma, St Louis, Missouri, USA)

Endothelial cell growth supplement (GIBCO BRL, Life Technologies)

Recombinant human VEGF (PeproTech Inc, USA)

Recombinant human FGF (R&D Systems)

Fetal bovine serum (FCS) Multi Ser™, (Thermo Trace, Melbourne, Australia)

2.1.3 Additional reagents

Fibronectin (Boehringer Mannheim) used at 50μg/ml diluted in PBS.

Gelatin (Sigma, St Louis, Missouri, USA)

EDTA (Univar, Asia Pacific Specialty Chemicals LTD, Sydney, Australia)

Matrigel® (Beckton Dickinson, MA, USA)

2.1.4 Solutions for cell culture

(i) HUVE Medium: M199 supplemented with 20% FCS, 20mM HEPES, 0.225% sodium bicarbonate, 2mM L-glutamine, 1% non-essential amino acids, 1mM sodium pyruvate, 1.2% penicillin/gentamicin.

(ii) HUVE Wash: medium199, 2% FCS, 10mM Hepes, 0.225% sodium bicarbonate, 2mM L-glutamine, 1% penicillin/gentamicin.

(iii) Complete DMEM: DMEM with 50 U/ml penicillin, 50μg/ml streptomycin sulphate, 10mM Hepes, 10% v/v FCS

(iv) Endothelial cell basal medium (EBM), Clonetics, BioWhittaker

2.1.5 Culture Flasks

Non pyrogenic, DNAase, RNAase free 25cm^2 or 75 cm^2 tissue culture flasks, CellStar®, Germany

6, 24, or 96 well trays, Nunclon™, Denmark

96 well non pyrogenic DNAase, RNAase free suspension culture plate (Cellstar®, Germany)

Lab-Tek® Chamber Slide™ System, Nalge Nunc International, Naperville, IL

2.1.2 Primary antibodies

Primary antibodies used in Western blotting (dilution as indicated) or flow cytometry are indicated in Table 2.1, along with the dilution at which they were used in Western blotting, the host, the source, as well as their characteristics.

2.1.3. Plasmids

The plasmids, pAdEasy-1 and pAdTrackCMV were obtained from Dr. R. Hannan, Molecular and Physiology Laboratory, Vic, Australia. The mammalian expression vector pRufNeo was obtained from Dr. T Gonda, Human Immunology, Hanson Institute, Institute of Medical and Veterinary Science.

2.1.4. Enzymes

*Eco*R I, *Hpa* I, Hind III, Bam H I, *Pme* I, *Xho* I were purchased from New England BioLabs, Ontario, Canada.

2.1.5. Inhibitors

LY294002 (PI-3kinase inhibitor) from Calbiochem, Darmstadt, Germany.

PD98059 and UO126 (MEK1 inhibitor) from Cell Signalling (Beverly, MA, USA).

PP1 (Src-family tyrosine kinase inhibitor) from Biomol Research Laboratories, PA, USA

AG-490 (JAK-2 tyrosine kinase inhibitor) from Biomol Research Laboratories, PA, USA

PMSF (protease inhibitor), Sigma, St Louis, Missouri, USA.

Protease inhibitor cocktail, Sigma, St Louis, Missouri, USA.

Pertussis Toxin, Sigma, St Louis, Missouri, USA

2.1.6 Dyes

Trypan blue, xylene cyanole FF, and bromophenol blue from Sigma, St Louis, MO

4'6-Diamidine-2'-phenylindole dihydrochloride (DAPI), Roche, Manheim, Germany.

2.1.7. Solutions and Buffers

Solutions and buffers are detailed in Table 2.2.

2.2 Cell culture

2.2.1 Cell Lines

Cell lines used in addition to HUVEC were as follows:

-HEK293 cells (ATCC Number: ATCC-CRL-1573), a transformed human kidney cell line

-NIH3T3 cells (ATCC Number ATCC-CRL1658), which are contact inhibited NIH Swiss mouse embryo-fibroblast cells.

- Bing Cells are a HEK293 packaging cell line containing the apparatus to produce a retrovirus when transfected with plasmid DNA. They were produced by Dr. Warren Pear in 1996 according to the method in (160), given to Dr. T Gonda, Hanson Institute, and subsequently sourced to the Vascular Biology Laboratory.

2.2.2 Isolation of HUVEC from cords

The umbilical vein was isolated, and irrigated with RPMI until the return was clear of blood. Collagenase solution 25ml was injected into one end of the vein, whilst elevating the other end to ensure uniform enzyme distribution. The ends of the vein were sealed with a stopcock, and the cord placed in a beaker of saline warmed to 37°C for 13 minutes. The cord was then removed from the saline, and the collagenase collected into two 50ml centrifuge tubes by injecting fresh medium into the vein. The process was then repeated with the stopcock closed this time massaging along the length of the cord 4 times. The stopcock was then opened and again the solution washed through four times. The medium was then collected into the tubes, followed by centrifuging at 1500 rpm for 5 minutes. The cells were resuspended in 12 ml HUVE wash, and pooled. Pelleting/ re-suspension was done a total of three times, and then the cells resuspended in 5 ml HUVE medium and dispensed into 25cm^2 gelatin coated flasks.

2.2.3 Cell culture

HUVEC were grown in 25cm^2 or 75cm^2 gelatin coated Costar flasks (Costar Corp., Cambridge, MA) in HUVEC Medium supplemented with heparin and ECGS at 150µg/ml. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured, each flask for 2 to 5 days, depending on the growth rate. The medium from the cells was aspirated, and the cells washed with EDTA 0.01M/PBS. Trypsin was pre-warmed in a 37°C water bath, and added to the cells (0.6ml and 1.2ml for 25 cm^2 and 75 cm^2 flasks respectively). The flasks were gently tapped until the cells were seen to be detaching, and then HUVE wash was used to neutralize the trypsin (5ml, 10ml for 25cm^2, 75 cm^2 respectively) (complete DMEM for HEK293 cells). The cells were pelleted by centrifugation

for 5 minutes at 1500 rpm. HUVEC were routinely passaged when confluent (each 3-4 days) with 1:2 split whereas HEK293 cells were split at 1:3 ratio when between 50-70% confluent. Exclusion of trypan blue (0.8% w/v in saline) diluted 9/10 was used to determine cell viabilities, and cell densities were calculated using a haemocytometer. HEK293 cells and NIH 3T3 cells were cultured in complete DMEM in uncoated flasks.

2.2.4 Cryopreservation of Cells

Cells were harvested at log phase and re-suspended in medium supplemented with 10% FCS. The cells were placed on ice, and an equal volume of ice-cold cryoprotectant (40% FCS, 20% dimethyl sulfoxide (MERCK Pty Ltd. Victoria, Australia) and 50% medium) was added drop wise to the cells. Cells in 1 ml aliquots were placed in cryotubes (Greiner, Labortechnik, Germany) and transferred to liquid nitrogen for long term storage.

2.3. DNA preparation

2.3.1 Retroviral construct

The retroviral vector used was pRUFNeo (5661bp)(161)which comprises the neomycin (G418) resistance gene (aminoglycoside phosphotransferase 3'(I). The cDNA of interest (wild type flag-epitope tagged sphingosine kinase, 1.2kb, cloned in our laboratory, or dominant negative SK, G82D, generated by site directed mutagenesis in our laboratory, and also flag epitope tagged(127)) was inserted into pRUFNeo by restriction digest (Fig 2.1). Empty vector (pRUFNeo without cDNA) was used as a control. Flag tag is an 8 amino acid structure (DYKDDDDK) with further details outlined as in (106).

2.3.2 Transfer of plasmids into bacteria

Electrocompetent cells (E Coli, DH5α) were thawed on ice. These bacterial cells had been grown in a low salt medium and desalted by washing in 10% glycerol in water to enhance efficiency of DNA transfer. Plasmid DNA (1 μL of 10ng/μL stock) was added to 40μl of cells and incubated on ice for 30 minutes. The cells were then placed into 42°C water bath for 2 minutes, and then placed in iced water for 1 minute. Luria Bertani broth (1ml) was added to the cells and the cells were incubated at 37°C for 30 minutes. After centrifugation at 6000 rpm for one minute, 1ml of broth was discarded, and the remaining 100μl was streaked onto an agar plate (containing 100μg/ml ampicillin) with a sterile (heat and alcohol treated) bent Pasteur tip. The plate was incubated overnight at 37°C. The following day, a single colony was picked with a sterile toothpick and used to contaminate 2 ml LB broth containing ampicillin 100μg/ml. The culture was grown overnight in a 37°C oven with vigorous shaking. The culture was then diluted by inoculating into 200 ml of LB broth containing ampicillin. This was cultured overnight at 37°C with vigorous shaking. The bacterial cells were then harvested by centrifugation at 5000rpm for 15 minutes at 8°C. The supernatant was discarded.

2.3.3 DNA Purification

The Qiagen Plasmid purification system (Hilden, Germany) was used according to the manufacturer's instructions to purify the DNA. The above bacterial pellet was resuspended in 4 ml of Buffer P1 by vortexing to ensure no clumps remained. To lyse the cells, Buffer P2 4ml was added and a viscous appearance was indicative of cell lysis. The solution was thoroughly mixed and incubated at room temperature for 5 minutes. To neutralize Buffer P2, ice cold Buffer P3 (4 ml) was added which resulted in the formation of a precipitate

containing genomic DNA and cell debris. A Qiagen-tip was equilibrated by the addition of 4 ml Buffer QBT and allowing gravity to aid the flow. The supernatant was filtered and loaded onto the QIAGEN-tip and allowed to enter the resin by gravity. At this stage, the DNA should bind to the QIAGEN Resin. The QIAGEN-tip was then washed with 2x10ml Buffer QC to remove contaminants. The DNA was eluted with 5ml Buffer QF and the eluate collected in a 10 ml tube. The DNA was precipitated with 3.5ml isopropanol at room temperature. The precipitated DNA was immediately centrifuged at 12000rpm for 30 minutes at 8°C. The supernatant was discarded and the DNA pellet was washed with 70% ethanol and then centrifuged at 12000 rpm for 10 minutes. The supernatant was decanted, and the pellet air-dried. The DNA was then dissolved in TE (10mM Tris, 0.1mM EDTA, pH 8.0), transferred to Eppendorf tubes, and stored at -20°C.

2.3.4 Determination of DNA concentration

UV spectrophotometry was used to determine the DNA concentration. The DNA was diluted in TE and loaded in a cuvette. Program 7 in the spectrophotometer (Beckman DU®-64) was used to determine the A260/280. TE was used as a blank solution. The DNA concentration (μg/μl) was the product of 0.05, A260 and the dilution factor. The amino acid sequence of the SK is indicated in Fig 2.2, and a map of cDNA for SK and G82D is shown in Fig 2.3.

2.3.5 Checking the purity of plasmid DNA

The DNA was re-dissolved in 20μL TE. One μL of DNA was run on a 1% agarose gel in TAE buffer (0.04M Tris, 1mM EDTA, glacial acetic acid to pH 8.0) at 100V for 40 minutes, stained with ethidium bromide, photographed and examined to ensure the presence of DNA. Restriction digestion using appropriate restriction endonuleases was undertaken using 1μg of

DNA, 5 units of restriction enzymes (less than 10% of final volume), 10X Buffer NEB2 (New England BioLabs, Ontario, Canada), and water to a total volume of 10μL. This was incubated at 37°C for one hour, and then 3μL of the digested DNA was mixed with 1μL 3X urea loading buffer (4M urea, 50% sucrose, 50mM EDTA pH 7.0, 0.1% bromophenol blue, 0.1% xylene cyanole), and run (alongside SPP1 molecular weight markers) on a 1% agarose gel in TAE buffer at 100V for one hour. The gel was stained with ethidium bromide and photographed.

2.4. Generation of retroviral supernatant

2.4.1 Calcium Phosphate Transfection of Bing Cells

Bing cells are a retroviral packaging cell line produced by Dr. W. Pear (according to the method of production of the BOSC 23 cell line(160)), with the only difference being the generation of amphotrophic as opposed to ecotrophic retrovirus. Bing cells at low passage were plated into 10 cm dishes at $2.5x10^6$ cells per dish in 10 ml of complete DMEM 36 hours prior to transfection. One dish was used per transfection, and one for a mock transfection. Transfection was undertaken at 80-90% confluence. One hour prior to transfection, the medium was changed to antibiotic free DMEM. For each dish, 15μg DNA was mixed with 250mM $CaCl_2$ and water to 1 ml. An equivalent volume of 2xHBSP pH 7.12 was added dropwise with bubbling. The mixture was allowed to stand for 2 minutes. Chloroquine 25μM was added per dish and subsequently the transfection mix was added dropwise to the cell medium. For each experiment, a mock transfection without DNA was undertaken. The formation of a dark precipitate was observed under light microscopy and the dishes were returned to incubate at 37°C for 7-11 hours after which the medium was replaced by fresh complete DMEM.

After 48 hours, the viral supernatant (media) was collected using a syringe and filtered using 0.22μm filter. The supernatant was aliquoted into Nunc vials, snap frozen in liquid nitrogen and then stored at -80°C.

2.4.2 Determining the Efficiency of Bing Cell Transfection

At the time of collecting the viral supernatant, the Bing cells were collected by washing with 0.01M EDTA/PBS and trypsin. Cells were divided into Eppendorf tubes at $1x10^6$ cells per tube in 500 μl FACS wash and the cells were collected by centrifugation at 2000 rpm for 2 minutes at 4°C. Methanol 500μl per tube was used to fix the cells on ice for 5 minutes. After spinning and aspirating the methanol, 100μl of 5% BSA/ PBS block solution was added to each tube to reduce non specific binding of antibody and the cells left at room temperature for 1 hour. The cells were then permeabilized with 100μl of antiflag antibody in 0.1% Triton X in PBS in the presence of the blocking solution, and incubated at 4°C overnight. For the negative control, 100μl of 0.1% Triton X in PBS was added without the antibody, and a control antibody reactive to irrelevant antigens (23-1F11) was used. The cells were then washed with 500μl FACS wash and incubated with anti-mouse FITC 1:100 in FACS wash on ice for one hour. The cells were washed using PBS, and after centrifugation at 2000 rpm for 2 minutes at 4°C were resuspended in 300 μl FACS Fix, transferred to FACS tubes and stored at 4°C in the dark until fluorescence was read using a flow cytometer.

2.4.3 Determination of the optimal viral titre

Infection of NIH3T3 cells was used to determine the optimal viral titre. Cells were plated at $3x10^5$ cells per 6 cm dish in 4 ml complete DMEM 24 hours before infection. The retroviral supernatant was thawed on ice, and log dilutions made in complete DMEM. Polybrene 4μg/ml was added to increase the efficiency of transfection. The medium from each dish was replaced by 1 ml of the viral dilutions and incubated for 3 hours at 37°C, after which time, a further 3 ml of complete DMEM was added to the cells. 24 hours after infection, the cells were washed twice with 0.01M EDTA/PBS and collected by the addition of trypsin 300μl and 700μl of complete DMEM. A 1:20 split was performed by taking 50μl of this cell mixture and seeding this into a new 6cm dish with 4 ml complete DMEM. At this time, G418 400μg/ml was added for selection of cells infected with the retrovirus bearing the G418 resistance gene. The medium was changed each 72 hours and fresh complete DMEM and G418 added. At approximately 10 days, colonies were visible under light microscopy, reflecting individual clones of cells successfully infected with the retrovirus.

The colonies were stained and counted when the negative control (uninfected) cells had died. The medium from the cells was aspirated, and the cells were fixed with 4ml 4% formaldehyde for 20 minutes. This was aspirated and the cells washed with 2 ml PBS. Two ml Giemsa stain was added to each dish for approximately 20 minutes or until colonies were visible. The stain was aspirated and the dishes allowed to dry on blotting paper. The colonies were then counted. Duplicate dishes were done for each retroviral construct. The test was considered valid if no colonies were seen in the mock transfections, and the maximum number of colonies seen with the lowest viral dilution.

2.4.4 Large scale Bing cell Transfection

To concentrate the retrovirus, large scale Bing cell co-transfection with the DNA of interest and pMDG was undertaken using six 10 cm dishes per construct. The viral supernatant was filtered as above, and then concentrated using the Vivaspin-20ml tubes (Vivascience, Sartorius group, Germany). This is a conical tube with twin vertical membranes and a thin channel concentration chamber designed to allow in the presence of a centrifugal force, solids to move to the bottom of the tube, and macromolecules and viral particles to concentrate between the membranes. The viral supernatant was placed in this tube, and centrifuged at 2500g for 30 minutes. Fresh complete DMEM was then added to the tube and centrifugation was repeated. The viral supernatant was then collected in a sterile manner using a pipette tip, aliquotted and snap frozen in liquid nitrogen and stored at -80°C.

2.4.5 Infection of HUVEC with retroviral supernatant

HUVEC cells at passage 2 or 3 were used for infection. Cells were plated 24 hours prior to infection in gelatin coated 6-well trays at $1.7x10^5$ cells per well in 2 ml HUVE medium with heparin and growth factors at 50 μg/ml. Cell density was found to be critical: sparsely plated cells were more readily infected, and for selection to occur, cells had to be dividing and thus subconfluent at the time of initiating selection. The optimal viral dilution determined using NIH3T3 cells was used for infection. For each well in the 6 well tray, the viral mixture was made using HUVE medium, heparin, growth factors, polybrene 4μg/ml and the retroviral supernatant to a total volume of 750 μl. The medium from the cells was aspirated and this viral mixture added, and the trays returned to incubate at 37°C for 3 hours. A further 2ml of HUVE medium with heparin and ECGS was added per well. A mock infection was done per experiment, using the reaction mix without the retroviral supernatant. At 48 hours post infection, the medium was replaced by selection medium containing 150μg/ml G418 sulfate (Promega, Madison, WI, USA). The medium was changed each 3 days and at approximately

10 days post infection, colonies were visible. The cells were harvested when the colonies were densely packed, and all cells in the mock infection had died.

2.5 Generation of adenoviral supernatant.

The AdEasy system was used to produce recombinant adenovirus according to the manufacturers instructions (**www.qbiogene.com/products/adenovirus/adeasy.shtml**). The initial step involves the insertion of the cDNA of interest into a transfer vector (pAdTrack-CMV), in the supercoiled plasmid form. This plasmid contains a copy of the CMV promoter allowing constitutive and high level expression of recombinant proteins. The plasmid is then linearized with restriction endonuclease Pme I. The second step involves the transfer of this linear vector into E Coli strain BJ5183 and is achieved by homologous recombination with an intact supercoiled viral DNA plasmid (pAdEasy-1). The transfer vector contains a kanamycin resistance gene, which allows for the selection of recombinants, which can be confirmed by restriction endonuclease analyses. Recombinant adenoviruses are then produced by transfecting the recombinant plasmid into an adenoviral packaging cell line (HEK293 cells).

2.5.1 Generation of recombinant adenoviral plasmids in bacterial cells

2.5.1.1 Preparing electrocompetent bacterial cells.

A fresh colony of BJ5183 cells was used to inoculate 10ml LB with 30ug/ml streptomycin. The cells were grown overnight at 37°C in a shaker. 1 ml of cells was diluted in 1000ml of streptomycin containing LB and grown for 5 hours in eight 1 litre flasks with vigorous shaking at 37°C. The cells were collected in four conical centrifuge tubes, incubated on ice for ten minutes and pelleted by centrifugation at 3000 rpm at 4°C for ten minutes. The cells

were washed with 1000ml sterile ice cold WB (10% ultrapure glycerol, 90% distilled water, v/v). The pelleting and washing steps were repeated twice. After the latter spin, all but 20 ml of supernatant was removed, and the cell suspension spun at 3000rpm for 10 minutes. The pelleted cells were then left in 2 ml of supernatant and mixed with the remaining WB. The cells were aliquoted (20µl/tube) and stored at –80°C.

2.5.1.2 Linearization of the shuttle plasmid

The shuttle plasmid (300ng) was linearized with the restriction endonuclease PmeI . The DNA was then phenol-chloroform extracted, precipitated with ethanol, and resuspended in 6.0µl H_2O.

2.5.1.3 Co-transformation of the plasmid

The digested shuttle plasmid (1µg) was co-transformed with 1.0ul (100ng/ul) of pAdEasy-1. E Coli BJ5183 cells (20µl) were added and electroporation undertaken at 2500V, 200 Ohms in a Bio-Rad Gene Pulser electroporator in 2.0mm cuvettes. The transformation mix was resuspended in 500µl LB and plated onto kanamycin containing plates and allowed to grow overnight at 37°C. The configuration of the pAdEasy-1-AdTrackCMV (pAEAT) is shown in Fig 2.4.

2.5.1.4 Mini-preps

Twenty smallest colonies were selected and grown in 2 ml LB containing kanamycin 25µg/ml for 10 hours. The alkaline lysis method was used for the mini-preps, and the size of the plasmid checked by running on a 0.8% agarose gel.

2.5.1.5 Linearization of the DNA

The DNA was linearized by Pac I restriction digest.

2.5.2. Adenovirus Production in HEK293 cells

HEK293 cells were seeded 24 hours prior to transfection into T-25 flasks at $2x10^6$ cells per flask to reach 90% confluence at the time of transfection. The recombinant adenoviral plasmids were digested with PacI (4μg/flask) on the day of transfection, ethanol precipitated, and resuspended in 20μl of sterile water. Transfection was done using Lipofectamine 2000 (GIBCO BRL) according to the manufacturers instructions. PacI digested DNA (4μg) was mixed in 500μl OptiMem 1 medium and incubated at RT for 30 minutes. Lipofectamine 2000 (20μL) was mixed with 500μL Optimem and incubated at RT for 30 minutes. The DNA/Optimem mixture and Lipofectamine2000/ Optimem mixture was combined, incubated at RT for 30 minutes. The medium from the flasks was aspirated, the cells washed with serum free DMEM, and then 2.5 ml antibiotic free DMEM was added to each T-25 flask. The DNA mix was added to the cells, and the flasks returned to incubate at 37°C. Forty-eight hours later, the DNA containing medium was replaced by 6 ml complete DMEM. The recombination of pAdEasy with pAdTrackCMV resulted in a vector with GFP (Fig 2.4) and thus the efficiency of transfection was monitored by GFP expression using immunofluorescent microscopy. At ten days post transfection, the cells were scraped using a rubber cell scraper, transferred to 50 ml conical tubes, and centrifuged at 1500 rpm for 5 minutes. The cell pellet was resuspended in 2.0 ml sterile PBS pH 8.0. Cells were frozen in liquid nitrogen, thawed in 37C water bath, and vortexed. This freeze/thaw/vortex cycle was done four times to disrupt cell membranes and allow the virus to be released from the cells. The samples were then centrifuged, and the viral supernatant aliquoted and stored at –20°C.

2.5.3. Amplification

The above transfection supernatant was used to infect two T-25 flasks of HEK293 cells at 70% confluence. 250uL of supernatant diluted in 2.5ml complete DMEM was added to HEK293 cells, incubated at 37°C for 2 hours and then a further 2.5ml of complete DMEM was added to the cells. Supernatants were collected as described above when 50-70% of cells were seen to be GFP-positive and detaching. The next round of amplification was done in T-75 flasks.

2.5.4. Generation of High Titre Viral Stocks

Thirty T-75 flasks containing HEK293 cells were used per construct. Cells were infected with the adenoviral supernatant at dilutions of 1:100 in 5 ml complete DMEM, and returned to incubate for two hours at which time a further 5 ml complete DMEM was added per flask. The efficiency of infection was monitored by GFP expression using immunofluorescent microscopy. When approximately 50% of the cells had been lysed and detached, the cells were scraped, combined and centrifuged for 5 minutes at 1500rpm. The pellet was resuspended in 8.0 ml PBS pH 8.0, and again four cycles of freeze/thaw/vortex was performed. The lysate was centrifuged in Sorvall HS4 rotor at 6000rpm at 4°C for 10 minutes. The supernatant was stored at –20°C until purified by cesium chloride gradient.

2.5.5. Cesium Chloride Purification

The double cesium chloride gradient method was used (a discontinuous gradient to remove cellular contaminants followed by a continuous gradient to separate infectious particles from

defective viral particles). A discontinuous gradient was prepared by placing 4ml CsCl density 1.4 (53gCsCl mixed with 87ml, 10mM Tris pH7.9) in a 12 ml polyallomer tube (Ultra-Clear™ Centrifuge tubes 14 x 89mm Beckman Coulter, Palo Alto, California), and gently overlayed with 4ml CsCl density 1.2 (26.8g CsCl mixed with 92mM Tris). The viral supernatant was added to the tube. Mineral oil was used to cover the surface, and a balance tube was prepared in a similar fashion. The tubes were centrifuged in SW41 rotor at 32,000rpm at 10°C for 2 hours, deceleration rate =0. The tubes were then removed and secured individually using a three pronged clamp and stand. Two bands were visualized, and the side of the tube was punctured using a 19G needle, and the lower band collected into a 5 ml syringe. The viral band was then placed onto a continuous CsCl gradient by mixing with 8ml CsCl density 1.35 (17.6g CsCl in 32ml 10mM Tris) in a 12 ml pollyalomer tube. The gradient was overlayed with mineral oil, and a balance tube was similarly prepared. The solutions were centrifuged in SW41 rotor at 32000 rpm, 4°C for 20 hours, deceleration rate =0. The viral band was then collected by needle puncture, and mixed with an equal volume of 2X storage buffer (10mM Tris pH=8.0, 100mM NaCl, 0.1% BSA, 50% glycerol, filter sterilized).

2.5.6. Virus Desalting

The virus was dialysed to remove the cesium chloride. The viral band was dialysed at 4C in a cellulose ester membrane (Slide-A- Lyzer® Cassette, Pierce, Perstorp Life Sciences, Rockford, IL) according to the manufacturers instructions, using 200x the volume of dialysis buffer (10mM Tris pH=8.0, 2mM MgCl2, 4% sucrose) and 3 changes of buffer over 24 hours. The virus was then collected using a 21G needle and 5ml syringe, aliquoted, and stored at –80°C.

2.5.7. Titration of Viral Particles

2.5.7.1 Determination of Tissue Culture Infectious Dose 50 ($TCID_{50}$)

Low passage HEK293 cells were plated in 96 well flat bottom trays at $2x10^4$ cells per well in 100µl DMEM containing 2% FCS two days prior to infection. Ten wells per construct were used, thus for three constructs, (EV, SK, G82D), three trays were used. Eight serial dilutions of the high titre adenovirus were prepared in duplicate, in 2% DMEM in sterile tubes, commencing at 10^{-3} and extending to 10^{-10}. Each of the first ten wells in the eight rows was infected by dispensing 100ul of the viral dilution to the wells, commencing at the highest dilution for the top row, and extending to the most concentrated viral mixture for the bottom row. The last two wells in each row were used as negative controls. The plates were then returned to incubate in a 37°C incubator for ten days.

At Day 10, microscopy was used to determine the number of wells at each viral dilution with cytopathic effect and plaque formation. Cytopathic effect was said to be present, if the cells had rounded up and detached from the bottom of the well, and plaque formation was evidenced by regions of cells with cytopathic effect with lysis of surrounding cells, reflecting the killing of neighbouring cells by released virus. The test was considered valid if plaque formation was seen in all the wells at the lowest dilution, and none of the wells infected with the highest virus dilution or the negative wells. The KARBER statistical method was then used to determine accurately the viral titre which was expressed as plaque forming units per ml (PFU/ml). Similarly, the titre was also determined by GFP expression using immunofluorescent microscopy.

2.5.7.2 Titration on HUVEC

Passage 3 HUVEC were plated into gelatin coated 24 well trays at $5x10^4$ cells per well in 500µl of complete HUVE Medium 24 hours prior to infection. The medium was then replaced with 250µl HUVE medium containing 2% FCS. Viral preparations were made in Eppendorf tubes in 2% HUVE medium to allow the infection of varying PFU/cell. Eleven wells per construct were used, to infect each at 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 PFU/cell, and allowing one well per construct. The trays were returned to the incubator for two hours, and then 250µl of HUVE medium containing 38% FCS was added to each well. Two days post infection, the cells were harvested by washing with EDTA 0.01M /PBS, 250ul/well warm trypsin, and 1.5 ml/well FACS wash. The cells were transferred to FACS tubes, pelleted, and resuspended in 300ul of FACS Fix. GFP expression was determined by flow cytometry. The parameters were adjusted such that < 1% of the uninfected control cells were in the area of positive fluorescence.

2.5.8. Adenoviral infection of HUVEC

HUVEC in gelatin coated flasks (T25 or T75) cultured in normal growth conditions were infected at 70-80% confluence. Adenoviral preparations were adjusted for either the same number of PFU/cell, or adjusted for the amount of GFP expression. For T75 and T25 flasks, the viral mix was prepared in 5 or 2.5 ml of 2% HM respectively, which was added to the cells, and supplemented two hours later by 5 and 2.5 ml respectively of HUVE medium containing 38% FCS.

2.6 SK Activity Assay

2.6.1. Sample preparation.

Cells were plated into gelatin coated 6 cm dishes at density 7.5 $X10^5$ cells per dish in 3 ml HUVE medium with heparin and ECGS. At 48 hours, the medium was changed to HUVE medium containing 2% FCS for 2 hours prior to cell lysis. Cells were then washed with ice cold PBS, and collected by scraping and centrifugation in 3 ml PBS. The cell pellet was re-suspended in 200µl ice-cold homogenizing buffer (20% glycerol, 25mM TRIS/HCl, pH 7.5, 1mM DTT, 1mM EDTA, 10mM MgCl, 10µg/ml leupeptin, 10µg/ml aprotinin, 1mM PMSF). The cells were homogenized by passing through a 26G needle 10 times and the cytosolic fraction was isolated by centrifugation at 13000rpm for 30 minutes at 4°C. The Bradford Reagents (BIO-RAD, Hercules, CA) were used to determine the protein concentration, and the samples were stored at –80°C until assayed.

2.6.2. Activity measurement

The sample (180µl) was combined with 20µl of reaction buffer (200µl PBS pH 7.4, 1mM ATP, 20µM sphingosine/5% Triton complex, γ-[^{32}P]ATP 2.5µl) and incubated in a pre-warmed water bath at 37°C for 30 minutes with gentle shaking. After 5 seconds of centrifugation, the reaction was stopped by the addition of 800µl $CHCl_3$/CH3OH (1:1, v/v) and 200µl 0.1M HCl/PBS. The mixture was vortexed for 1 minute and centrifuged for 2 minutes. The $CHCl_3$ phase was collected, transferred to a new tube, and 40µl was spotted onto a TLC plate. The plate was run in solvent containing 1-butanol/ methanol/ acetic acid/ water (8:2:1:2 v/v), and the radioactive spots corresponding to S1P were quantified using the phosphorimager.

2.7 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

2.7.1. Preparation of cell lysates.

Cell lysates were prepared by washing adherent confluent cells with ice cold PBS, scraping the cells in RIPA Buffer using a rubber cell scraper, and homogenizing the lysate by passing through a 26G needle ten times. The homogenized lysate was centrifuged at 4°C at 13000 rpm for 25 minutes, and the supernatant collected. The lysate was stored at –20°C.

2.7.2 Determination of protein concentration of cell lysates

The protein concentration was determined using the Bradford Reagents (BIO-RAD, Hercules, CA) according to the manufacturers' instructions. Five µl of each sample was added per well in a 96-well microtitre plate (in duplicate). Twenty µl of Reagent A/S mixture (20µl Reagent A in 1 ml Reagent S) was added to each well, and then 200 µl of Reagent B was added to each well. The absorbance was measured on a microplate reader (Biorad) at a wavelength of 595nm. The protein concentration was determined against a standard curve generated from known serial dilutions of BSA using Microplate Manager software (Biorad).

2.7.3. Protein electrophoresis

Electrophoresis was undertaken using Biorad Protean II electrophoresis apparatus assembled according to the manufacturers' instructions. Electrophoresis was done using 10-12% SDS polyacrylamide (29.2:0.8 acrylamide:bisacrylamide) gels comprising 375mM Tris-HCL pH 8.8, 0.1% ammonium persulphate, 0.1% SDS, 0.08% TEMED). The separating gel was poured between the glass plates, and the gel was overlayed with mQ water. The water was poured off when the gel had polymerized, and then the stacking gel (5% polyacrylamide,

125mM Tris-HCL pH 6.8, 0.1% SDS, 0.1% ammonium persulphate, 0.1% TEMED) was poured over the top. The comb was inserted. After the gels had polymerized (approximately thirty minutes), the combs were removed and the wells were rinsed with 1X running buffer. Cell lysates were prepared in 5 X sample loading buffer and boiled on a 100°C heating block for 5 minutes. The samples were loaded into the stacking gel, in a tank of 1X running buffer. Prestained protein molecular weight standards (GibcoBRL) were included in each gel. Electrophoresis was performed at 150V for 45-60 minutes or until the dye front had run off the gel. Proteins were then transferred to a nitrocellulose membrane (S&S Protran BA, Schleicher &Schuell, Keene N.H, USA). The transfer cassette was assembled as follows, from the black side towards the clear side: gauze pad, blotting paper, gel, membrane, blotting paper, gauze pad. Each layer was rolled using pipettes to prevent bubbles forming between the layers. The cassettes were placed in a transfer tank 80% filled with transfer buffer. An ice pack and magnetic stirrer were used to prevent localised overheating. Transfer was performed at 4°C at 100V for one hour. The membrane was then blocked using blocking buffer for one hour at RT.

2.7.4. Detection of Proteins

The membrane was then probed with primary antibody overnight at 4°C. The membrane was washed three times for five minutes using PBS/0.1% Tween 20 prior to the addition of secondary antibody conjugated to horse radish peroxidase (anti-mouse-HRP, or anti-rabbit HRP, 1/12000). The membrane was again washed as above. All antibodies were diluted in PBS/0.1% Tween 20 except for anti-flag antibody for which all steps were done using blocking buffer. Enhanced chemiluminescence (ECL, Amersham Pharmacia, Buckinghamshire, UK) was used according to the manufacturers instructions and the membrane exposed to X-Ray film. Alternatively, ECL-+Plus Amersham Pharmacia,

Buckinghamshire, UK) was used, and the membrane exposed to a phosphorscreen (Typhoon Phosphorimager) and the image quantified using ImageQuant software (Molecular Dynamics). To re-probe the membranes, the membrane was stripped using Reblot Plus (Chemicon International, Temecula, CA) according to the manufacturer's instructions, then washed three times in PBS 0.1% Tween 20, blocked in blocking buffer for 30 minutes and re-probed with antibodies as above.

2.8. Flow cytometry

2.8.1 Staining for cell surface/ transmembrane proteins

HUVEC were plated at $5x10^5$ cells per well in 500µl complete HUVE Medium in gelatin coated 24 well trays. At 24 hours, the cells were washed with FACS Wash (RPMI supplemented with 10% FCS and 1% sodium azide), and incubated on ice for 30 minutes with primary antibody (10-20µg/ml) diluted in FACS Wash (250µl/well). This was then aspirated, the cells washed with FACS Wash, and then the secondary antibody added (FITC-conjugated, anti-mouse Ig (Fab2, DAF; Silenus Laboratories, Hawthorn, Australia, or for GFP-expressing cells, goat anti-mouse IgG R-phycoerythrin conjugate at 1/50, Southern Biotech Birmingham, AL, USA). Again the cells were incubated on ice for 30 minutes. The cells were then harvested by EDTA/ trypsin treatment, pelleted, and resuspended in 300µl of FACS Fix (2% glucose, 1% formaldehyde, 5mM sodium azide made in PBS). For each assay, a non-relevant isotype matched Ig (Keyhole Limpet Hemacyanin, 23-1F11) was used as a negative control. The tubes were stored in the dark at 4°C until read using a Coulter Epics Profile XL flow cytometer. A minimum of 1000 events per test was analysed, and the results were expressed as median fluorescence intensity, after subtracting the accompanying value for the negative control Ig.

Alternatively, the cells were harvested initially, and the staining performed in suspension in Eppendorf tubes containing $1X10^6$ cells per tube, and a total volume of antibody of 100µl /tube.

The effect of pretreatment of HUVEC with recombinant human TNFα 0.5ng/ml (R&D Systems Inc) for four or eighteen hours, and 5µM S1P, D-*erythro* (Biomol Research Laboratories Inc, Plymouth Meeting, PA, USA) for various time points was assessed.

2.8.2 DNA Flow Cytometry

Flow cytometric staining for apoptotic cells was performed using Annexin V-PE and 7-AAD. Cells were detached by EDTA/trypsin treatment, and washed with cold PBS. The cells (1 X 10^6) were re-suspended in 50µl of Annexin V Binding buffer. The cells were incubated at RT in the dark for ten minutes with 4µl of Annexin V-PE and 1µg of 7-AAD (10µl of 0.1µg/µl solution). The cells were transferred to FACS tubes and a further 400µl of Annexin V Binding buffer was added to each tube. Unstained HUVEC (uninfected with virus) of the same cell line were used as a negative control for PE and 7-AAD. HUVEC stained for PECAM-1 using anti-PECAM-1 antibody (51-6F6) and goat anti-mouse IgG R-phycoerythrin conjugate were used as the positive control for PE. HUVEC lysed in 80% ethanol (1 minute treatment with gentle vortexing) were used as the positive control for 7-AAD. The cytometer was calibrated using these controls, and the samples stained with Annexin V and 7-AAD were then analysed within one hour of staining. Apoptotic cells were those which stained positive for Annexin V and negative for 7-AAD. The mean fluorescence in this apoptotic cell population was determined. The analysis was performed using cells grown in normal culture conditions, and those subjected to 24 hours of serum deprivation.

2.9 Immunoprecipitation

Immunoprecipitation was undertaken using confluent cells in 75cm^2 flasks. Cells were treated for 5 minutes with 100mM sodium orthovanadate and washed with cold PBS. RIPA buffer (750 μL) to which was added 1/10 Protease inhibitor cocktail (Sigma, St Louis, Missouri, USA), and 1/100 (v/v) 100mM sodium pervanadate was added to the monolayers. After fifteen minutes, the cells were scraped using a rubber cell scraper, and transferred to Eppendorf tubes. Three cycles of freezing/thawing (using liquid nitrogen and a 37°C water bath respectively) were undertaken to lyse the cell membranes. The cell solution was centrifuged at 13000 rpm at 4°C for 20 minutes, and the supernatant taken. The relevant antibody (1-2 μg) was added to the supernatant, which was placed on a rotating device (Clay Adams® Brand) at 4°C for three hours. Twenty μL of goat-anti-mouse sepharose IgG (Zymed, San Fransisco, California, USA) together with 40μL of protein A sepharose (50% slurry) (Amersham Pharmacia Biotech, Uppsala, Sweden) was added and the cell lysate incubated as above for three hours. The solution was centrifuged briefly to pellet the beads, and the supernatant discarded. The beads were washed with RIPA buffer (1mL) three times, and then boiled together with 2x sample loading buffer, for three minutes at 100°C. The immunoprecipitate was stored at -20°C and re-boiled prior to detection of proteins by Western blot analysis.

2.10 Measurement of Caspase-3 Activity

Cell lysates were prepared using confluent cells in a 24 well plate. The cells were detached using 50μl of cold caspase-3 lysis buffer. The solution was transferred to an Eppendorf tube, homogenized by passing through a 26G needle, and spun at 1300 rpm for 25 minutes at 4°C.

The supernatant was stored at –20°C after determining the protein concentration using the Bradford reagents. Ten µl of lysate was placed onto a 96 well tray. In a separate tube, 10 ml of caspase-3 buffer was mixed with 15.45mg DL-dithiothreitol, (Sigma Chemical Co, St Louis, USA) and 10µl of 2.5mM fluorogenic substrate DEVD-aminofluoromethylcoumarine (DEVD-AFC) substrate (Calbiochem-Novabiochem, Darmstadt, Germany). Two hundred µl of this mixture was added to each well, and the tray left to incubate in the dark at room temperature for five hours. The assays were done in duplicate, and for each assay, a negative (normal HUVEC) and positive control (HUVEC treated for 16 hours with 10 ng/ml TNFα and 2µg/ml cycloheximide) were used. Fluorescence was then measured with a well plate reader using excitation and emission wavelengths of 385nm and 460nm respectively, and filter 515nm cut off. The mean of the duplicates was calculated, and normalized for the protein concentration.

2.11 Measurement of Cell Permeability

Endothelial cells were seeded into fibronectin coated 3.0µm transwells with a polycarbonate membrane (Corning Inc, NY, USA) at $10x10^4$ cells per well in 150µL HUVE medium supplemented with ECGS and heparin. Six hundred µl of HUVE medium was added to the bottom of the transwell and the cells incubated at 37°C for 24 hours. The medium from the top and bottom of the transwell was then replaced with 2% HUVE medium with growth factors and heparin and the cells incubated for a further 4 hours. Stimulation with thrombin (Sigma, St Louis, MO) at a concentration of 0.2units/ml (in serum free HUVE medium) was done immediately prior to the assay whereas sphingosine 1 phosphate (1µM) was added 20 minutes earlier. FITC-dextran (500µg/ml diluted in serum free HUVE medium) was added to each transwell and then 20µL medium collected from the bottom of each transwell at

predetermined time points (commencing at time =0). This was dispensed into a 96 well flat bottom tray containing 60μL serum free medium per well. The fluorescence was then quantified with a well plate reader using excitation and emission wavelengths of 485nm and 530nm respectively.

2.12 Measurement of Cell Proliferation

Endothelial cells were plated into gelatin coated 96 well flat bottom trays at $3x10^3$ cells per well in 150μL of culture medium (with varying concentrations of serum and in the presence or absence of growth factors/ heparin). A separate tray was used for Day 0 and Day 3 readings to indicate the number of cells plated, and the amount of cell proliferation respectively, and the assays were done in triplicate. Two hours after the cells had been plated, and were seen to be attaching, 30μL MTS (Promega, WI, USA) was added to the wells for the Day 0 reading and the cells incubated for a further 2 hours. The optical density (reflected by the absorbance) was determined using an ELISA plate reader using a single wavelength (490nm), and subtracting the accompanying absorbance for a blank solution containing cell-free medium with MTS. At day 3, the amount of proliferation was noted by microscopy, and then quantified using MTS as above. The mean of the triplicate values was calculated, and the rate of proliferation determined as the ratio of Day 3: Day 1 absorbance.

2.13 Measurement of Cell survival

Cells were plated as for proliferation, this time in the absence of serum or growth factors/heparin. The amount of cell accumulation in serum-free conditions was determined using MTS as above at predetermined time points.

2.14 Cell suspension

Cell survival in suspension was determined with cells plated into non-adhesive 96well trays and the MTS reagents as above. Cells were plated in varying concentrations of serum, at $8x10^3$ cells per well, using time points of Day 0 and Day 1.

2.15 Cell attachment

Microtitre trays (96 well flat bottom) were coated with gelatin, fibronectin (50µg/ml), vitronectin (10µg/ml), (Promega, Madison, WI, USA) or collagen (50µg/ml) for one hour at 37°C. Cells were seeded at $1x10^4$ cells per well in 100µL of serum-free HUVE medium. At the predetermined time points of 20 minutes, one hour, and three hours, the wells were washed with serum-free HUVE medium to remove any unattached cells. Serum-free HUVE medium (100µL) and 20µL MTS was added to each well. A separate tray was used for the three time points, and all assays were done in triplicate. A negative control with cell free medium and MTS was used as a blank. After the addition of the MTS, the trays were incubated at 37°C for a further two hours, and then the optical density determined using an ELISA plate reader and single wavelength of 490nm.

2.16 Cell migration to fibronectin

The lower surface of a 6.5mm, transwell with a polycarbonate membrane with a pore size of 8µm (Corning Inc, NY, USA) was coated with 30 µl of fibronectin (50µg/ml in HBSS with Ca^{++}/Mg^{++}) for 30 minutes at 37°C. This was then aspirated, and the wells were blocked with 0.5% BSA in HBSS with Ca^{++}/Mg^{++} for 15 minutes. For the negative control, the wells were coated with HBSS with Ca^{++}/Mg^{++} without fibronectin, and then blocked as above. At the

same time, 12 wells in a 96 well flat bottom tray were also treated with fibronectin as above, for a standard curve. All samples were done in triplicates. The medium from HUVEC was replaced with fresh medium 4 hours before harvesting to ensure log phase. The cells were resuspended in HBSS containing 0.5% BSA with Ca^{++}/Mg^{++}, and GF/H, and then $1x10^5$ cells in 100µl was added to the top well. To the lower well was added 500µl of HBSS with 0.5% BSA, Ca^{++}/Mg^{++}, GF/H. The standard curve was plated in the fibronectin coated 96 well tray, commencing with 100 $x10^3$ cells with 6 subsequent doubling dilutions in HBSS with 0.5% BSA, Ca^{++}/Mg^{++}, GF/H. The cells in the transwells and the standard curve were incubated overnight at 37°C. The following day, the cells from the transwells were aspirated, washed with PBS, and any adherent cells were gently removed by wiping the membrane with a cotton bud soaked in PBS. The wells were washed a further two times with PBS to ensure the removal of any cells on the top surface. The cells that had migrated to the lower surface of the membrane were fixed by the addition of 500µl of cold methanol to the lower wells for 15 minutes. The methanol was aspirated and the cells were stained with 0.1% crystal violet in 0.1M borate buffer, pH 9.0. The dye was aspirated, and the upper and lower wells were washed with PBS three times. The standard curve was fixed, stained and washed in the same way using 100 µl of methanol, crystal violet and PBS. The stained cells in the lower transwells were eluted with 300ul of 10% acetic acid for 30 minutes, and 300µl duplicate samples were transferred to a 96 well tray. The standard curve was eluted in the same way, and the 300µl volume was transferred to clean wells. The optical density was determined using an ELISA plate reader and a single wavelength of 595nm.

2.17 Tube formation in Matrigel

Matrigel Basement Membrane Matrix (Beckton Dickinson Labware, Bedford, MA, USA) was thawed at 4°C and used to coat individual wells in a pre-cooled 96 well flat bottom tray (using

cooled pipette tips). The tray was incubated at 37°C for thirty minutes. Cells were prepared at a concentration of $3x10^5$ cells/ml in HUVE medium and 140µl of this cell solution was added to each well. The assays were done in duplicates. The cells were visualised at regular intervals by microscopy to observe for cell movement through the Matrigel and tube formation. Photographs were taken at baseline, and at various time points.

2.18 Immunofluorescent staining of apoptotic cells

The DAPI dye (4'6-Diamidine-2'-phenylindole dihydrochloride, Roche, Manheim, Germany) was used to stain apoptotic cells. Cells were seeded into fibronectin coated (50µg/ml) LabTek slides at 6 $X10^4$ cells per well in medium comprising varying concentrations of FCS (250µL per well). The cells were incubated at 37°C for 24 hours. The medium from the cells was aspirated and the cells washed with 100µL DAPI-methanol (1µg/ml). This was aspirated, and the cells incubated at 37°C with an additional 150µL DAPI-Methanol for 15 minutes. The solution was aspirated, and the cells washed with 150µL of methanol at RT. The slide was mounted in 2% propylgallate and stored in the dark at 4°C until visualized by fluorescent microscopy. Apoptotic cells were visualized to stain very brightly with fragmented nuclei, while live cells had intact nuclei and less intense staining. The percentage of apoptotic cells in consecutive fields was calculated, and the cells photographed.

2.19 Immunolocalization of PECAM-1

Cells were seeded into Lab-Tek slides coated with fibronectin (50µg/ml) at 3 $x10^4$ cells per well in HUVE medium supplemented with ECGS. At pre-determined time points (15 minutes and 1 hour) the medium was aspirated and the cells fixed with 4% paraformaldehyde for 10 minutes at RT. After washing with PBS, cells were incubated for one hour at RT with an

antibody directed to PECAM-1 (51-6F6) diluted to 10 μg/ml in 2% BSA in PBS. A non-relevant isotype-matched antibody directed to Keyhole Limpet Hemacyanin (23-1F11) was used as a negative control. After washing with PBS, the cells were incubated for 30 minutes at RT with goat anti-mouse IgG Alexa Fluor (Molecular Probes, Jomar Diagnostics) diluted 1:500 in PBS. The antibody was removed by washing, and the slide mounted in 2% propylgallate in glycerol. Localization of PECAM-1 at cell junctions was visualized by immunofluorescent microscopy.

2.20 Determination of neutrophil adhesion to endothelial cells

2.20.1 Isolation of neutrophils

Neutrophils were isolated from blood as follows: citrated blood was added to 1% dextran T500 (Pharmacia), and incubated at RT for 25 minutes to allow erythrocyte sedimentation. The buffy coat was placed onto an equivalent volume of cells comprising mononuclear cells and T-cells. The neutrophils were pelleted by centrifugation for 30 minutes at 1800 rpm. The supernatant was discarded, and the neutrophils re-suspended in 0.2% cold NaCl. After 40 seconds of gentle agitation, an equal volume of cold 1.6% NaCl was added. The neutrophils were again pelleted by centrifugation at 1800 rpm for 5 minutes, and then counted using crystal violet.

2.20.2 Neutrophil adhesion assay

HUVEC were seeded into fibronectin-coated Lab-Tek slides at $3x10^4$ cells per well in 300μl HUVE medium with ECGS, and incubated at 37°C for 24 hours. The medium from the cells was aspirated and the cells washed in HUVE Wash. Neutrophils isolated as above, were

added to each well at $1X10^5$ cells per well, in 150µl of HUVE medium. The cells were incubated at 37°C for 30 minutes, and then any non-adherent neutrophils were removed by washing three times with HUVE wash. The endothelial cells were then fixed with methanol treatment for five minutes at RT, and then the slide mounted using 2% propylgallate in glycerol. Adhesion of neutrophils to the endothelial cells was observed by microscopy, and photographs taken. The number of adherent neutrophils in consecutive fields was determined. The effect of endothelial cell stimulation with TNFα on neutrophil adhesion was determined.

2.21 Statistical analysis

2.21.1 Normal variables

For variables which followed a normal distribution, the Student's t-Test (paired) was used to test the null hypothesis, that there is no real difference between the two variables. In each instance, a two-sided significance test was employed, thus allowing for departures from the null hypothesis in either direction. The probability that the null hypothesis is true, and that any observed difference is due to chance variation, (the p-value derived from the Student's paired t-Test) was indicative of the significance level of the result. A probability smaller than 5% ($p<0.05$) was considered significant evidence to reject the null hypothesis.

2.21.2 Non parametric variables

Ratios do not follow a normal (parametric) distribution, and thus analysis using the student's t-Test (a parametric test) was not appropriate. Log transformation of random values however results in a normal distribution, and hence the approach below was utilized.

Analyses, stratified over replicate experiments, were performed by ANOVA style regression using Statistica Version 6.1 (Statsoft, Inc.). In general, the outcome measurements were all log transformed which ensured the predicted values were always positive and enabled interpretation of the analysis as the median fold change relative to a chosen baseline. In some cases, analysis of the untransformed outcome measurement described the data better, and the interpretation in these instances was the mean difference from the specified baseline.

The predictor variables were all treated as categorical and included experiment identifier, vector and depending on the experimental design, treatment, treatment dose or treatment time. As a general rule, interaction terms involving experiment ID were set to zero but all other interaction terms were included in the analyses. However, for some analyses (e.g. cell proliferation assays) it was appropriate to include two-factor experiment ID*vector interaction terms in the analysis to allow for random variation in the starting cell numbers. Sigma-restricted coding was used for the predictor variables to enable interpretation of the regression coefficients as mean effects, independent of other predictor variables in the analysis.
The majority of the analyses were performed by normal linear regression and the reported p-values were determined by the t test with appropriate degrees of freedom. For the VCAM-1, E-Selectin, TNFα and S1P dose response curves, there was evidence of increasing error with increasing outcome measurements in these data, therefore these analyses were performed by Poisson regression (log link) with adjustment for overdispersion. For these analyses, the reported p-values were from the Type 3 Log Likelihood Ratio Test.

Mean (μ) effects, relative to a specified baseline, and their associated standard errors (s.e.) were determined by appropriate linear contrasts of the regression coefficients. For analyses of untransformed outcome data, these were reported directly and represent the mean difference from the specified baseline. For analyses of log transformed outcome data, approximate large

sample 95% confidence intervals (CI) were obtained using the formula: μ+/- 1.96*s.e. The median fold change (relative to the specified baseline) with approximate 95% CI, were then obtained by back-transformation (i.e. exponentiation).

Table 2.1 shows primary antibodies used in either Western blot analysis or flow cytometry, the host, type, i.e. monoclonal (M) or polyclonal (P), the dilution used in Western blotting, and the source.

Antibody directed to	Host	Type	Dilution	Source
Flag epitope	Mouse	M	1:4000	Sigma, St Louis, MO
Shc	Rabbit	P	1:1000	BD Transduction Laboratories, Lexington, KY
ERK1/2	Rabbit	P	1:5000	Promega, WI, USA
active MAPK®	Rabbit	P	1:5000	Promega, WI, USA
Akt	Rabbit	P	1:1000	Cell Signaling Technology, Beverly, MA
Phospho-AKT (Ser473)	Rabbit	P	1:1000	Cell Signaling Technology, Beverly, MA
Phosphotyrosine	Mouse	P	1:1000	Cell Signaling Technology, Beverly, MA
Cyclin D1 (DCS-6)	Mouse	M	1:1000	Santa Cruz Biotechnology, California
Cyclin E (HE12)	Mouse	M	1:1000	Santa Cruz Biotechnology, California
E Selectin (49-1B11)	Mouse	M		Hanson Institute, Adelaide, Australia
VCAM-1 (51-10C9)	Mouse	M	1:500	Hanson Institute, Adelaide, Australia
VE cadherin (55-7H1)	Mouse	M	1:500	Hanson Institute, Adelaide, Australia
PECAM-1 (51-6F6)	Mouse	M	1:500	Hanson Institute, Adelaide, Australia
β1 integrin (61-2C4)	Mouse	M	1:500	Hanson Institute, Adelaide, Australia
CD34, R-phycoerythrin (R-PE)- conjugated	Mouse	M		BD Pharmingen, San Diego, CA
Actin	Mouse	M	1:1000	Santa Cruz Biotechnology, California
HA	Mouse	M	1:1000	Santa Cruz Biotechnology, California
A1	Rabbit	P	1:1000	Santa Cruz Biotechnology, California
Bim	Rabbit	P	1:1000	BD Pharmingen, San Diego, CA
Bcl-2	Rabbit	P	1:1000	BD Pharmingen, San Diego, CA
Bax	Rabbit	P	1:1000	BD Pharmingen, San Diego, CA

Table 2.2 Solutions and Buffers

Solution	Composition	Preparation and Storage
PBS	137mM NaCl, 2.7mM KCl, Na_2HpO_4 12.5mM, KH_2PO_4 1.5mM, pH 7.3	Filter sterilized
HBSS	400mg/L KCL, 60 mg/L KH2PO4, 8g/L NaCl, 350g/L NaHCO3, 47.5mg/L Na2HPO4,1g/L D-glucose	Filter sterilized , and stored at 4°C
RIPA buffer	50mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS	Stored at 4°C. 1X Protease cocktail inhibitor added immediately prior to use.
Caspase-3 lysis buffer	10% Nonidet P-40, 1M Tris-HCL, 1M EDTA	Stored at 4°C
Caspase-3 substrate buffer	12g/L Hepes, 100g/L sucrose, 1g/L Chaps, pH 7.4	Stored at -20°C
5 X sample loading buffer	50mM Tris/HCL, pH 8.0, 5mM EDTA, 100mM DTT, 5% SDS (w/v), 50% glycerol, 0.1% bromophenol blue	Stored at 4°C
10X Running Buffer	30g Tris, 144g glycine, 10g SDS in water to make up to 1 litre	Stored at RT. To use, 100 mls was diluted with 900 mls of water
Transfer Buffer	3.03 g Tris,14.4 g glycine, 200 mL methanol, 800ml mQ water	Stored at 4°C.
5% Blocking Buffer	5g non fat dried milk in 100mls PBS/ 0.1% Tween 20	Made immediately prior to use.
Annexin V Binding buffer	HBSS with 5mM Hepes, 2.5mM $CaCl_2$	Stored at 4°C.
2X sample loading buffer	20mM Tris/HCl pH 8.0, 2mM EDTA, 40mM DTT, 2% SDS, 40% glycerol, 0.1% bromophenol blue	Stored at 4°C
LB	10g/L NaCl, 10 g/L trypticasc peptone, 5g/Litre yeast extract	pH to 7.0, Autoclave, and stored at RT
LB-Agar	20g bacto-agar in 1L LB	pH to 7.0, Autoclave, and stored at RT

FACS Wash	RPMI with 10% FCS and 1% sodium azide	Stored at 4°C
FACS Fix	2% glucose, 1% formaldehyde, 5mM sodium azide, in PBS	Stored at 4°C

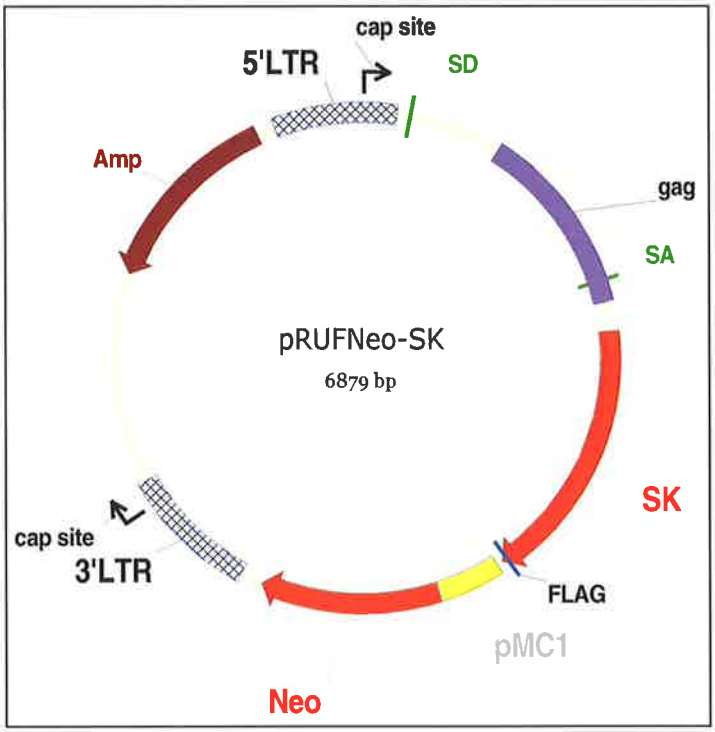
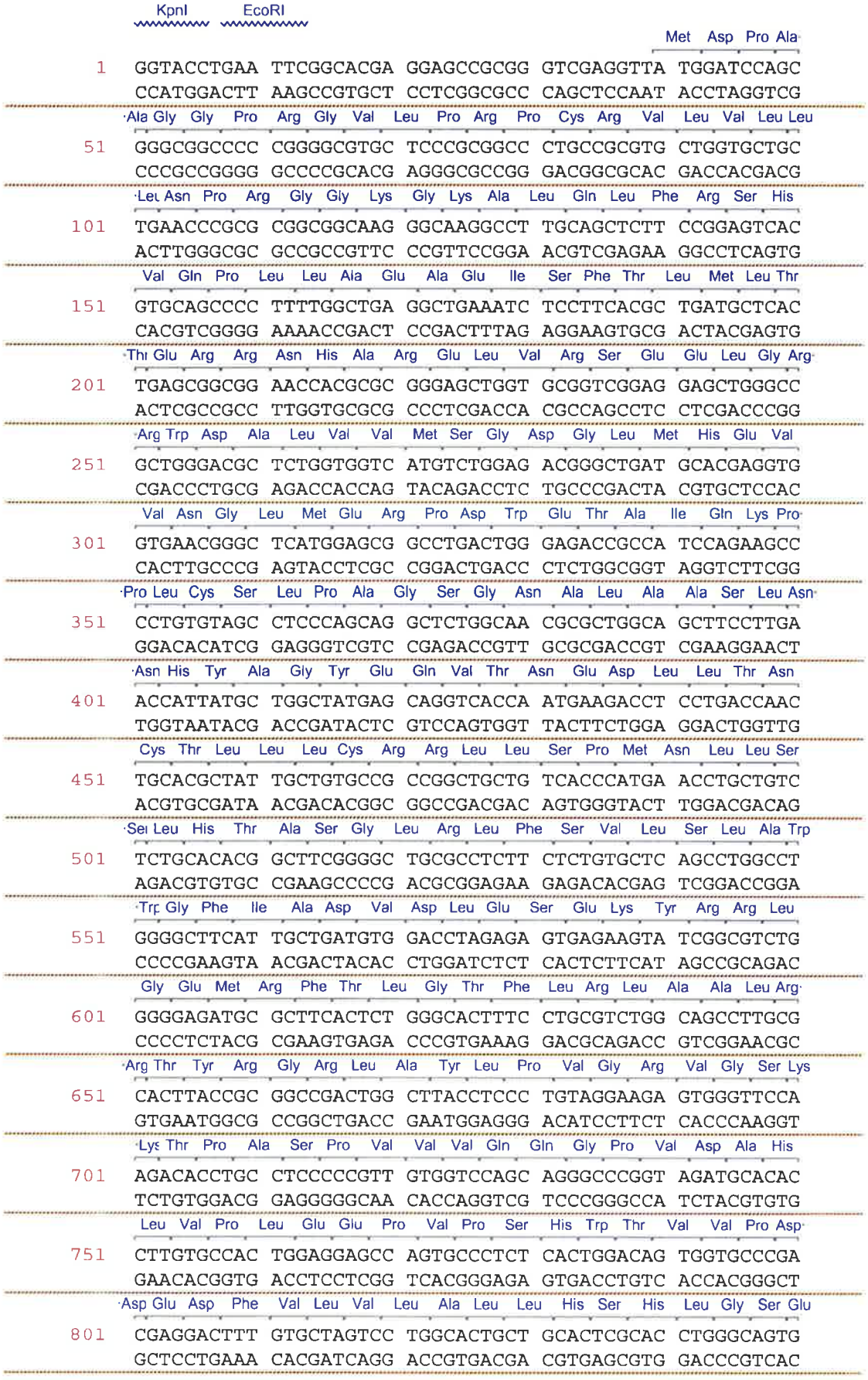


Fig 2.1 shows the PrufNeo vector into which has been cloned SK, created by Paul Moretti, Signal Transduction Laboratory, Hanson Institute, Adelaide. For pRufNeo-G82D, the SK sequence was substituted with the G82D sequence{427}. Neo denotes the neomycin resistance gene, Amp denotes ampicillin resistance gene, LTR denotes long terminal repeat, bp denotes base pairs, cap site denotes a transcription initiation site.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Ala Gly Gly Pro Arg Gly Val Leu Pro Arg Pro Cys Arg Val Leu Val Leu Leu
51
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CCCGCCGGGG GCCCCGCACG AGGGCGCCGG GACGGCGCAC GACCACGACG
Leu Asn Pro Arg Gly Gly Lys Gly Lys Ala Leu Gln Leu Phe Arg Ser His
101
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ACTTGGGCGC GCCGCCGTTC CCGTTCCGGA ACGTCGAGAA GGCCTCAGTG
Val Gln Pro Leu Leu Ala Glu Ala Glu Ile Ser Phe Thr Leu Met Leu Thr
151
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CACGTCGGGG AAAACCGACT CCGACTTTAG AGGAAGTGCG ACTACGAGTG
Thr Glu Arg Arg Asn His Ala Arg Glu Leu Val Arg Ser Glu Glu Leu Gly Arg
201
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ACTCGCCGCC TTGGTGCGCG CCCTCGACCA CGCCAGCCTC CTCGACCCGG
Arg Trp Asp Ala Leu Val Val Met Ser Gly Asp Gly Leu Met His Glu Val
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301
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351
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501
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Trp Gly Phe Ile Ala Asp Val Asp Leu Glu Ser Glu Lys Tyr Arg Arg Leu
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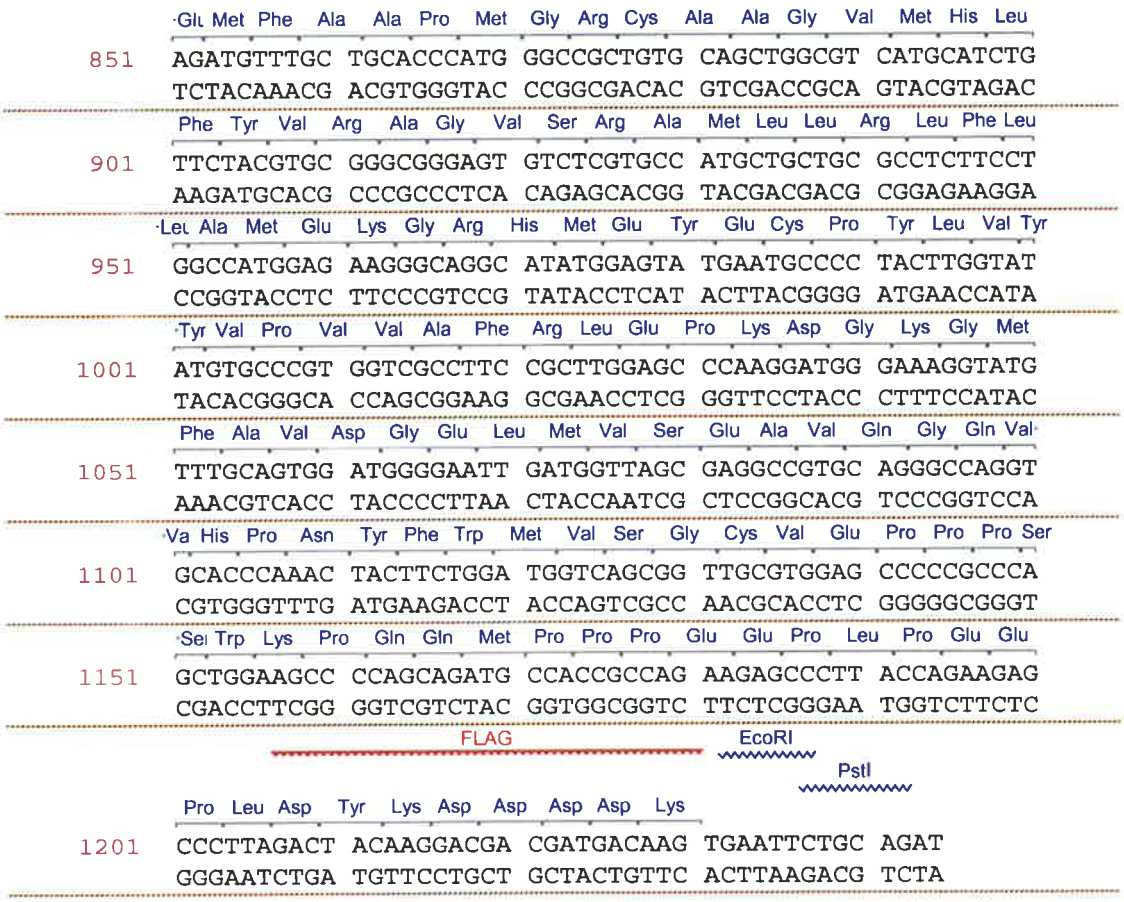
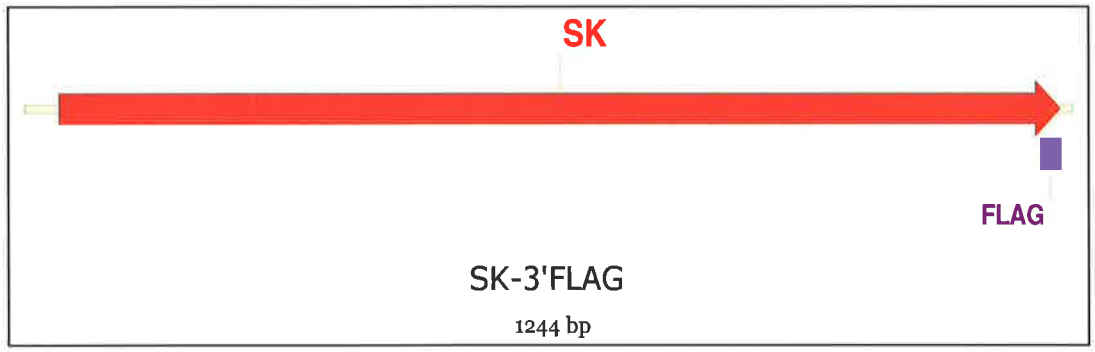
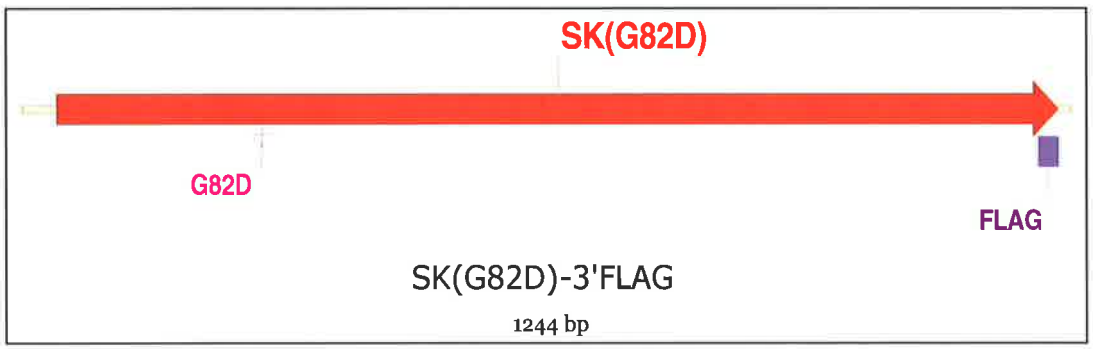


Fig 2.2 shows the nucleotide sequence (black) and corresponding amino acid sequence (blue) of SK 5'-3'. The restriction sites Kpnl and EcoR1 are indicated along with the 3' FLAG sequence of eight amino acids.



2.3(a)



2.3(b)

Fig 2.3 shows the orientation of the DNA for SK (a) and G82D (b). Both constructs have a FLAG-tag at the 3'end. bp denotes base pairs

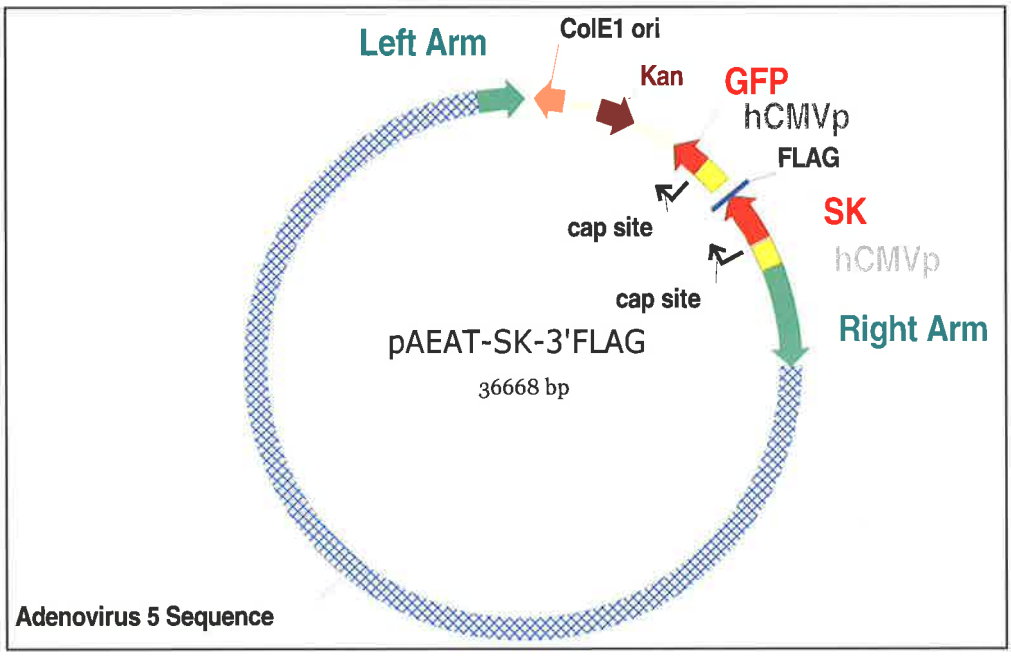


Fig 2.4 shows the configuration of the vector resulting from recombination of pAdEasy-1 with pAdTrackCMV. The insertion of SK is indicated. Alternatively, G82D was substituted for SK{427}. The vector was created by Dr. Chris Hahn and Michelle Parsons. Kan denotes kanamycin resistance gene, GFP denotes green fluorescent protein gene, bp denotes base pairs, hCMVp denotes the human CMV promoter gene.

CHAPTER 3

The phenotypic consequences of over-expression of sphingosine kinase in HUVEC achieved by retroviral-mediated gene transfer

INTRODUCTION

The endothelium provides a cellular lining to all blood vessels in the circulatory system, and forms a structural barrier between the vascular space and the tissues. This cellular layer is no longer viewed as an inert structure but rather has been recognized to be dynamic organ, important in several house-keeping functions and in disease. Endothelial cells are between 25-50μm in length, 10-15μm in width and up to 5 μm in depth. The location of the endothelium at the interface between the blood and the vessel endows upon it an obligatory role in vasoregulation, the provision of an antithrombotic surface that sustains laminar blood flow, and selective permeability to haematopoietic cells and nutrients. These activities are most evident in the microcirculation where the endothelial cell surface area: blood volume ratio is maximal.

The development of in vitro endothelial cell culture techniques in 1973 (162-164) has provided a useful tool for the investigation of endothelial function in both health and disease. The degree to which this model resembles the in vivo environment has been questioned, and thus the extent to which results may be extrapolated to the clinical setting has been a subject of much debate. Indeed it is now recognized that endothelial cell culture in itself activates the cells, with an increase in replicative rate from the negligible in vivo rate of 0.1% replications per day, to 1%-10% replications per day(165). Moreover certain integrins ($\alpha v\beta 3$ integrin) which are not expressed by quiescent endothelial cells in vivo become detectable in culture systems (165). A further concern with the use of HUVEC as a model of all endothelial cells is the extent to which these vascular cells (of venous origin) are representative of endothelial cells from the microvessels and arteries. Certain properties of endothelial cells however are

uniformly displayed regardless of the vascular bed of origin, such as the response to TNFα in up-regulation of adhesion molecules. It is more the differences in vasomotor regulation which vary from endothelial cells from one vascular bed to another(166). The above limitations of endothelial cell culture systems not discounted, endothelial cell culture has allowed advances in the understanding of inflammatory diseases, cancer/metastases, and in the development of gene therapy for vascular disorders such as restenosis. The use of HUVEC has the advantage over endothelial cells from other sources as it is human in origin, supply is not generally problematic, and complicated, time-consuming preparation of cells is not required.

Aberrant angiogenesis is recognized to be a key feature of many inflammatory diseases such as RA, as well as diabetic retinopathy, tumour growth/ metastasis hence much research has been directed to the pathophysiology of the vascular endothelium. Moreover, the endothelium is an attractive candidate tissue for gene transfer which may potentially provide promising therapeutic strategies for vascular diseases. Delivery of gene expression constructs into the endothelium in vitro, however, is the initial stage of development of these therapeutic strategies, and is a useful means of delineating the function of a particular gene. Indeed the effects of over-expression or deletion of certain genes has expanded knowledge of the normal functions of these genes.

Traditionally, either bovine aortic endothelial cells (BAEC), bovine retinal endothelial cells or HUVEC have been used for such studies. Unlike many other cell types, human endothelial cells are notoriously difficult targets for gene transfer and are often relatively refractory to traditional methods of gene delivery.

There are numerous methods for introducing a gene of interest into a cell (transfection) and these are categorized as non-viral methods or viral methods (infection or transduction). The method chosen depends largely on the cell type involved, the transfection efficiency required, and whether the cells need to be actively replicating.

There are various non-viral methods of gene transfer. Calcium phosphate transfection involves the phagocytosis of DNA contained within a calcium phosphate precipitate. This method is inexpensive and simple to perform, but is exquisitely dependent on pH as well as the size of the precipitate and has variable efficiency (0.1-10%) (167;168). Efficiency is enhanced by an osmotic insult to the cells (15% glycerol) 4-16 hours after transfection, however this is toxic to endothelial cells. DEAE dextran and polybrene have also been used for many years. Both are poly-cations and thus complex with negatively charged DNA and, due to the relative excess of positive charge, adhere to cell membranes and are taken up by endocytosis.

Lipid-mediated gene delivery uses the principle of cationic lipid particles complexing with negatively charged DNA, and the subsequent delivery of the lipid-DNA complex into the cell. This method allows a gene construct of unlimited size to be delivered, requires relatively small quantities of DNA, and requires no carrier DNA. Although transfection efficiencies for many cells have been reported as high, the situation with HUVEC remains disappointing. Indeed many groups have not been able to obtain any transfection in HUVEC(169) and others have had limited success, for example approximately 5% transfection efficiency (167). The specific liposome used influences the transfection efficiency, and the liposome found to be the best delivery vehicle into HUVEC is the Perfect Lipid (pFx-7) mixture(170). The adsorption of apolipoprotein E onto lipid particles facilitates uptake into the cells by specific cell surface receptors and has enhanced transfection efficiency in HUVEC to 22.6%(171)

Electroporation involves the delivery of DNA into the cell by providing a high voltage current to disrupt the plasma membrane. This has been employed in HUVEC, however not without significant cytotoxicity(172;173). Microinjection of DNA into the cells is an alternative approach, however transfection of a large number of cells is not generally feasible.

Viral methods for gene transfer include those utilizing adenoviral and retroviral vectors. Retroviral gene transfer involves the insertion of the gene of interest (insert capacity is approximately 8kb) into a replication-defective retrovirus containing a drug resistance (e.g. neomycin, G418) gene(174). The long terminal repeats intrinsic to the retrovirus may be used as the promoter or various internal promoters, intrinsic to the virus may be used to drive the production of the gene of interest. The replication-defective retrovirus containing foreign DNA is then transfected into a packaging cell line. The packaging cell line contains *gag, pol,* and *env* proteins which allow the production of complete viral particles containing the defective RNA of the foreign gene. The infectious retrovirus then is used to infect the cell line of interest, eg HUVEC. The transgene randomly integrates into the host genome in a stable and permanent fashion in a small proportion of cells, which are then amplified by selection for drug resistance. One proviso is that the cells must be replicating actively for retroviral infection to proceed. Using this method, viral titres of 10^6-10^8 cfu/ml have been obtained, and yield can be augmented by ultrafiltration of the retrovirus. In HUVEC, transfection efficiencies of 57% using retroviral vectors have been described (175).

AIM

The aim here is twofold, firstly, to establish a method for the generation of stable endothelial cell lines over-expressing SK using a retroviral vector, and secondly, to determine the

phenotype of these cells, and specifically determine whether they have an angiogenic and inflammatory phenotype as hypothesized.

RESULTS

3.1 Development of Retroviral mediated gene transfer into HUVEC

The retroviral vector used was PRufNeo (161)into which was cloned flag-epitope tagged sphingosine kinase or flag-epitope tagged dominant-negative (catalytically inert) sphingosine kinase (G82D)(127). This DNA vector was transfected by calcium phosphate precipitation into Bing cells (a HEK293 packaging cell line). At the time of collection of the retroviral supernatant (48hours) the Bing cells were permeabilised and stained with anti-flag antibody to determine the transfection efficiency by flow cytometry.

3.1.1 Efficiency of Bing Cell Transfection

3.1.1.1 Titration of anti-flag antibody

Efficiency of Bing cell transfection was assessed by flow cytometry by permeabilising and staining the cells with anti-flag antibody. The "gates" for positive and negative fluorescence on the flow cytometer were arbitrarily assigned by calibration using unstained, un-transfected Bing cells (negative control), and SK-HEK293 cells (positive control). The gates for positive fluorescence were set to encompass the region of mean fluorescence for ≥95% of SK-HEK cells stained with anti-flag antibody, but < 5% of unstained, un-transfected Bing cells. Conversely, the gate for negative fluorescence was defined as the region where the mean

fluorescence of ≥95% unstained, untransfected Bing cells overlay the region where < 5% of SK-HEK cells stained with anti-flag antibody.

The optimal working dilution of anti-flag antibody (4.4mg/ml stock concentration) to be used in assessing the efficiency of Bing cell transfection was determined by flow cytometry to be 1/6000. At this dilution, 71.8% of cells transfected with SK showed positive fluorescence, with a background of non-specific staining of 4.2% in un-transfected Bing cells, and 5.8% in Bing cells transfected with empty vector (Fig 3.1)

3.1.1.2 Efficiency of Bing Cell Transfection

The efficiency of Bing cell transfection as indicated (by flow cytometry) by permeabilizing and staining with anti-flag antibody (1/6000 dilution) ranged from 40% to 71.8% in three separate transfections (Fig 3.2).

3.1.2 Determining Working Retroviral Titre

Viral titration of the retroviral supernatant on NIH 3T3 cells yielded best results with a starting dilution of 1/10. At this dilution, numerous colonies had formed, and had merged together (Table 3.1). At further dilutions lower colony yields were observed.

3.1.3 Retroviral infection of HUVEC

Factors found to be critical in retroviral infection of HUVEC

1. Cell density at the time of infection: HUVEC were most readily infected at a low cell density, however, if cells were plated too sparsely, cell death occurred. Seeding

$1.3x10^5$ cells per well in a six well tray 24 hours prior to infection yielded optimal results, however it was estimated that only 1-3 in every 10^5 HUVEC became infected by retrovirus, i.e. the efficiency of HUVEC transfection was exceedingly low and in the order of 0.001%.

2. Dose of G418 for selection: Traditional doses of G418 used for selection of resistant colonies in NIH3T3 cells (400μg/ml) were toxic to HUVEC. A dose of 150μg/ml allowed the emergence of resistant clones, whilst maintaining toxicity to non-infected cells. The window for the dose of G418 was narrow; a dose of 200μg/ml showed marked reduction in colony formation, and conversely, 100μg/ml allowed non-infected cells to escape selection.
3. Timing of G418 selection: G418 was toxic to uninfected cells only if actively replicating, and thus a need for selection to be initiated whilst cells were subconfluent. Conversely, initiating selection too early, 24 hours post infection when cells were approximately 50% confluent, did not allow colony formation. The optimal time for selection to proceed was 48 hours post-infection, at which time, cells were slightly subconfluent, however sufficient time had elapsed for DNA integration to occur.
4. Time for harvesting: Yield was maximized by harvesting cells when colonies were tightly packed, beginning to merge, and when all cells in the mock transfection had been killed. This was generally achieved 10-14 days post infection.
5. Cell culture: Once harvested, cell growth was most efficient in the absence of G418 however, the cells were treated with intermittent pulses of G418 150μg/ml (48 hours each week) to maintain selection pressure.

3.1.4 Detection of over-expression of SK in HUVEC

3.1.4.1 Confirmation by Western blot

Expression of SK in HUVEC was confirmed by Western blot using anti-flag antibody (Fig 3.3). Definite expression was confirmed in cells over-expressing SK in all cell lines used for further experiments.

3.1.4.2 Determination of SK activity in cells over-expressing SK

Stimulation of endothelial cells with TNFα results in approximately 165% increase in SK activity above basal which is transient, returning to basal levels within thirty minutes after stimulation (122) In order to determine whether over-expression of SK confirmed by Western blot resulted in enhanced enzymatic activity, SK activity was measured in cells over-expressing SK and control.

Over-expression of SK resulted in a moderate (but significant) increase in SK activity above control (Fig 3.3). The mean fold increase in SK activity above control was 3.5 (95% confidence interval 1.93-6.41) in five separate endothelial cell lines. These SK activity assays were performed by Dr. Stuart Pitson in the Division.

3.1.5 Conclusions

The methodology for the generation of HUVEC over-expressing SK using retroviral vectors was thus established. This resulted in moderately enhanced SK activity (approximately fourfold above basal) and set the scene for an investigation of the phenotype of these cells.

3.2 Does over-expression of SK result in features of enhanced angiogenesis?

It is known that S1P in endothelial cells serves as an angiogenic regulator by virtue of its actions on EDG-1 and EDG-3, as well as its actions as an intracellular second messenger. It

was thus sought to determine whether raised intracellular levels/ activity of SK promote angiogenesis. The cardinal features of angiogenesis are enhanced cell survival, proliferation, reduction in apoptosis and up-regulation of specific integrins. Hence these phenotypic alterations were used as surrogate measures of angiogenesis in cells over-expressing SK.

3.2.1.1 Over-expression of SK enhances cell accumulation

The MTS colorimetric assay was used to measure cell proliferation. In this assay the absorbance (optical density) is proportional to the number of viable cells and thus cell accumulation over a defined period can be assessed. Relative cell accumulation was defined as the ratio of the optical density at Day 3 and Day 0, and is recognized to reflect a sum of cell proliferation and cell death.

Over-expression of SK did not alter cell accumulation compared with control in normal culture conditions comprising 20% FCS ($n=3$, $p=0.719$).

Cell accumulation was measured in medium supplemented with 2% FCS. As shown in Fig 3.4(a), although there was no difference in the numbers of cells over-expressing SK compared with control that were seeded at Day 0 ($p=0.60$), at Day 3, cells over-expressing SK showed significantly enhanced cell numbers (greater cell accumulation) compared with control. Analysis of 18 observations derived from six separate experiments revealed the mean fold increase in relative cell number over three days for EV to be 1.8 (95% confidence interval 1.44-2.27), $p<0.001$, and for SK 4.4 (95% confidence interval 3.54-5.58), $p<0.001$, as shown in Fig 3.4(b). Cells over-expressing SK showed a 2.5-fold increase in relative cell accumulation compared with control (1.78-3.39), $p<0.001$, after adjusting the value for Day 0, as indicated in Fig 3.4(c).

3.2.1.2 Over-expression of SK enables cell proliferation in the absence of serum

Serum is a recognized trophic and survival factor for endothelial cells, and routine culture of endothelial cells is thus undertaken in medium supplemented with FCS. In order to determine whether the enhanced cell accumulation seen in cells over-expressing SK was dependent on serum, cell accumulation was measured (using the MTS assay) over three days in serum-free medium supplemented with ECGs.

Cells over-expressing SK showed significantly enhanced cell accumulation in the absence of serum unlike control cells which did not increase in relative cell number ($p=0.31$) (Fig 3.5).

3.2.1.3 The effect of trophic factors on SK-mediated enhanced cell accumulation

3.2.1.3(a) Over-expression of SK enhanced proliferation in response to FGF or VEGF alone

Cytokine growth factors are involved in the process of angiogenesis. In HUVEC, VEGF and FGF enhance mitogenesis and are critical to cell proliferation and angiogenesis. To delineate whether cells over-expressing SK were dependent upon either FGF or VEGF for enhanced cell accumulation, cell proliferation was measured over three days in the presence of either FGF or VEGF alone (in medium containing 2% FCS). The concentration of the growth factors used was based on prior dose-response studies undertaken in our laboratory by Dr. C. Hahn, in which the optimum working dose for cell proliferation had been ascertained. This dose was 1 ng/ml for FGF, and 10 ng/ml for VEGF.

Cells over-expressing SK maintained significantly enhanced cell accumulation over control when cultured in the presence of VEGF or in the presence of FGF (Fig 3.6).

3.2.1.3(b) Over-expression of SK leads to a preferential response to FGF over VEGF

Given the enhanced cell accumulation of cells over-expressing SK in the presence of either VEGF or FGF, it was sought to determine whether these cells respond preferentially to either trophic factor. Cells over-expressing SK showed significantly enhanced cell accumulation when cultured in the presence of FGF compared with VEGF ($p<0.001$), while the EV showed no preferential response ($p=0.43$), as shown in Fig 3.6(c).

3.3 Conclusions

I thus concluded that over-expression of SK enhances cell accumulation above control over three days in 2% FCS. This effect is independent of the trophic effects of serum, and is maintained in the presence of either FGF alone or VEGF alone. Furthermore cells over-expressing SK show a preferential response to FGF over VEGF in terms of cell accumulation, and this may provide an insight into the underlying mechanisms.

3.4 The effect of over-expression of SK on interactions of the endothelial cells with the extracellular matrix

3.4.1 Over-expression of SK targets specific integrins

The integrin group of cell surface receptors mediate the attachment of endothelial cells with the extracellular matrix. Many (especially β1 integrins) have been linked to cell proliferation

and regulation of angiogenesis. Hence cell surface expression of integrins in endothelial cells over-expressing SK was measured by flow cytometry to determine the median fluorescence intensity.

Over-expression of SK resulted in a significant increase in β1 integrin expression and α2β1 integrin (Table 3.2). There was also a trend to increased αvβ3 integrin which was not statistically significant.

Over-expression of SK did not alter α5β1 integrin ($p=0.38$) or α1β1 integrin ($p=0.12$) expression. Thus over-expression of SK targets selected integrins on endothelial cells.

3.4.2 Over-expression of SK increases adhesion to extracellular matrix

To determine whether the up-regulation in β1 integrin expression induced by over-expression of SK had functional consequences, attachment of cells to various matrices was measured using the colorimetric MTS assay. The pooled data of three separate endothelial cell lines each measured in triplicate showed that SK increased adhesion to gelatin ($n=9$, $p<0.05$), fibronectin ($n=9$, $p<0.05$), and collagen ($n=9$, $p<0.05$) at 20', 1hr, and 3hour time points consistent with up-regulation of β1 integrin. Attachment to laminin ($n=9$, $p<0.05$) at these time points was also increased.

The profile for attachment to fibronectin is shown in Fig 3.7. Cells over-expressing SK significantly enhanced cell adhesion to fibronectin compared with control, at all time points tested.

3.5 Over-expression of SK modulates cell junctional proteins

Endothelial cell viability depends not only on integrin-mediated attachments with the extracellular matrix but also on cell-cell contact, which is regulated at least in part by the junctional molecules VE cadherin and PECAM-1. Cell surface expression of VE cadherin and PECAM-1 was measured by flow cytometry.

Over-expression of SK consistently increased the cell surface expression of PECAM-1 and VE cadherin as shown in Fig 3.8. Analysis of the pooled data from these endothelial cell lines over-expressing SK depicted in Fig 3.8, showed the mean fold increase above control for PECAM-1 expression was 1.42 (95% CI 1.05-1.91), n=3, p=0.08, and for VE cadherin expression was 1.41 (95% CI 1.17-1.69), n=5, $p< 0.05$.

The lack of a statistically significant increase in PECAM-1 expression by over-expression of SK may reflect the small sample size, and the large sample confidence intervals used, which were not adjusted for the degrees of freedom. Despite the marginally non-significant p-value, the consistent up-regulation of PECAM-1 by SK is likely to be of biological significance, as will be discussed later.

3.6 Over-expression of SK results in a resistance to apoptosis

Total cell accumulation represents the sum of cell proliferation and cell death. In view of the SK-induced enhanced cell accumulation, it was sought to determine whether this was in part attributable to a reduction in cell death. The caspase family of proteolytic enzymes are the executers of cell death, and in this cascade, caspase-3 plays an important role, representing the point at which the death signal becomes irreversible and commitment to death occurs.

Caspase-3 activity was measured (based on caspase-3-mediated cleavage of the fluorogenic substrate DEVD-AFC) in cell lysates made in normal culture conditions.

Over-expression of SK significantly reduced mean basal caspase-3 activity compared with control (Fig 3.9). This indicates that the enhanced cell accumulation seen with over-expression of SK (compared with control) is at least in part due to less cell death in this population.

3.7 Three atypical cell lines in which phenotypic consequences of over-expression of SK were not evident

There were three HUVEC lines in which attempted over-expression of SK did not confer the phenotypic change of enhanced cell accumulation. These cells were able to survive in selection medium, but on Western blot analysis using anti-flag antibody, showed comparatively weak expression. Consistent with this there was no appreciable increase in SK activity in these three lines ($p=0.77$).

Phenotypic analysis of these atypical cell lines

Attempted over-expression of SK in these three atypical lines did not up-regulate β1 integrin ($p=0.28$), VE Cadherin expression ($p=0.61$), or caspase-3 activity ($p=0.35$), as shown in Fig 3.10. Attachment to gelatin, fibronectin, collagen, and laminin was measured in one of these atypical cell lines and there was no change in the adhesive properties of these cells. PECAM-1 expression was measured in one of these three lines and was not altered.

Phenotypic analysis of these three cell lines served as a useful negative control for endothelial cell lines in which enhanced SK activity was evident.

3.8 Conclusions

I concluded that over-expression of SK enhances cell accumulation, up-regulates selected integrins, alters the cell junctional proteins and in addition, confers a resistance to apoptosis as indicated by reduction in caspase-3 activity. These are all features seen in angiogenesis, and thus I concluded that moderately raised intracellular levels and activity of SK favour angiogenesis.

3.9 Does over-expression of SK enhance inflammatory potential?

The cardinal features of endothelial activation, at least when induced by inflammatory cytokines are enhanced expression of adhesion molecules such as VCAM-1 and E Selectin. Cell surface expression of these adhesion molecules, along with the response of endothelial cells to stimulation with TNFα was examined in order to test the hypothesis that over-expression of SK converts the quiescent endothelium to the activated, pro-inflammatory state.

3.9.1 The effect of over-expression of SK on endothelial activation

The normal quiescent endothelium does not express E Selectin or VCAM-1. Stimulation of endothelial cells with TNFα results in adhesion molecule expression (VCAM-1 and E Selectin), which has been shown to be mediated by SK. To determine whether over-expression of SK results in changes to the endogenous phenotype of endothelial cells, adhesion molecule expression on cells over-expressing SK was determined by flow cytometry.

In five separate endothelial cell lines, over-expression of SK resulted in a consistent, small, but statistically significant increase in basal VCAM-1 expression compared with control (Fig 3.11), indicating endothelial activation. There was however no consistent change in basal E Selectin expression in four separate endothelial cell lines over-expressing SK (p=0.44) (Fig 3.11).

3.9.2 The effect of over-expression of SK on the adhesion molecule response to TNFα

3.9.2.1 Over-expression of SK augments the magnitude of the response to TNFα

Stimulation of endothelial cells with TNFα results in between a ten and fifteen-fold increase in the cell surface expression of VCAM-1 which peaks at 8-24 hours and a fifteen to twenty-fold increase in the cell surface expression of E Selectin which peaks at 4-6 hours (122). To delineate whether cells over-expressing SK altered the response to TNFα, cells were stimulated with TNFα (0.5ng/ml) for four hours, and basal and stimulated VCAM-1 and E Selectin levels were measured by flow cytometry. As dominant-negative SK (G82D) blocks agonist-induced SK activation, the effect of over-expression of G82D on the endothelial response to TNFα was also investigated.

Cells over-expressing SK showed a significantly greater VCAM-1 response to TNFα (p=0.016) compared with control and, over-expression of G82D resulted in significant inhibition of this response compared with EV (p=0.023) (Fig 3.12).

There was a trend towards augmenting the magnitude of the E Selectin response to TNFα in cells over-expressing SK, which did not reach statistical significance (p=0.08). It is noted however that in all four endothelial cell lines tested, over-expression of SK resulted in a

consistent increase (range 15-80% above control) in the magnitude of the E Selectin response to TNFα. The lack of statistical significance needs to be interpreted in the context of the small sample size (n=4), and the large sample confidence intervals used. Over-expression of G82D caused a marked inhibition of the E Selectin response to TNFα (p=0.015).

3.9.2.2 The effect of over-expression of SK on the duration of the E Selectin response to TNFα

TNFα induction of E Selectin on endothelial cells peaks at 4-6 hours and declines to near basal levels by 18-24 hours. An alteration of this time course in cells over-expressing SK was sought. Cells were stimulated with TNFα for 18 hours (0.5ng/mL) and E Selectin levels measured by flow cytometry.

In three endothelial cell lines over-expressing SK there was a consistent but statistically insignificant increase in the duration of the TNFα- induced E Selectin response (p=0.09), (which ranged from 8% to 360% above control) as shown in Fig 3.12. Over-expression of G82D significantly inhibited the response to TNFα in two of these same cell lines (p=0.014).

3.9.3 Conclusions

I concluded that over-expression of SK causes endothelial activation in its own right as evidenced by up-regulation of VCAM-1 expression. It acts to further potentiate the pro-inflammatory effects of TNFα by augmenting the VCAM-1 response to TNFα and with a trend towards augmenting the magnitude and duration of the E Selectin response to TNFα. The inhibition of the TNFα-induced adhesion molecule response by over-expression of dominant-negative SK confirms that the observed effects are mediated specifically by SK,

and do not represent an aberrant response to over-expression of a non-specific protein in endothelial cells.

DISCUSSION

HUVEC are relatively refractory to many traditional transfection techniques, and hence the need to resort to intricate and time-consuming methods of transfection. Retroviral- mediated sphingosine kinase over-expression in HUVEC allowed the development of stable cell lines which in itself has implicit virtues. However there are several disadvantages of this system. As the infection efficiency of HUVEC was so low ($\approx$1 in 10^5 cells), there was a time delay in culture (approximately one month) until cell numbers were sufficient for functional assays, and thus cells of older passage were used. Prolonged cultivation allows the development of secondary changes in endothelial cell phenotype, and resembles less and less the physiological in vivo state. Secondly, the process of selection introduces a bias in selecting for cells which may grow under the influence of the gene. Indeed cells which had become transfected with very high level expression of SK may not have been allowed to grow in this system. Thirdly, the selection process with G418 may itself induce unrecognized changes in the cells and introduce other confounding factors.

Notwithstanding these potential limitations, retroviral-mediated gene delivery of sphingosine kinase achieved, for the first time, endothelial cell lines with stable and chronic over-expression of sphingosine kinase. This provided a novel model which allowed the effects of long term intracellular over-expression of sphingosine kinase in HUVEC to be delineated. Moreover concerns that retroviral-mediated gene delivery may unfavourably influence EC phenotype have not been substantiated by the work by Inaba M et al which showed that

HUVEC transduced by retroviral vectors were not activated, had normal cell growth, and did not show changes in inducible cell adhesion molecule expression(175).

It has been shown here that stable over-expression of SK in HUVEC confers upon the cells an angiogenic and inflammatory phenotype as hypothesized.

The mechanisms and intracellular regulation of cellular survival and proliferation have been the focus of much interest, and it is clear that the integrin group of cell surface receptors play a critical role. Integrins are heterodimeric transmembrane receptors which mediate attachment of endothelial cells to the extracellular matrix. Furthermore, integrin ligation triggers intracellular signals which regulate cell survival, proliferation and differentiation.

β1 integrin plays a critical role in cell proliferation, and disruption of β1 integrin function has led to reduced epithelial cell proliferation in mouse mammary gland (176).
SK-induced up-regulation of β1 integrin is consistent with enhanced cell accumulation, and the induction of α1β1 integrin is novel, as this integrin is normally present on microvascular endothelium, and absent from large vessel endothelium (such as HUVEC). Although the magnitude of the changes induced by SK were relatively small, there were functional consequences evidenced by heightened cell attachment to specific extracellular matrices.

The integrin αvβ3 plays a critical role in angiogenesis, and inhibitors of this integrin have been shown to block angiogenesis in the chorioallantoic membrane model for angiogenesis(177). There is negligible expression of αvβ3 integrin on quiescent endothelial cells, but high levels of expression in rheumatoid synovium (178) Over-expression of SK caused an increase in αvβ3 integrin expression in each of three cell lines tested. The increase in αvβ3 integrin in cells over-expressing SK is consistent with the preferential response to

FGF over VEGF in terms of cell accumulation, as FGF is known to signal through αvβ3 integrin (whereas VEGF signals through αvβ5 integrin (177).

Over-expression of SK provided protection against apoptosis, as reflected by a reduction in basal caspase-3 activity. This is consistent with the documented anti-apoptotic effects of S1P(97;130;131), and is in keeping with the enhanced cell accumulation, a critical feature of angiogenesis. The caspase family of proteolytic enzymes constitute the cell's machinery for executing cell death. In this sequential cascade(179) caspase-3 plays a particularly important role, being an effector caspase, and representing the point at which the death signal becomes irreversible(180).

Cell-cell interactions are recognized to regulate diverse cellular events including survival and proliferation (181), and in endothelial cells such interactions also regulate the transendothelial migration of leukocytes (182). Indeed over-expression of SK was shown to influence positively the expression of the cell junction proteins VE Cadherin and PECAM-1. The consistent upregulation of PECAM-1 expression in three separate endothelial cell lines over-expressing SK is intriguing, as PECAM-1 is an otherwise stable, long- lived protein. PECAM-1 is a 130kDa transmembrane glycoprotein of the Ig superfamily, which is constitutively expressed, and concentrated at sites of cell-cell contact. PECAM-1 is regulated by cell-associated mechanical forces, fluid shear stress, osmotic shock and TGFβ (183),(184),(185). In myeloid cells, PECAM-1 is regulated by modulators of NFκB (TNFα, PMA), as there are two consensus NFkB-binding sites in the PECAM-1 promoter. PECAM-1 has a role in cell-cell adhesion (186;187), and leukocyte transmigration (188). It protects against apoptosis induced by serum deprivation by both up-regulation of protective genes A20 and A1 (189), and by virtue of homophilic PECAM-1-PECAM-1 interactions (190). The SK-

induced induction of PECAM-1 expression may thus provide a mechanism for reduction in apoptosis.

Over-expression of SK up-regulated basal VCAM-1 expression in HUVEC, thus inducing a pro-inflammatory phenotype in its own right. It further augmented the endothelial responses to the pro-inflammatory effects of TNFα. The eventual up-regulation of E Selectin with very long term over-expression of SK in the single EC line which became immortalised is further support for a role of SK in mediating inflammation. Clearly VCAM-1 and E Selectin are regulated independently. Upregulation of PECAM-1 by over-expression of SK may also alter the inflammatory potential of these cells, given the recognized role of PECAM-1 in leukocyte transmigration. During leukocyte transendothelial migration, homophilic PECAM-1-PECAM-1 interactions between adjacent EC are temporarily replaced by EC-leukocyte PECAM-1 interactions to allow passage of the leukocyte(182).

In summary, moderately raised intracellular SK activity induces an angiogenic phenotype (enhanced cell accumulation, up-regulation of integrins, resistance to apoptosis), and an inflammatory phenotype, as hypothesized. The alterations in integrin expression and junctional molecules, along with the reduction in caspase-3 activity may provide a partial insight into the cellular changes responsible for the phenotypic changes described. Indeed there is cross talk between integrins and PECAM-1, and integrin engagement is known to result in PECAM-1 dephosphorylation in endothelial cells (191). It is hypothesized that SK-mediated regulation of cell junctional molecules and in particular, PECAM-1, plays a central role in mediating enhanced cell accumulation and resistance to apoptosis. The results described here not only confirm the hypothesis that intracellular SK activity is involved in the regulation of angiogenesis and inflammatory parameters, but raise a number of important questions. Firstly, is the SK-mediated enhanced cell accumulation a result of increased cell

proliferation, reduction in cell death, or a combination of the two? Secondly, what role do the junctional molecules play in mediating such changes and, do similar phenotypic alterations (of enhanced cell accumulation and resistance to apoptosis) occur when signalling via the junctional molecules is inhibited? Thirdly, does raised SK activity alter other measures of angiogenesis, such as cell migration and cellular remodeling? Fourthly, as the magnitude of the changes in endothelial adhesion molecule expression consequent upon raised intracellular SK activity were small, do they have functional consequences in terms of enhanced leukocyte binding?

Clearly such questions warrant further investigation. However, in light of the limitations of retroviral-mediated gene delivery which have been discussed, and in particular, the low transfection efficiency of HUVEC with retrovirus, this system was not the appropriate system for addressing the questions raised above. Thus prior to further enquiry regarding the intracellular mechanisms and signalling pathways involved, the question can similar changes be reproduced with alternate methods of SK gene delivery is to be addressed first.

Viral Dilution	EV	SK
1/10	Colonies merged	Colonies merged
1/100	9, 13	17, 20
1/1000	5, 0	2, 1
$1/10^4$	0, 0	1, 0

(a)

Viral dilution	EV	SK	G82D
1/10	58, 54	54, 50	60,51
1/100	42, 30	18, 22	26,18
1/1000	8, 6	15, 22	10,6
$1/10^4$	0, 2	4, 6	0,0,
$1/10^5$	0,0	0,0	0,0

(b)

Table 3.1 shows the number of infected colonies of NIH-3T3 cells formed (in duplicates trays) when infected with varying dilutions of two different batches of retroviral supernatant (a,b) carrying EV, SK or G82D. Colonies were manually counted after staining with Giemsa stain.

Integrin	MFI and 95% CI	
β1 integrin	1.5 (1.39-1.71)	n=5, p< 0.001
α2β1 integrin	1.5 (1.15-1.86)	n=3, p< 0.05
αvβ3 integrin	3.3 (1.23-8.55)	n=3, p=0.07
α1β1 integrin	1.8 (1.01-3.07)	n=3, p=0.12
α5β1 integrin	1.4 (1.02-3.15)	n=3, p=0.38

Table 3.2 shows the SK-induced mean fold increase above control (MFI) in median fluorescence intensity for integrins β1, α2β1, αvβ3, α1β1 and α5β1. The 95% confidence intervals associated with the mean are indicated along with the corresponding p values and number of experiments (n).

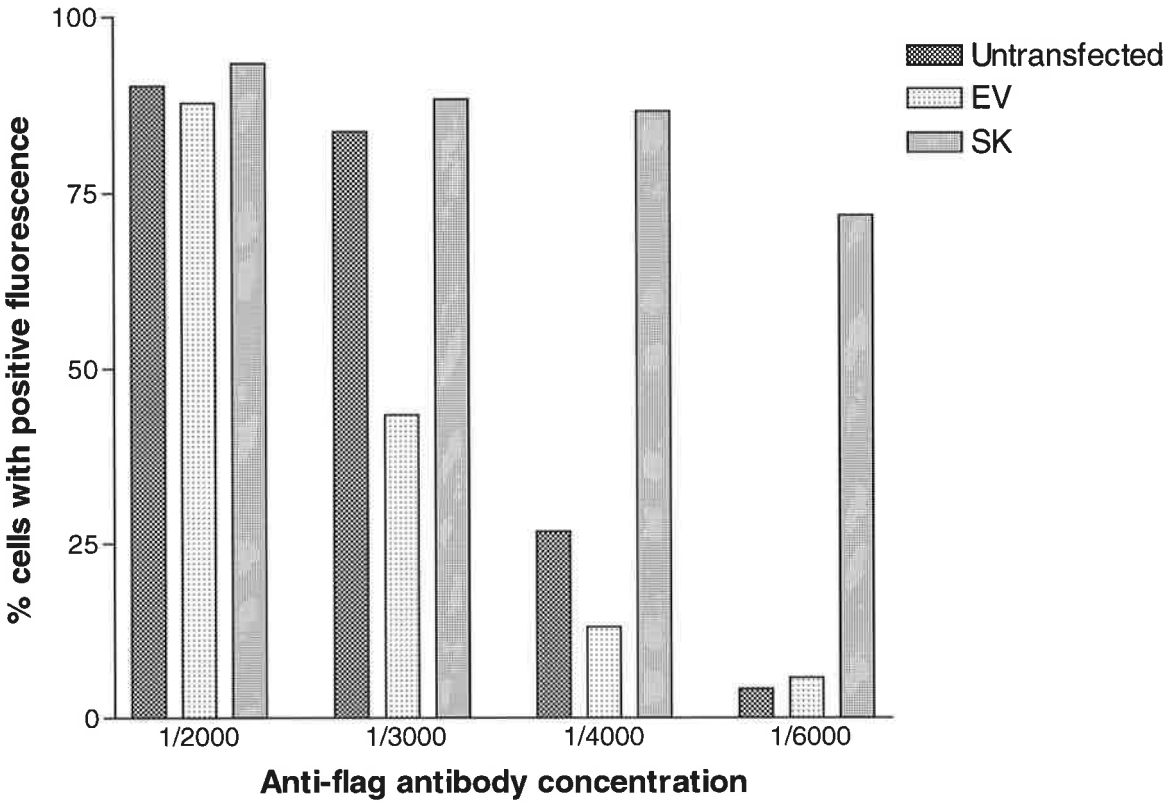


Fig 3.1 shows the percentage of untransfected Bing cells, and Bing cells transfected with SK, or empty vector (EV) which showed positive fluorescence when permeabilised and stained with varying concentrations of anti-flag antibody. Background fluorescence resulting from staining with a non-relevant isotype matched control antibody (23-1F11) has been subtracted.

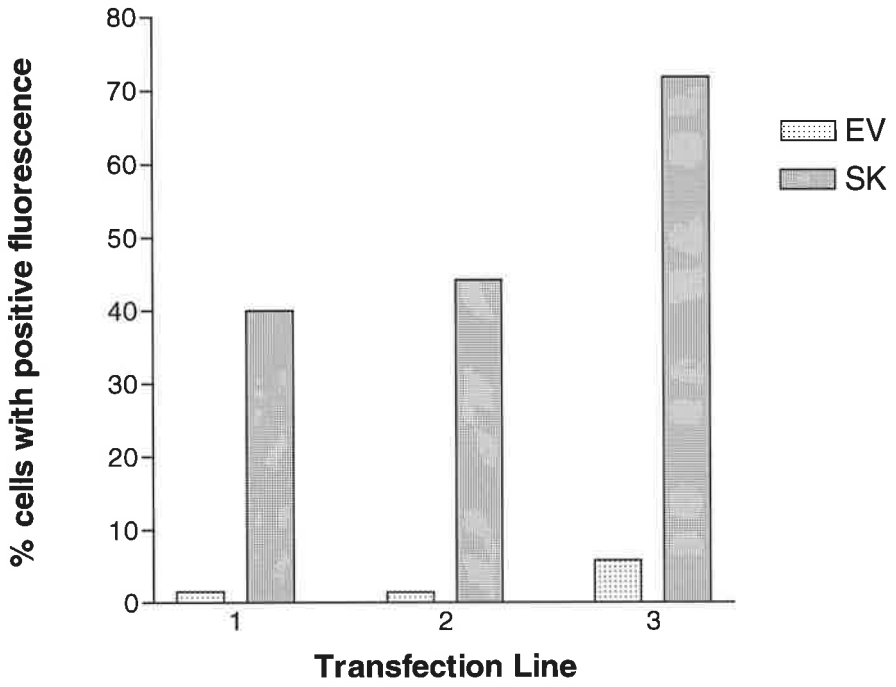


Fig 3.2 shows the percentage of Bing cells transfected with SK or control (EV) which showed positive fluorescence when permeabilised and stained with anti-flag antibody in three separate transfection experiments. Background fluorescence resulting from staining with a non-relevant isotype matched control antibody (23-1F11) has been subtracted.

(a)

(b)

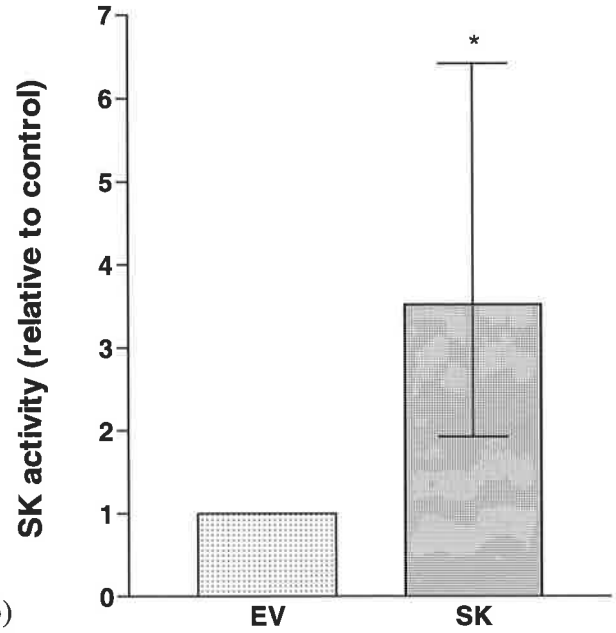


Fig 3.3 Confirmation of expression of SK in endothelial cells by Western blot (a) and by SK activity (b). The Western blot was performed using anti-flag antibody on cell lysates using 30μg of protein. The membrane was blotted with anti-actin antibody to ensure equivalent protein loading. (b) shows the composite analysis of five separate endothelial cell lines normalized to the EV, using Statistica Version 6.1 (Statsoft, Inc.). Bars represent 95% confidence intervals. *$p<0.05$ SK versus EV.

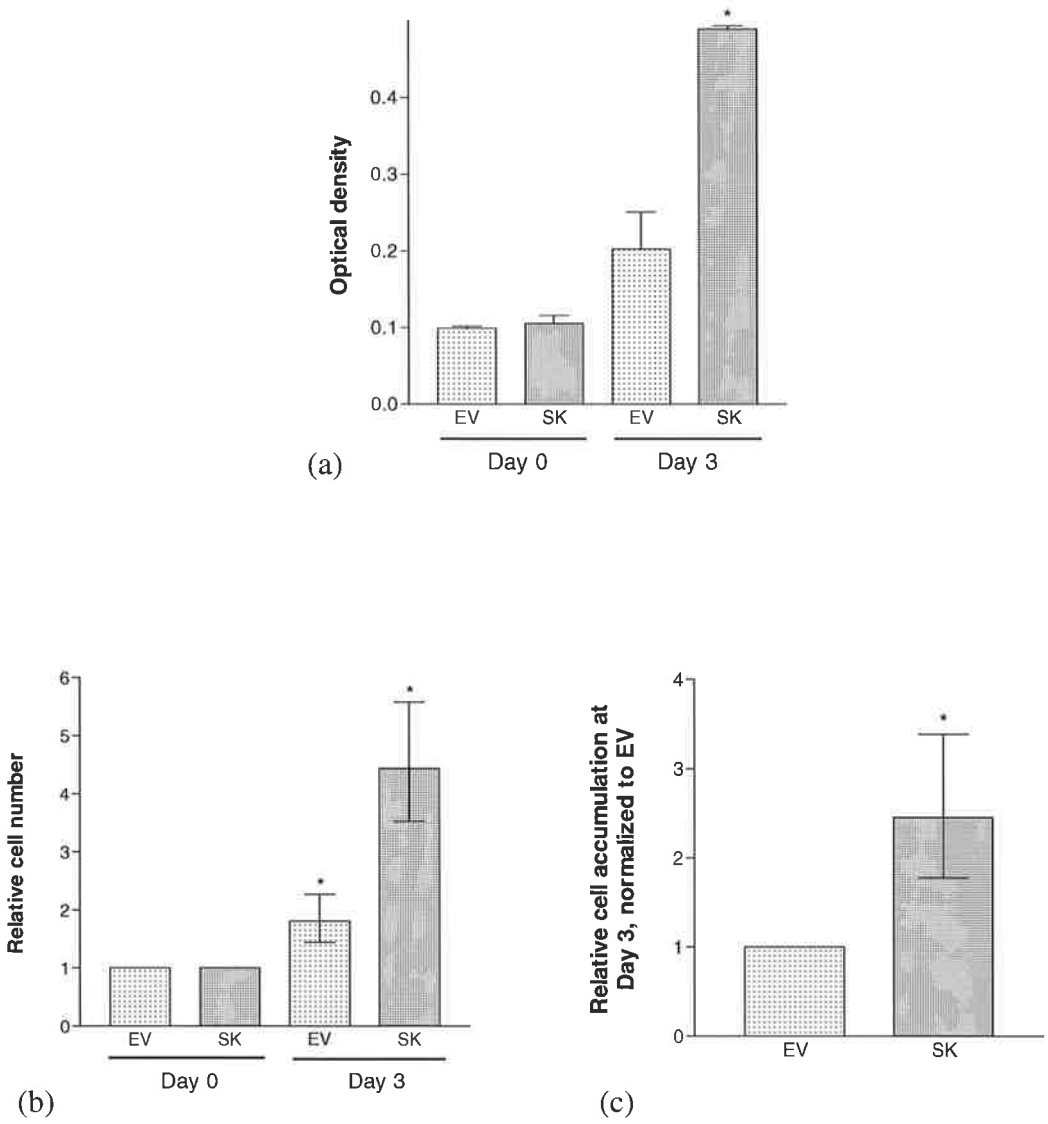


Fig 3.4 shows cell accumulation over three days for cells over-expressing SK and control (EV) in medium containing 2% FCS. (a) shows the number of cells (reflected by the optical density) seeded at Day 0 and the number of viable cells at Day 3. * $p<0.05$ SK compared with EV at Day 3, by Student's t-Test. (a) shows the results from a single experiment performed in triplicate which is representative of six separate experiments performed in triplicate (bars represent SEM), (b) and (c) show the composite analysis of these six experiments using Statistica Version 6.1 (Statsoft, Inc.). Bars represent 95% CI. (b) shows the fold increase in relative cell number, normalized for Day 0=1, for cells over-expressing SK compared with EV. * $p<0.001$ for the corresponding vector at Day 0. (c) shows the relative cell accumulation at Day 3 for cells over-expressing SK, normalized to EV, *$p<0.001$.

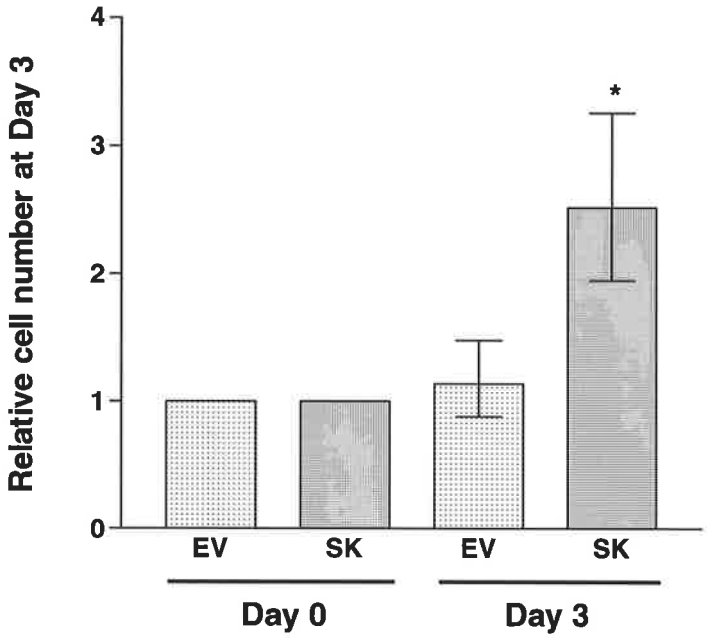


Fig 3.5 shows relative cell accumulation over three days in serum-free medium for cells over-expressing SK and control (EV), normalized to Day 0=1. *p<0.001 compared with SK at Day 0. Bars represent 95% CI. The figure shows the composite analysis of four separate experiments (each performed in triplicate) using Statistica Version 6.1 (Statsoft, Inc.) using different isolates of endothelial cells.

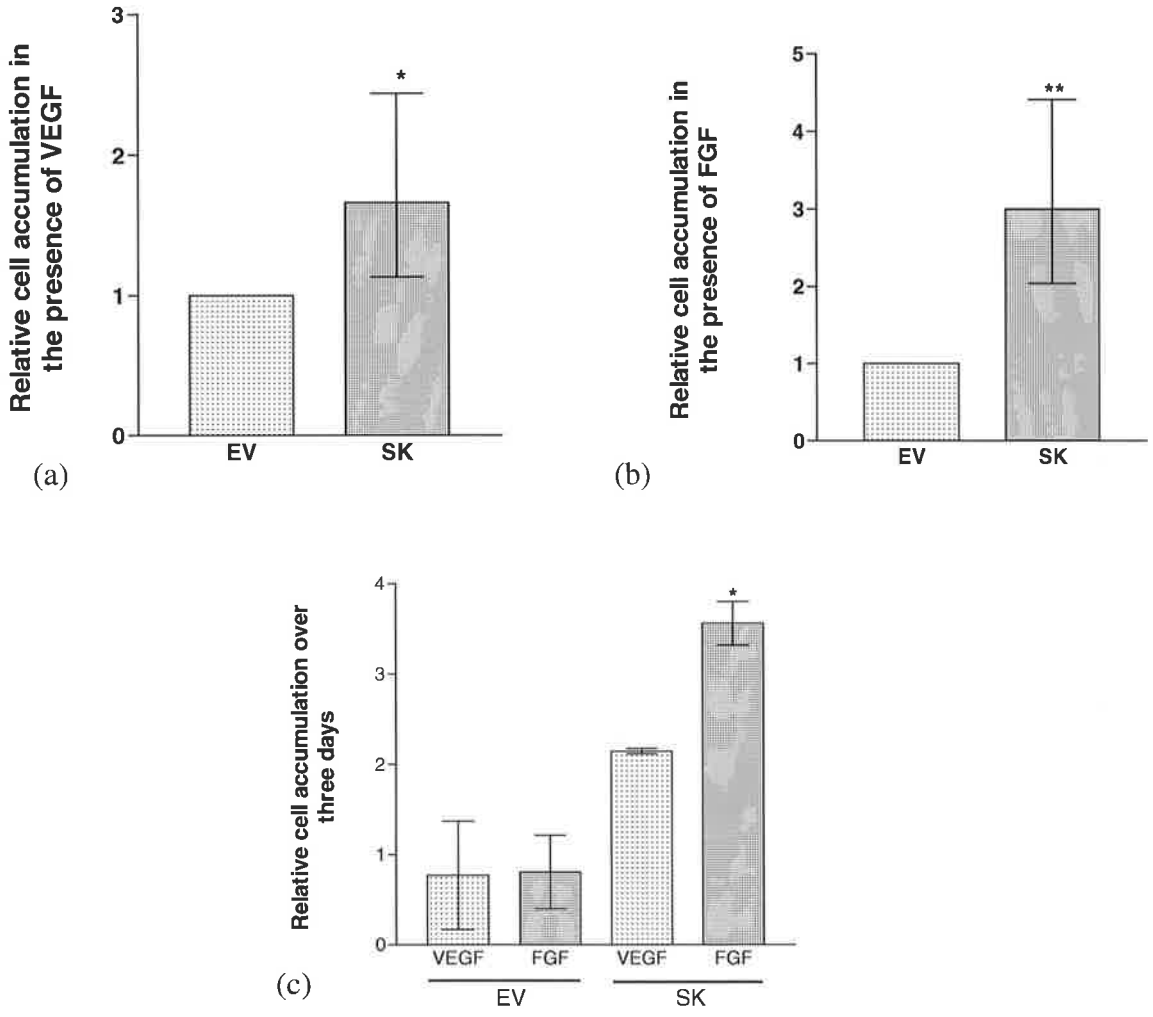


Fig 3.6 shows relative cell accumulation over three days for cells over-expressing SK and control (EV), when cultured in medium supplemented with 2% FCS, to which was added VEGF (10 ng/mL) (a) or FGF (1 ng/mL) (b). *$p<0.05$ SK compared with EV, **$p<0.001$ SK compared with EV. The figure depicts the composite analysis of five observations derived from two separate experiments using Statistica Version 6.1 (Statsoft, Inc.). (c) shows a comparison of the response of EV and SK, to VEGF and FGF. *$p<0.001$ SK-FGF compared with SK-VEGF. The figure shows the composite analysis of three separate experiments each performed in triplicate using Statistica Version 6.1 (Statsoft, Inc.). Bars represent 95% CI.

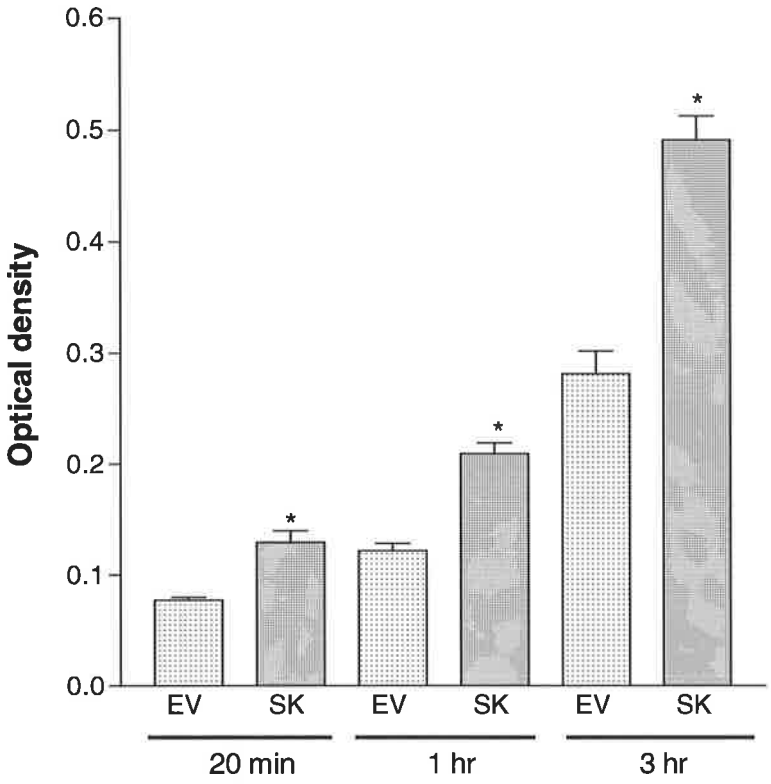


Fig 3.7 shows the attachment of HUVEC over-expressing SK and control (EV) to fibronectin (50μg/ml) at 20 minutes, 1 hour, and 3 hours. *$p<0.05$ SK compared with EV at each time point by Student's t-Test. The figure shows the result from a single experiment performed in triplicate, which is representative of three separate experiments. Bars represent SEM.

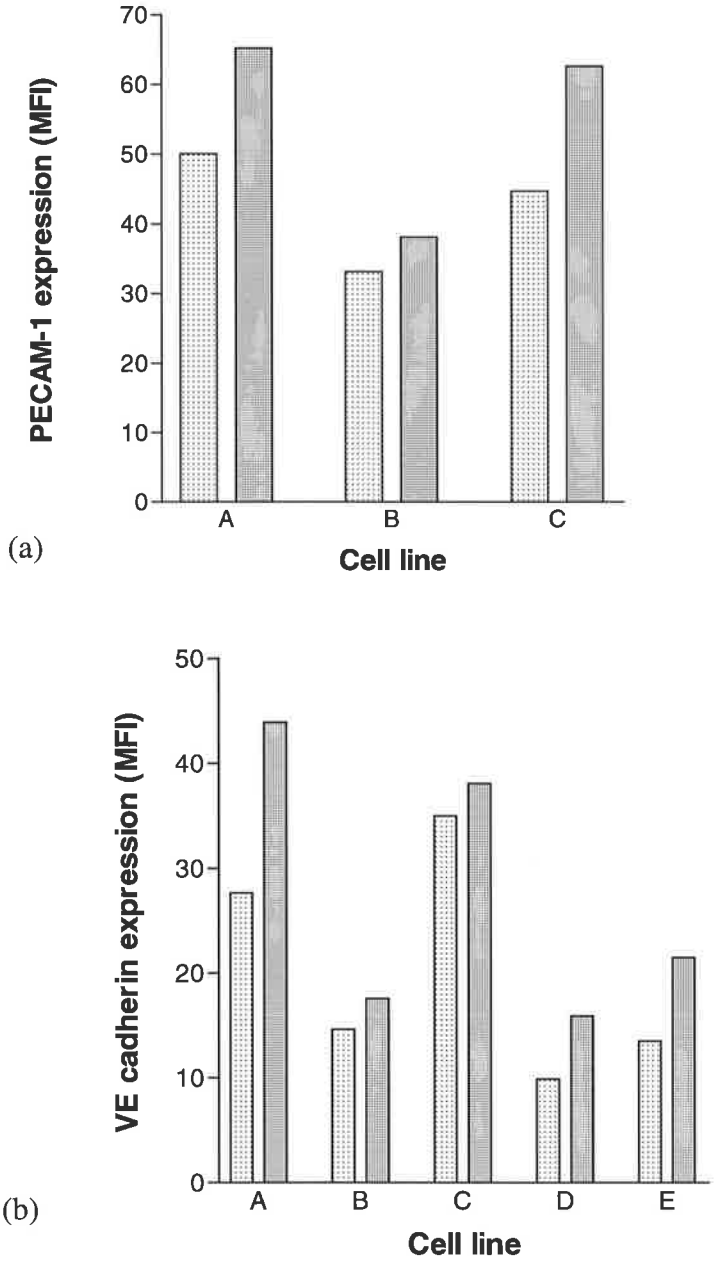
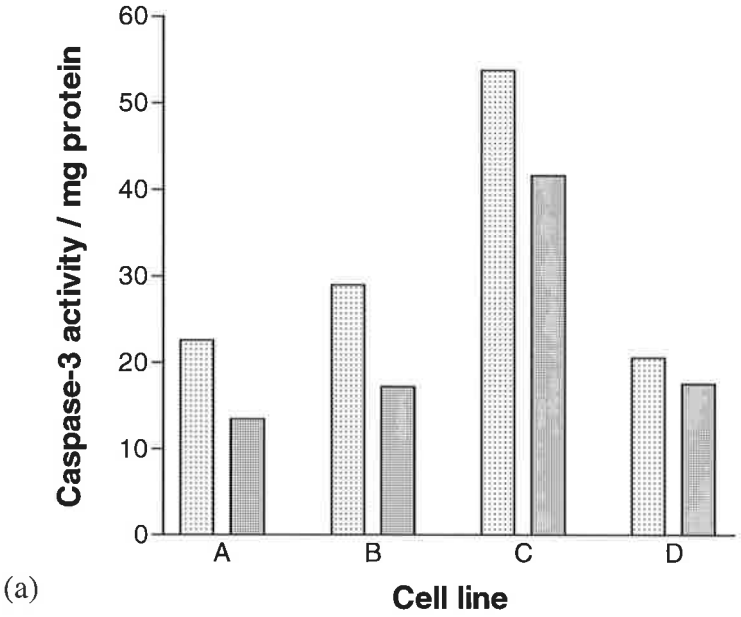
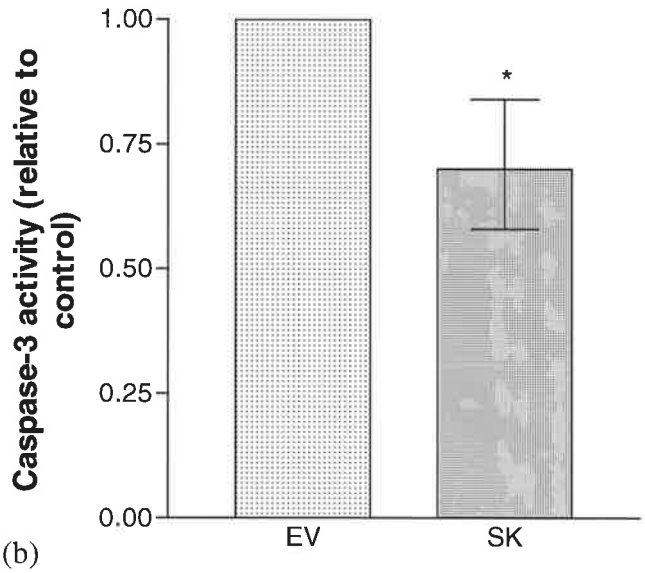


Fig 3.8 shows PECAM-1 expression in three endothelial cell lines (a) and VE-cadherin expression in five endothelial cell lines (b), as reflected by the median fluorescence intensity (MFI) in cells over-expressing SK (dense dots) and the corresponding values for the EV control (sparse dots). Fluorescence resulting from staining with a non-relevant isotype matched antibody (23-1F11) has been subtracted.



(a)



(b)

Fig 3.9 shows the effect of over-expressing SK on caspase-3 activity. (a) shows caspase-3 activity (normalized for the protein concentration) in four HUVEC lines over-expressing SK (dense dots) compared with EV control (sparse dots). (b) shows the mean fold change in caspase-3 activity for SK normalized to control, based on the composite analysis of four separate experiments each performed in duplicate, using Statistica Version 6.1 (Statsoft, Inc.). *$p<0.05$ SK versus EV. Bars represent 95% confidence intervals.

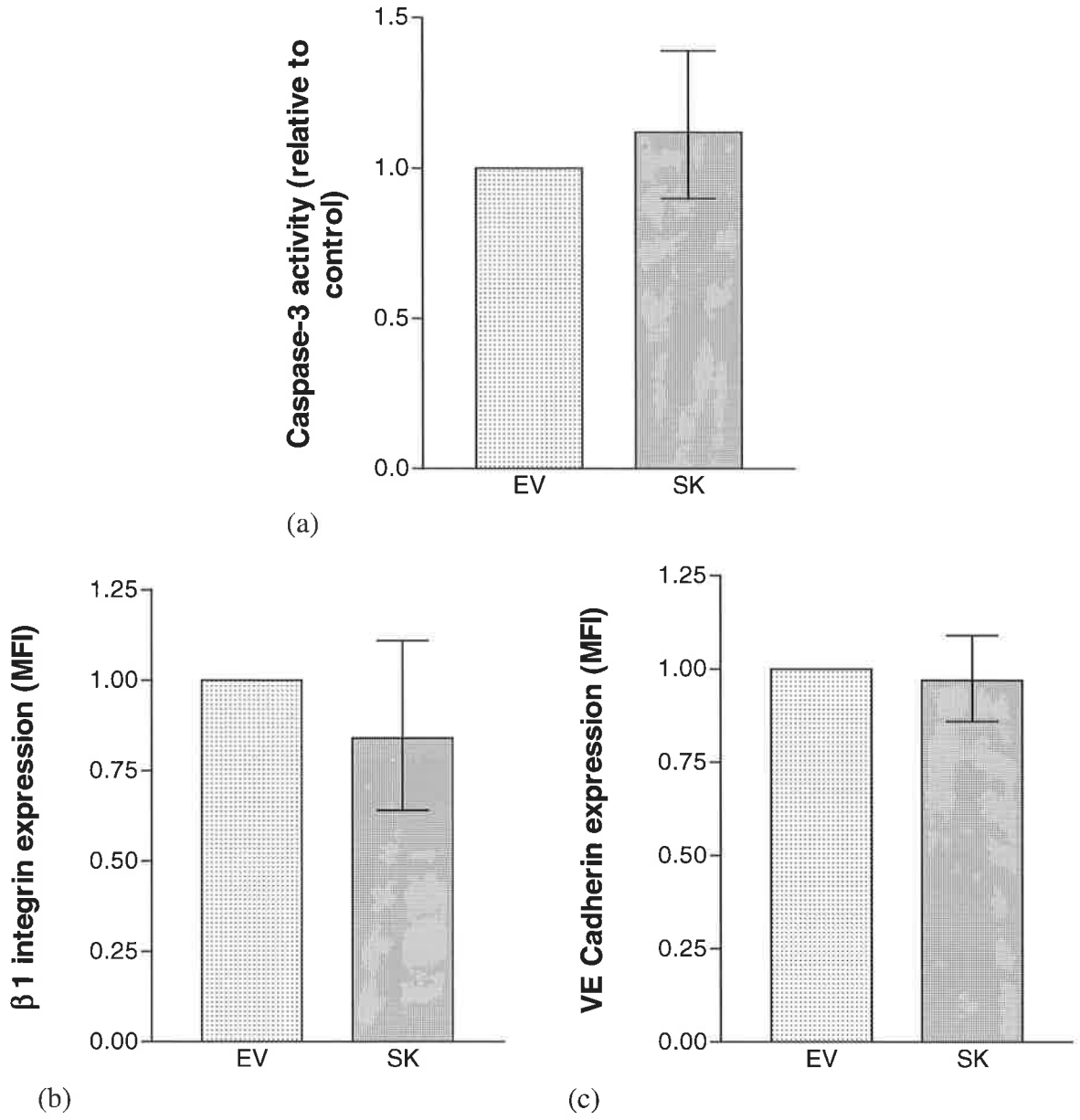


Fig 3.10 shows caspase-3 activity (a), β1 integrin expression (b), and VE Cadherin expression (c) in three atypical endothelial cell lines in which attempted over-expression of SK did not result in enhanced enzymatic activity. (a) shows the composite analysis of six observations derived from these same three cell lines (b) and (c) show the composite analysis of three observations derived from these cell lines and normalized to the control, using Statistica Version 6.1 (Statsoft, Inc.). Bars represent 95% confidence intervals.

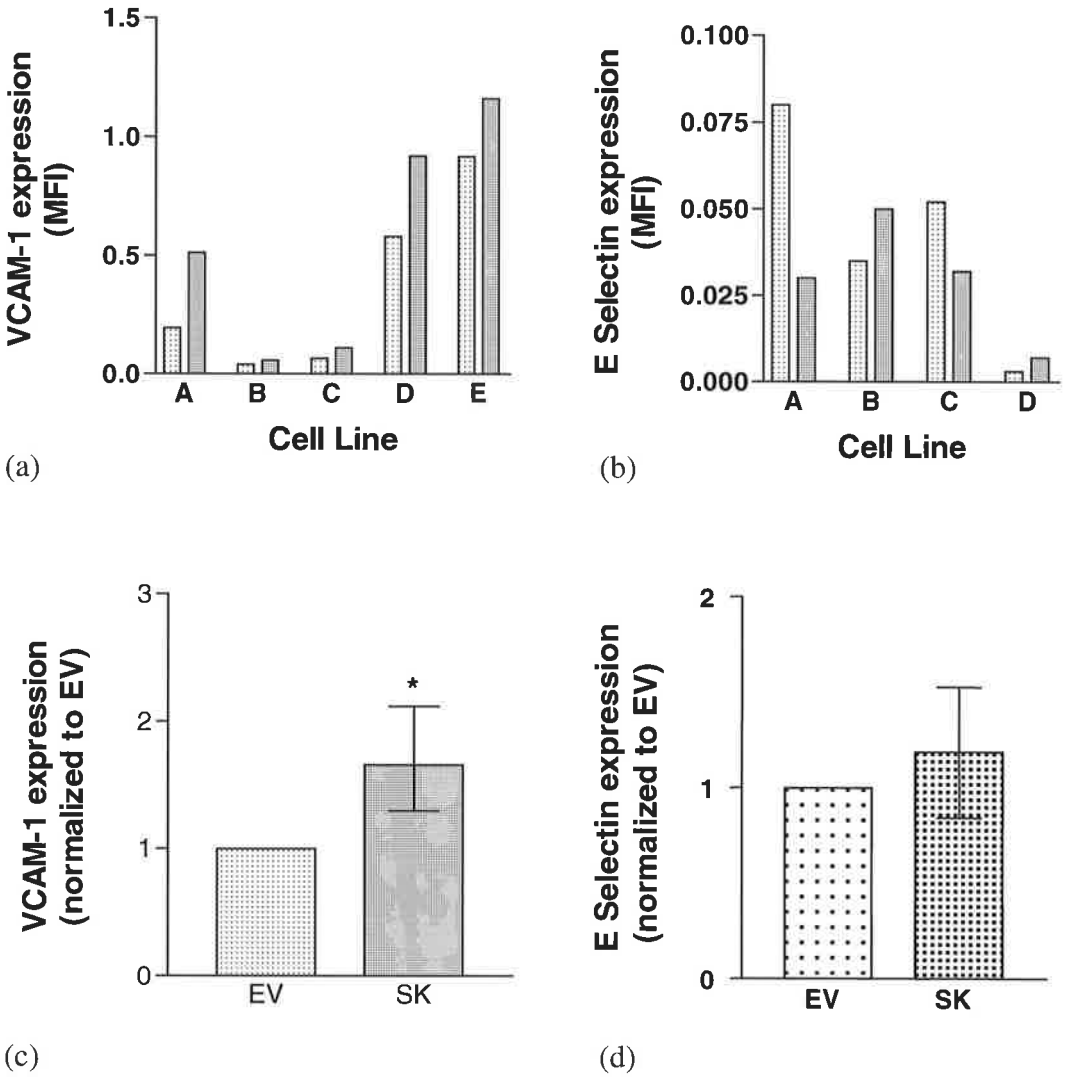


Fig 3.11 shows adhesion molecule expression in un-stimulated endothelial cells over-expressing SK (dense dots) and the corresponding values for the EV control (sparse dots) as indicated by the median fluorescence intensity (MFI). (a) shows VCAM-1 expression in five different cell lines, (b) shows E Selectin expression in four separate endothelial cell lines, after subtracting fluorescence resulting from staining with a non-relevant isotype matched antibody (23-1F11). The mean fold increase above control in VCAM-1 expression (c) and E Selectin expression (d) for cells over-expressing SK, represents the composite analysis of the cell lines depicted in (a) and (b), normalized to EV, using Statistica Version 6.1 (Statsoft, Inc.). *$p<0.05$ SK versus EV. Bars represent 95% confidence intervals.

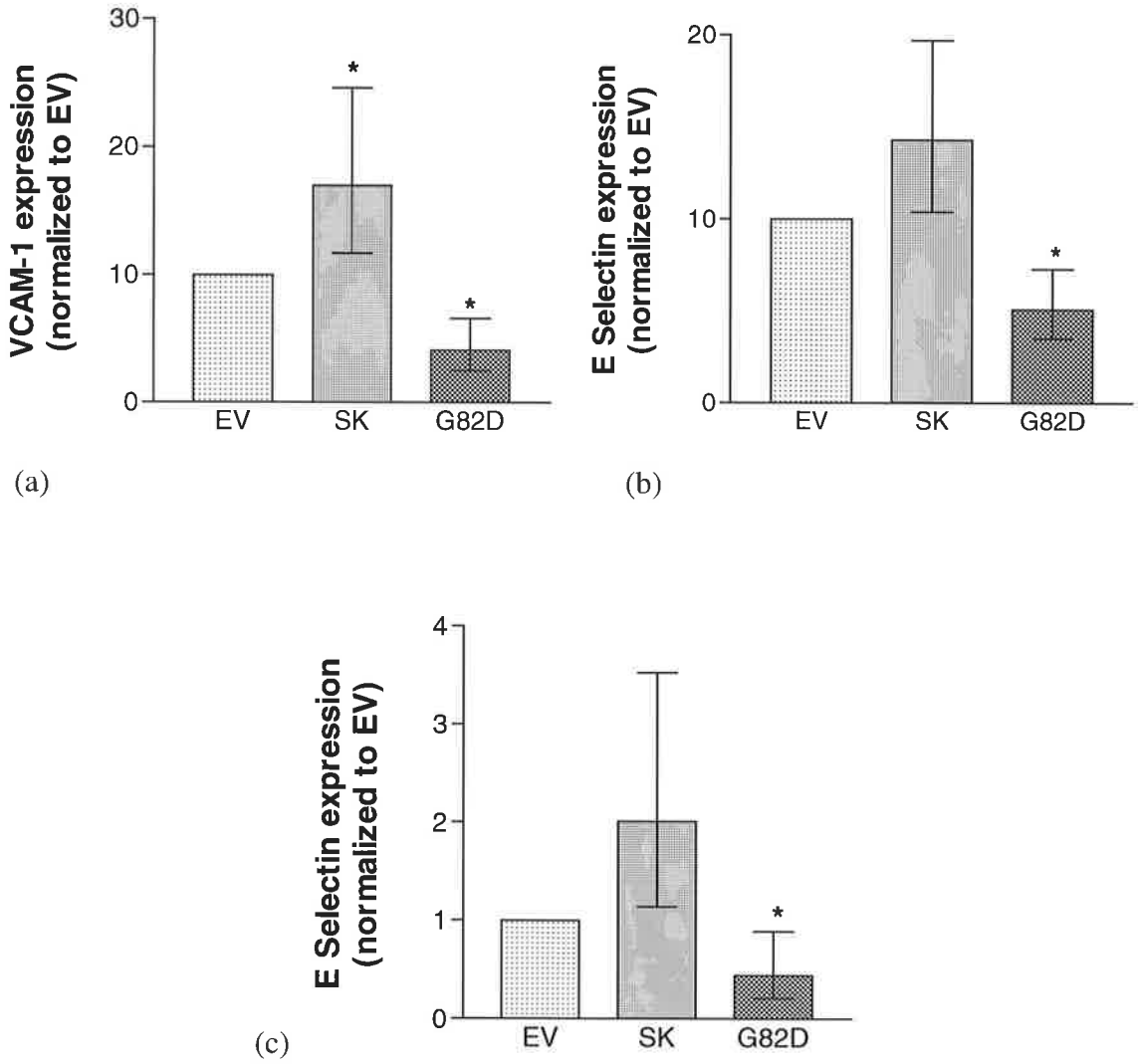


Fig 3.12 shows the adhesion molecule response (as measured by the median fluorescence intensity) of cells over-expressing SK, dominant-negative-SK (G82D) and control (EV) to stimulation with TNFα (0.5ng/mL) for four hours (a,b) or eighteen hours (c). VCAM-1 expression is shown in (a), and E Selectin expression is shown in (b,c). (a), (b) and (c) show the composite analysis of five, four, and three separate experiments respectively, normalized to the control, using Statistica Version 6.1 (Statsoft, Inc.). *$p<0.05$ SK or G82D compared with EV, **$p<0.001$ G82D versus EV. Bars represent 95% confidence intervals. Fluorescence resulting from staining with a non-relevant isotype matched antibody (23-1F11) has been subtracted.

CHAPTER 4

The phenotypic consequences of over-expression of Sphingosine Kinase in HUVEC achieved with adenoviral-mediated gene delivery

INTRODUCTION

The phenotype of long-term over-expression of SK in HUVEC achieved by retroviral-mediated gene delivery has been described in Chapter 3. Retroviral-mediated gene delivery provided a relatively crude tool for transfection of cells, as the gene dose was unable to be regulated, and integration of the viral genome introduced yet another confounding factor. Hence it was thought necessary to pursue a complementary method of transfection of HUVEC with SK, to ensure that the phenotypic alterations described were not artifacts of the method of transfection, but rather were mediated specifically by SK.

Adenoviruses have been used for gene transfer. The vector is created by replacing the E1A or E1B genes from the wild type adenoviral genome (~36kb in size) with the gene of interest (this reduces viral oncogenic and lytic properties). The helper line used for the production of replication-defective infectious viral particles is the HEK293 cell line (a human embryonic kidney cell line containing 11% of Ad5 genome). Viral titres in the range of 10^{12}-10^{13} plaque forming units (pfu)/mL may be achieved.

There are several important differences between adenoviral and retroviral-mediated gene delivery. Adenoviruses infect both replicating as well as non-dividing cells and a high level of transfection of all cells can be achieved. Indeed the level of gene expression achieved is higher than with the retroviral system, however expression is more transient and negligible after a period of 2 weeks. Titration of the adenoviral supernatant enables regulation of the gene dose delivered. Whilst the entire retroviral genome integrates in a stable fashion, the

adenovirus remains essentially episomal. These differences make adenoviral-mediated gene delivery a useful technique to complement retroviral-mediated transfection of HUVEC.

The limitations of retroviral-mediated gene delivery, in particular poor transfection efficiency, have been discussed (Chapter 3). An investigation into the signalling pathways engaged by raised intracellular levels of SK requires a system in which accurate titration of the gene dose is possible, and one which enables the generation of large numbers of cells over-expressing SK. It was envisaged that adenoviral-mediated over-expression of SK in HUVEC would allow the generation of sufficient cells over-expressing SK for use in assays to determine the mechanisms underlying the phenotypic consequences of moderately raised SK activity in HUVEC.

AIM

My aim now is to optimize the conditions for over-expressing SK in HUVEC using adenoviral vectors and secondly, to ascertain the phenotypic properties of these cells, focusing on factors involved in angiogenesis and inflammation. A third aim is to delineate the mechanisms underlying the phenotypic changes resulting from moderately raised SK activity in HUVEC.

It is hypothesized that delivery of SK using adenoviral vectors (to achieve a similar degree of enhanced SK activity as the twofold increase in SK activity resulting from endothelial cells stimulation with TNFα and the 3.5-fold increase in SK activity achieved with retroviral-mediated gene delivery) will result in similar phenotypic alterations as those seen with retroviral-mediated gene delivery, i.e. those of enhanced angiogenesis and inflammatory potential.

RESULTS

4.1 Optimization of adenoviral-mediated gene delivery into HUVEC

4.1.1 Adenoviral titration on HEK293 cells

Two separate preparations of adenoviral supernatant were generated using the pAdEasy system as described (Chapter 2.5). The viral titre as reflected by plaque forming units/mL (pfu/mL), and GFP expression were determined on HEK293 cells and is shown in Table 4.1(a). In respective viral supernatants, the titre for both SK and EV preparations was similar with regard to both pfu/ml and GFP expression, while the titre for G82D preparation was one log lower for both estimates. Thus infection of HUVEC with equivalent pfu/cell of adenoviral supernatant carrying SK or EV yielded a similar degree of GFP expression.

The number of infectious viral particles per mL of viral supernatant as reflected by the OD_{260} and outlined in the pAdEasy system manual for both EV and SK was one per 100.

4.1.2 Titration of adenoviral supernatant on HUVEC

The viral titre was estimated on HUVEC by harvesting the cells 48 hours after infection with varying pfu/cell, and then determining the level of GFP expression by flow cytometry. The median fluorescence intensity is shown in Table 4.1(b). At the level of sensitivity for which the flow cytometer was calibrated, the saturation limit for median fluorescence intensity was 1012.

For infections in HUVEC, conditions were optimized such that cells were infected with an equivalent number of pfu/cell of viral supernatant. This correlated with an approximately

equivalent level of GFP expression, in favor of slightly excess GFP expression for the empty vector (Table 4.1a).

4.1.3 Detection of over-expression of SK in HUVEC

4.1.3.1 Confirmation of expression using Western blot

Expression in HUVEC was detected by immunofluorescent microscopy for GFP expression, and was confirmed by Western blot using anti-flag antibody (Fig 4.1). Definite expression was observed in endothelial cells infected with 1 pfu/cell of adenoviral supernatant carrying SK, with a much more intense band noted in endothelial cells infected with 50 pfu/cell.

4.1.3.2 Determination of SK activity

To determine whether cells over-expressing SK (as confirmed by Western blot) had enhanced SK activity compared with control, and to delineate whether infection with greater pfu/cell of SK caused a greater increase in SK activity, SK activity was measured in both cells infected with 1 pfu/cell and 50 pfu/cell of adenoviral supernatant carrying SK or EV.

Over-expression of SK resulted in increased enzyme activity (Fig 4.1). Infection with 1 pfu/cell of adenoviral supernatant carrying SK resulted in moderately raised SK activity (mean fold increase above control 5.2, 95% confidence interval 4.86-5.51), which was statistically significant. This was noted to be similar to the degree of enhanced SK activity achieved with retroviral-mediated gene delivery. Infection with 50 pfu/cell of adenoviral supernatant carrying SK resulted in a very large increase in SK activity (mean fold increase above control 100, 95% CI 94.1-106.3). Infection of HUVEC with the greater EV viral load (50 pfu/cell compared with 1 pfu/cell of EV) did not alter SK activity ($p=0.18$) however

infection with the greater amounts of adenovirus carrying SK (50 pfu/cell compared with 1 pfu/cell) resulted in a further significant increase in SK activity ($p<0.05$).

4.1.4 Conclusion

A system utilizing an adenoviral vector for over-expressing SK (and G82D) in HUVEC was thus established. This system allowed the rapid generation of cells over-expressing SK, and unlike the retroviral system (Chapter 3), cell numbers were not limited. This system had the additional advantage of enabling accurate titration of gene dose, and thus set the scene for an investigation of the effects of varying gene dose.

Henceforth infection with 1 pfu/cell and 50 pfu/cell will be referred to as low levels and high levels of over-expression of SK respectively.

4.2 Low versus high levels of sphingosine kinase have opposite effects on cell accumulation

Cell proliferation was measured over three days using the colorimetric MTS assay, and cell accumulation was defined as the mean absorbance value at Day 3 divided by the mean absorbance value at Day 0. The effect of varying SK gene dose in HUVEC on cell accumulation is shown in Fig 4.2(a). Low levels of over-expression of SK significantly enhanced cell accumulation in 2% FCS compared with control. In contrast, high levels of SK in HUVEC significantly inhibited cell accumulation, such that cell numbers at Day 3 were significantly lower than EV. Importantly, infecting HUVEC with less than one viral particle per cell (1/10 pfu/cell) did not result in an appreciable increase in SK activity, and had no demonstrable effect on cell accumulation.

The increase in cell number of cells over-expressing SK (infected with 1 pfu/cell) over a three day period, compared with control cells is shown in Fig 4.2(b). It is noted that in 2% FCS, control cells increase in cell number, although to a lesser degree than cells over-expressing SK.

4.3. The phenotype of cells over-expressing low levels (1 pfu/cell) of SK

As the aim is to confirm (and extend) the phenotypic alterations resulting from retroviral-mediated delivery of SK into HUVEC using adenovirus, the investigation will be directed to the effects of over-expression of low levels of SK in HUVEC. This level of infection (1 pfu/cell) results in a similar degree of enhanced enzymatic activity as is achieved by stimulation of normal endothelial cells with TNFα (97), and also corresponds to the increase in enzymatic activity as is achieved with the retroviral system (Chapter 3.1.4.2).

4.3.1 Over-expression of low levels of SK confers a resistance to apoptosis

Apoptosis or programmed cell death is a fundamental biological process which involves the removal of unwanted cells in order to maintain cellular homeostasis. Apoptotic cells in contrast with necrotic cells have reduced cell size, condensed nuclei and membrane blebbing. In contrast to necrosis, apoptosis involves an orderly fragmentation of the cell, which is a slow process requiring 6-48 hours. The process of apoptosis is observed during normal physiological events such as embryogenesis, and it also plays a role in cancers. It is of particular relevance to the autoimmune diseases, as deletion of self-reactive T-cells in the thymus and B-cells in the bone marrow occurs by apoptosis. Hence there has been considerable interest in understanding the cellular regulation of apoptosis, and in techniques to detect apoptosis in vitro.

S1P is known to promote cell growth and inhibit apoptosis, and in endothelial cells is responsible for providing protection from TNFα-induced apoptosis (97). Cell accumulation represents a balance between cell proliferation and cell death. In light of the enhanced cell accumulation observed in cells over-expressing SK compared with control cells, it was sought to determine whether this was attributable to a reduction in cell death.

Apoptosis was visualized by DAPI staining, and also measured by the fluorogenic caspase-3 activity assay and by flow cytometric detection of Annexin V binding.

4.3.1.1 Over-expression of SK confers resistance to serum deprivation induced apoptosis as determined by DAPI staining.

DAPI is a fluorescent dye with high affinity for DNA and binds A-T rich sequences of DNA, to form a complex which can be visualized by fluorescence microscopy(192).

Endothelial cells over-expressing SK or EV taken from normal culture conditions, in 2% FCS and those subjected to 48 hours of serum deprivation were stained with DAPI. Apoptotic cells were defined as those with intense bright nuclear staining. The percentage of apoptotic cells in five consecutive fields was determined.

Under basal conditions there were only occasional apoptotic cells (Fig 4.3a). In 2% FCS, few of the control cells were apoptotic, but a negligible number of those over-expressing SK were apoptotic (Fig 4.3b). The most profound differences were detected in those cells subjected to 48 hours of serum deprivation, where many of the control cells were apoptotic, but very few of those over-expressing SK (Fig 4.3c). Quantification is shown in Table 4.2, and it is noted

that after 48 hours of serum deprivation, there are significantly more apoptotic control cells compared with those over-expressing SK ($p<0.001$).

It is acknowledged that DAPI staining is a subjective method of assessing apoptosis, and that inter-observer error is likely to occur in quantification. Therefore an investigation into whether the enhanced cell accumulation resulting from raised intracellular levels of SK is attributable to reduction in apoptosis, and whether SK provides resistance to serum deprivation-induced apoptosis was furthered by alternative, more reliable measures of apoptosis.

4.3.1.2 Over-expression of sphingosine kinase reduces Caspase-3 activity

The caspase family or proteolytic enzymes are the effectors of cell death, with caspase-3 playing a particularly important role in this cascade. In this cascade, activation of caspase-3 reflects the point at which the death signal becomes irreversible and commitment to cell death occurs.

Caspase-3 activity was measured as an indicator of cell death under normal culture conditions and in cells subjected to serum deprivation for 24 hours.

In the five endothelial cell lines shown (Fig 4.4(a)) there was a consistent reduction in caspase-3 activity in cells over-expressing SK when compared with control in basal conditions and when subjected to serum deprivation (Fig 4.4(b)). Analysis of the pooled data confirmed that over-expression of SK significantly reduced basal caspase-3 activity and conferred further resistance to caspase-3 activation induced by serum deprivation (Fig 4.4(c,d)).

4.3.1.3 Over-expression of SK causes a resistance to increased Annexin V-PE uptake in response to serum deprivation

A critical event in apoptosis is the translocation of phosphatidylserine (a membrane phospholipid) from the inner to the outer leaflet of the cell membrane. Annexin V, a phospholipid-binding protein binds to phosphatidylserine with high affinity in a calcium-dependent manner. Staining for apoptotic cells using fluorophore conjugated-Annexin V takes advantage of the accessibility for binding of phosphatidylserine that has become exposed on the outer cell membrane (193). By contrast, the use of 7-actinomycin D (7-AAD) allows the distinction between dead and viable cells to be made on the basis of membrane integrity (the membrane of dead but not live cells is permeable to 7-AAD) (194).

Apoptosis in cells over-expressing SK and control (EV) was measured using Annexin V-PE and 7-AAD under basal conditions, and in response to serum deprivation, and the mean fluorescence intensity for Annexin V-PE staining determined by flow cytometry. Using the coulter Epics profile flow cytometer, laser channel 2 was used to detect phycoerythrin, and laser channel 4 for detection of 7-AAD.

Although there was no significant difference between cells over-expressing SK and EV cells in Annexin V-PE staining under basal conditions ($p=0.6$), with serum deprivation for 24 hours, cells over-expressing SK showed significantly lower Annexin V-PE binding (Fig 4.5).

This is consistent with the lower caspase-3 activity seen with serum deprivation in cells over-expressing SK (above). It however does correlate with the reduction in basal caspase-3 activity (discussed below).

4.3.1.4 Conclusion

As evidenced by less DAPI staining of DNA, significantly less caspase-3 activation, less annexin-V binding in response to serum deprivation, I concluded that moderately raised intracellular SK activity confers a resistance to apoptosis induced by serum deprivation. The conjoint use of these three techniques to detect apoptosis provides strength to this data. The reduction in basal caspase-3 activity in cells over-expressing SK does not translate to increased Annexin V binding or DAPI staining. It is possible that these assays are not as sensitive as the caspase-3 activity assay and that reduction in caspase-3 activity may not necessarily translate into other events associated with cell survival in unstressed cells. In view of the lack of enhanced cell accumulation noted under basal culture conditions, I concluded that there is no biologically significant reduction in apoptotic tendency in cells over-expressing SK under basal conditions.

4.3.2 Over-expression of SK enhances cell survival

Endothelial cells require serum for survival, and thus normal culture conditions (HUVEC Medium) comprise 20% FCS. The lesser degree of apoptosis in response to serum deprivation seen in cells over-expressing SK (Section 4.3.1) prompted an enquiry into whether this had functional consequences and enabled these cells to survive in the absence of serum. Cell survival was measured in serum-free medium supplemented with ECGs by the MTS colorimetric assay.

Over-expression of SK resulted in enhanced cell survival at Day 2 (Fig 4.6(a)). There was no difference in the numbers of cells over-expressing SK compared with EV seeded at Day 0 ($p=0.27$). At Day 1 cells over-expressing SK showed enhanced cell numbers (reflecting cell proliferation) compared with control ($p< 0.001$). It is possible that this reflects reduction in

apoptosis in cells over-expressing SK, and that the differences in cell numbers compared with control cells are not due to a difference in the rate of proliferation. At Day 2 there were significantly more viable cells over-expressing SK than control ($p< 0.001$), and this was similar in number to the time of seeding the cells (reflecting cell survival). By Day 3 however, there was no significant difference between SK and control ($p=1$). Significant reduction in cell numbers occurred at Day 2 for EV cells ($p< 0.05$) and at Day 3 for cells over-expressing SK ($p< 0.001$).

Thus I concluded that cells over-expressing SK are able to survive in the absence of serum for longer compared with control cells.

4.3.3 Over-expression of SK allow cells to survive in suspension

Endothelial cells have a survival requirement for attachment to extracellular matrix. The enhanced serum-free cell survival seen in cells over-expressing SK (Section 4.3.2) prompted an enquiry into whether these cells were able to survive the further stress of loss of attachment to the extracellular matrix. Cell survival in suspension was measured using non-tissue culture, non-adhesive 96 well trays coated with BSA, in serum-free conditions using the colorimetric MTS assay.

Over-expression of SK enhanced the ability of the cells to survive unattached as shown in Fig 4.6(b). Although equivalent numbers of cells over-expressing SK and control were seeded at Day 0 ($p=0.42$), by Day 1, cells over-expressing SK showed enhanced cell numbers. Control cells were able to survive until Day 1 ($p=0.68$). By Day 2, while control cells had shown significant reduction in viable cell numbers compared with the time of seeding ($p<0.001$), cells over-expressing SK maintained cell numbers (cell survival). This effect was lost by Day

3 at which time both cells over-expressing SK and control cells showed reduction in viable cell numbers such that there was no difference between SK and EV ($p=0.1$).

I concluded that over-expression of SK allowed the cells to survive unattached for longer periods.

4.3.4 Over-expression of SK enhances attachment to extracellular matrices

A cardinal feature of endothelial cells is a survival requirement for attachment to extracellular matrix. Attachment to extracellular matrix mediated by the integrin group of cell surface receptors confers signals from the exterior to the interior of the cell and is known to modulate cell survival and proliferation. Thus in light of the enhanced cell accumulation and cell survival seen in cells over-expressing SK, the attachment profile of these cells to various matrices was measured using the colorimetric MTS assay. This is based on the principle of washing off any unattached cells at pre-determined time points, and quantifying by MTS the number of residual (attached) cells.

Over-expression of SK increased adhesion to various matrices including fibronectin, gelatin, collagen, and vitronectin. The attachment of cells to fibronectin at 20 minutes, 1 hour, and 3 hours is shown in Fig 4.7. For the three time points tested, cells over-expressing SK showed significantly greater adhesion to fibronectin compared with control cells ($p<0.05$). Cells over-expressing SK also enhanced adhesion to gelatin, collagen, and vitronectin compared with control cells (data not shown).

4.3.5 The effect of over-expression of SK on permeability

One of the pivotal functions of the vascular endothelium is to provide a structural barrier which is selectively permeable to nutrients. In addition to attachment with extracellular matrix, endothelial cell survival and endothelial integrity is also dependent on cell-cell contacts. The role of S1P in endothelial cell morphogenesis and enhancing the development of adherens junctions is well established (136). Hence the effect of intracellular over-expression of SK on cell-cell junctions was determined by measuring permeability of endothelial cell monolayers to FITC-dextran.

4.3.5.1 Basal cell permeability

As indicated in Fig 4.8(a) cells over-expressing SK showed a significant reduction in permeability to FITC-dextran when the average permeability across all time points was analysed. There was however no significant difference in basal permeability at any individual time point, due to the considerable variability in measurement reflected by the large confidence intervals.

4.3.5.2 The permeability response to thrombin stimulation

Thrombin is known to induce endothelial cell contraction (195) and disrupt cell –cell junctions. S1P has been shown to reverse the disruptions in endothelial cell junctions induced by thrombin(196). In view of the enhanced structural integrity of endothelial cell monolayers (as reflected by reduction in permeability) over-expressing SK, an enquiry into whether these cells are resistant to the effects of thrombin was made. Cells were stimulated with thrombin (0.2units/mL) immediately prior to measuring permeability to FITC-dextran.

Although low levels of SK reduced basal permeability (Fig 4.8(a)), in the presence of thrombin, there was no significant difference between cells over-expressing SK and control cells ($p=0.37$) (Fig 4.8(b)). Both control cells and cells over-expressing SK responded to thrombin with a significant increase in permeability to FITC-dextran (Fig 4.8(c,d)).

Thus I concluded that over-expression of SK tightens cell junctions in the basal state. These cells are not however resistant to the effects of thrombin as anticipated, and respond with an increase in permeability to thrombin equivalent to that in control cells. The lack of resistance to thrombin-induced increase in permeability in these cells argues against the reduction in basal permeability being mediated by S1P acting on the EDG receptor, but rather suggests the reduction in permeability conferred by SK is mediated by other mechanisms, possibly at an intracellular level. It is however possible that in these cells over-expressing only low levels of SK, extracellular S1P may be in a range sufficient to promote structural integrity of the monolayers in the basal state, but may be insufficient to overcome the opposing effects of thrombin.

4.3.6 Over-expression of SK enhances cell migration

Cell migration is a cardinal feature of angiogenesis and a role for S1P in stimulating endothelial cell migration is well established (197;198). Hence it was sought to determine whether cells with intracellular over-expression of SK also show enhanced cell migration. Cell migration towards fibronectin was measured using a colorimetric assay in which the cells were stained with crystal violet.

Indeed cells over-expressing SK showed significantly increased migration towards fibronectin at 20 hours as shown in Fig 4.9. This is consistent with the recognized role of S1P in

stimulating cell migration, and again supports a role for moderately enhanced intracellular levels of SK in angiogenesis.

4.3.7 Over-expression of SK enhances tube formation in Matrigel

The ability of endothelial cells to arrange into capillary like networks, or tubes is an in vitro correlate of angiogenesis, and endothelial cell stimulation with S1P is known to enhance cellular remodeling, by virtue of its actions on Rac and Rho small GTPases. In light of the enhanced cell survival, reduction in apoptosis, and enhanced cell migration seen with over-expression of SK, it was important to determine whether raised intracellular levels of S1P/ SK also stimulate cellular remodelling. To this end, tube formation in the complex basement membrane matrix Matrigel, was qualitatively assessed as a measure of angiogenesis.

Results are shown in Fig 4.10. The single cell population of endothelial cells seeded at time =0 is shown. By 15 minutes cells over-expressing SK had already commenced alignment whereas control cells remained disorganised. There was a clear difference at 30 minutes and at one hour, with cells over-expressing SK showing greater alignment into tube-like structures compared with control. At 24 hours when tube formation was complete in both cells over-expressing SK and control, there was no difference in the extent of tube formation.

It was thus concluded that over-expression of SK increases the rate of cellular remodeling and tube formation.

4.3.8 Conclusions

I concluded that over-expression of low levels of SK (which results in moderately enhanced SK activity) enhances cell accumulation in 2% FCS, and cell survival not only in the absence of serum, but also in the absence of attachment to extracellular matrix. This is consistent with the observed resistance to stress-induced apoptosis, as evidenced by caspase-3 activity, annexin V binding, and DAPI staining. Adhesion to extracellular matrix is also enhanced. These results are in keeping with those of enhanced cell accumulation, resistance to apoptosis, and increased attachment to extracellular matrix seen with retroviral-mediated delivery of SK (Chapter 3). Thus the phenotypic alterations resulting from retroviral-mediated over-expression of SK have been confirmed using adenoviral vectors, and have been extended to show that cells over-expressing low levels of SK also showed evidence of increased cell migration, reduced permeability of cell monolayers, and enhanced tube formation, features all consistent with an angiogenic phenotype. On this basis it is concluded that moderately raised intracellular levels of SK stimulate angiogenesis.

4.4 Hallmarks of inflammation

It was previously shown (Chapter 3.9) that stable over-expression of SK causes an inflammatory phenotype as evidenced by induction of VCAM-1 expression and augmented responses to TNFα. To test the hypothesis that over-expression of SK using adenoviral vectors will also cause endothelial activation and sensitize the cells to the effects of TNFα, adhesion molecule expression (basal and stimulated) in cells over-expressing low levels of SK (1 pfu/cell) was measured as a marker of inflammation.

4.4.1 Over-expression of SK up-regulates basal VCAM-1 expression

Over-expression of SK resulted in a small increase in VCAM-1 expression above control cells in six separate endothelial cell lines as shown in Fig 4.11(a). The mean fold increase in VCAM-1 expression of SK above control was of marginal statistical significance (p=0.052),

based on a conservative analysis using large sample confidence intervals (Fig 4.11(c)). Analyzing the data as a mean difference rather than as a ratio, revealed a p-value of 0.041. The consistent increase in VCAM-1 expression seen across six separate endothelial cell lines was also confirmed by non-parametric rank sum analysis ($p<0.05$) and was therefore considered significant. By contrast, E Selectin expression was not consistently altered in the five separate endothelial cell lines examined ($p=0.52$), as shown in Fig 4.11(b,d).

4.4.2 The effects of SK on TNFα -induced adhesion molecules

4.4.2.1 The effect of high dose of TNFα

To determine whether over-expression of SK altered the pro-inflammatory responses to TNFα, cell surface expression of VCAM-1 and E Selectin was measured after endothelial cell stimulation with TNFα 500ng/μL for four hours.

Cells over-expressing SK showed a significant exaggeration of the normal TNFα-induced up-regulation of VCAM-1 ($p=0.009$) and E Selectin ($p=0.008$) (Fig 4.12).

4.4.2.2 Over-expression of SK shifts the dose-response curve of VCAM-1 expression in response to TNFα

In light of the exaggeration in TNFα-induced VCAM-1 and E Selectin seen with over-expression of SK, it was sought to determine whether these cells alter the dose-response curve to TNFα. Endothelial cells over-expressing SK or transfected with EV were stimulated with TNFα at a concentration of 500ng/μl, 100 ng/μl, 20 ng/μl, or 4 ng/μl for four hours, and cell surface expression of VCAM-1 and E Selectin was measured by flow cytometry.

In three separate experiments, cells over-expressing SK consistently responded with higher levels of VCAM-1 and E Selectin than control cells, to each of the doses of TNFα used (Fig 4.13). There was however considerable variability in the degree of response between the different cell lines, hence the wide confidence intervals for the mean. The response of VCAM-1 to the lowest (4 ng/μl) and highest (500ng/μl) dose of TNFα tested, was significantly greater in cells over-expressing SK compared with control ($p<0.05$) (Fig 4.13(a)). Analysis of the VCAM-1 response to TNFα across all doses of TNFα revealed that over-expression of SK caused a significant shift in the curve to the left ($p=0.02$).

Over-expression of SK appeared to produce a modest up-regulation of E Selectin compared with EV across all doses of TNFα. This did not reach statistical significance at any individual dose of TNFα, or when analysed across all doses ($p=0.3$), as shown in Fig 4.13(b).

Both VCAM-1 and E Selectin followed a sigmoidal dose-response curve in response to stimulation with TNFα. A determination of the EC50 (the dose required to elicit 50% of the maximal response) was not possible as data points across smaller dose increments were not obtained, and the dose-response curve was not extended to a saturating dose (plateau). It is however suggestive that cells over-expressing SK will peak at a higher plateau than control cells suggesting a shift in the maximal response.

4.4.2.3 Over-expression of SK sensitizes HUVEC to the pro-inflammatory effects of TNFα

The most marked difference in induction of VCAM-1 and E Selectin by cells over-expressing SK in response to varying doses of TNFα were seen with the lowest (4ng/μl) dose of TNFα. This dose of TNFα did not induce VCAM-1 or E Selectin in control cells, however cells over-expressing SK responded with a substantial up-regulation of both VCAM-1 (in 2 of 3 experiments) and E Selectin (in each of 2 experiments) (Table 4.3). Over-expression of SK

resulted in a 4.42 (95%CI 1.51-12.94)-fold increase in VCAM-1 expression above EV, when stimulated with a low dose of TNFα ($p<0.05$), suggesting that these cells are sensitized to the effects of TNFα. Again, however, there was marked variability in the magnitude of the response between different isolates of endothelial cells, as indicated in Table 4.3.

4.4.3 Over-expression of SK enhances leukocyte adhesion to endothelial cells and augments TNFα-induced leukocyte adhesion

The magnitude of the changes in adhesion molecules induced by SK were modest, raising uncertainty whether these alterations resulted in biologically-relevant sequelae. Neutrophil adhesion to endothelial cells is the ultimate measure of inflammatory potential and therefore neutrophil adhesion was measured under basal and stimulated conditions, in cells over-expressing SK and in EV control cells. As over-expression of SK was shown to sensitize the endothelium to low doses of TNFα in terms of adhesion molecule response, it was sought to determine whether endothelial cell stimulation with doses of TNFα below the threshold for direct stimulation, would result in enhanced leukocyte binding. As dominant-negative SK (G82D) blocks agonist-induced activation of SK (127) and had been shown to inhibit TNFα-induced VCAM-1 and E Selectin expression (Section 3.9.2.1), it was to be ascertained whether over-expression of G82D also inhibited TNFα-induced neutrophil adhesion to control cells. The number of neutrophils adhering to each 100 endothelial cells within a single microscopic field of view at 120X magnification was determined. Consecutive fields were photographed.

Photographs are shown in Fig 4.14, and quantification in Fig 4.15. Under basal conditions control cells did not bind neutrophils, consistent with the known non-adhesive surface of the quiescent endothelium. Cells over-expressing SK however showed significantly enhanced

neutrophil binding compared with EV in the basal state ($p<0.001$). In response to stimulation with TNFα (0.04 ng/ml) for four hours, cells over-expressing SK showed greater enhancement in neutrophil binding than control cells, $p<0.001$. Cells over-expressing G82D did not alter neutrophil adhesion compared with EV in the basal state ($p=0.18$) but significantly reduced the number of adherent neutrophils per 100 endothelial cells in response to stimulation with TNFα compared with EV ($p<0.05$).

4.4.4 Conclusions

I concluded that over-expression of SK causes endothelial activation and, further potentiates the pro-inflammatory effects of TNFα to result in enhanced neutrophil binding in the basal and stimulated states.

4.5 Extracellular versus intracellular effects of S1P

4.5.1 Effect of pertussis toxin on basal levels of adhesion molecules

Stimulation of endothelial cells with exogenous S1P is known to upregulate adhesion molecules by virtue of its interaction with the EDG receptors. Hence, it was to be determined whether the alterations in adhesion molecule expression resulting from intracellular over-expression of SK could be explained by secretion of S1P (and hence EDG receptor ligation). The EDG receptor being a G protein-coupled receptor (GPCR) is pertussis toxin sensitive and hence pertussis toxin was used as a tool to inhibit signalling from GPCRs.

Pertussis toxin increased VCAM-1 expression in control cells ($p<0.05$) and cells over-expressing SK ($p=0.07$) as shown in Fig 4.16(a). Although the E Selectin reponse to pertussis toxin was more variable between cell lines, both control cells and cells over-expressing SK consistently responded with an increase in E Selectin expression (Fig 4.16b).

4.5.2 The effect of pertussis toxin on TNFα-induced adhesion molecules

To determine whether the augmentation of the TNFα-induced adhesion molecule response in cells over-expressing SK was due to S1P signalling through EDG receptors, cells were pre-treated with pertussis toxin (50ng/ml) for 18 hours and then stimulated with TNFα (0.5ng/ml) for four hours in the presence of pertussis toxin. Adhesion molecule expression was measured in these cells.

In two separate endothelial cell lines, pre-treatment with pertussis toxin did not alter TNFα-induced VCAM-1 expression in control cells ($p=0.28$), or cells over-expressing SK ($p=0.23$), as indicated in Fig 4.16(c). Similarly, TNFα-induced E Selectin expression was unaltered with pre-treatment with pertussis toxin (Fig 4.16(d)).

4.5.3 Over-expression of SK: its effects on the adhesion molecule response to the exogenous addition of S1P

The adhesion molecule response of cells over-expressing SK to exogenous stimulation with S1P (5μM) was examined. There was no significant difference between cells over-expressing SK and control, in SIP-induced VCAM-1 expression in four separate experiments ($p=0.31$), as shown in Fig 4.17(a) indicating that the EDG receptor pathway is intact in these cells. Cells over-expressing SK responded with a significantly greater up-regulation of E Selectin than control cells in response to stimulation with S1P, $p<0.001$ (Fig 4.17(b)).

The augmented E Selectin response to S1P stimulation in cells over-expressing SK was of interest, and it was sought to determine whether SK alters the dose-response curve to S1P. Cell surface expression of VCAM-1 and E Selectin in response to stimulation with 0.6μM 1.2

μM, 2.5 μM, or 5.0 μM S1P was measured by flow cytometry. Cells over-expressing SK consistently showed greater VCAM-1 expression across all doses of S1P tested, however there was no significant difference at any individual dose (Fig 4.17(c)). Overall, there was a trend to shift of the dose response curve with over-expression of SK ($p=0.08$). The dose response curve for E Selectin was significantly shifted to the left in cells over-expressing SK compared with control ($p=0.002$), as indicated in Fig 4.17(d). On average, the E Selectin response to S1P was 30% greater in cells over-expressing SK compared with EV.

Determination of the EC50 was not possible due to the limited data points obtained.

4.5.4 Conclusions

I concluded that the adhesion molecule response to intracellular over-expression of SK is not GPCR-dependent. It is acknowledged however that there are other confounding factors in measuring the adhesion molecule profile after blocking GPCR with pertussis toxin and that pertussis toxin is a non-specific tool which blocks other GPCR as well as EDG receptors.

DISCUSSION

In this Chapter, the findings of enhanced angiogenesis and inflammatory potential resulting from retroviral-mediated delivery of SK into HUVEC (Chapter 3) have been confirmed using adenoviral vectors to transfect SK in relatively transient manner. In addition to demonstration of resistance to serum deprivation-induced apoptosis and enhanced cell survival, these observations have been extended to measure the integrity of cell junctions (permeability), cell migratory capacity, as well as the ability to undergo cellular remodeling. Over-expression of SK was shown to regulate each of these additional measures of angiogenesis. In terms of inflammation, the investigation has been extended from induction of VCAM-1 expression by SK, to demonstrating that SK shifts the dose-response curve to TNFα, and sensitizes the cells

to very low doses of TNFα which do not alter the expression of adhesion molecules in control cells. An investigation into the functional significance of these changes has shown that modest elevation in SK activity results in a substantial increase in leukocyte adhesion to the endothelium in the absence of cytokine, and enhancement of neutrophil adhesion to cytokine-stimulated endothelial cells. These functional changes were seen despite the small magnitude of the changes in adhesion molecules. Together, these results suggest that modest elevations in SK activity in endothelial cells result in substantial functional changes, with endothelial activation, amplification of response to cytokine, and stimulation of angiogenesis.

Stimulation of endothelial cells with TNFα results in approximately a twofold increase in SK activity above basal which is transient, returning to basal levels within thirty minutes after stimulation (122). By manipulating the dose of adenovirus, cell lines that had an approximately five-fold increase in activity of SK were generated. These levels corresponded to the transient increase in endogenous SK activity achieved after stimulation of normal HUVECs with TNFα, and also to the degree of enhanced SK activity achieved with retroviral-mediated gene delivery (Chapter 3.1.4.2). Therefore, this system allowed an investigation of the effects of chronic over-activity of SK in what is estimated to be within the ambit of normal physiological responses.

Adenoviral vector-mediated gene delivery provided a more sophisticated method of transfection (than retroviral methodology), enabling accurate titration of gene dose, and allowing the rapid generation of large numbers of transfected cells. This resulted in the use in functional assays of cells of young passage (passage 3-4), which are more representative of normal endothelial cells and the physiological in vivo state. The dogma that retroviral-mediated gene transfer produces a chronic over-expression of the gene, whilst adenoviral-mediated gene transfer produces a transient over-expression of the gene is theoretical, and not

relevant in the over-expression of SK, as both methods achieve a long-term over-expression of SK, in comparison to the brief activation achieved with TNFα.

Modestly raised intracellular levels of SK in primary endothelial cells (HUVEC) resulted in a resistance to serum deprivation-induced apoptosis, as determined by three independent measures of apoptosis (Fig 4.3, 4.4, 4.5). Consistent with this, there was an alteration in the time course of cell survival with the cellular stresses of serum deprivation, and lack of attachment to extracellular matrix, as cells over-expressing SK were able to survive for two days while control cells were able to survive for one day only. The degree of over-expression of SK to achieve the phenotype was modest (approximately five-fold) in comparison to what is seen in established cell lines such as Swiss 3T3 fibroblasts in which thousand fold over-expression is not unusual (199). Nevertheless the phenotype seen in the endothelial cells over-expressing SK was robust and is likely to correspond to the type of changes seen under physiological circumstances (132). The eventual cell death in cells over-expressing SK by Day 3 when cultured under each of these conditions may be due to declining SK activity, or it may be that the resistance to apoptosis conferred by raised intracellular SK activity is of insufficient magnitude to overcome the cellular stresses over a more prolonged time period. In favour of the cell death being attributable to declining SK activity is the demonstration of cell survival (and proliferation) at Day 3 in cells infected with retrovirus carrying SK (Chapter 3.3.1.2), which does produce more stable gene expression. Whether cell death after Day 2 may be aborted in cells over-expressing SK by further trophic factor stimulation, introduction of serum, or exogenous stimulation with S1P remains to be seen. Clearly an investigation into trophic factors which may prolong the ability of cells over-expressing SK to survive will be of major importance, since if these cells can be rescued from cell death which ensues, it is foreseeable that they may be used therapeutically (e.g. to seed vascular grafts).

Survival of endothelial cells requires sustained contact both with the extracellular matrix and with neighbouring cells. Raised intracellular levels of SK were shown to stimulate cell-matrix attachments and strengthen cell-cell contacts. Binding to the extracellular matrix mediated by the integrin family of cell surface receptors is known to transduce signals from the exterior to the interior of the cell, resulting in the activation of cellular pathways involved in proliferation and survival. A role for SK in targeting the cell junction is indicated by enhanced structural integrity of the endothelial cell monolayers, as reflected by the reduction in basal permeability.

Cell migration to the perivascular space is a requirement for angiogenesis to proceed, and over-expression of SK stimulated cell migration to fibronectin. The ability of endothelial cells to align into capillary like-tubes is an in vitro correlate of angiogenesis, and the exaggerated induction of capillary tube formation by cells over-expressing SK suggests their sensitization to angiogenic factors. The enhanced cell migration and stimulation of tube formation is consistent with the recognized angiogenic actions of S1P (143;144).

Increased attachment to extracellular matrix as seen with raised intracellular SK activity would normally be predicted to inhibit cell migration, however it has been shown here that over-expression of SK stimulated both adhesion to extracellular matrix and also migration. This discrepancy is intriguing and demands an investigation into the mechanisms of SK-mediated cell migration. In endothelial cells, the serine threonine kinase Akt is involved not only in preventing apoptosis, but is also recognized to regulate cell migration(200). Indeed cell migration mediated by exogenously added S1P is mediated by the phosphatidylinositol 3-kinase/ Akt pathway (201;202), and the question is raised whether this pathway is also activated by raised intracellular levels of SK/ S1P. Involvement of this pathway would be consistent with the reduction in apoptosis and survival advantage conferred by raised SK

activity. In addition to Akt, the cell junctional protein PECAM-1 is known to regulate endothelial cell chemotaxis, via stimulation of Rho activity, in a pertusis toxin-sensitive manner (203). A role for SK in targeting the cell junction is suggested by the observed reduction in basal permeability (Fig 4.8), and also by the upregulation of PECAM-1 expression resulting from retroviral-mediated over-expression of SK (Section 3.5). That PECAM-1 plays a role in S1P-mediated cell migration is suggested by the observation that endothelial cells lacking PECAM-1 fail to undergo chemotaxis in response to exogenous S1P (203). Thus, it will be interesting to determine whether activation of the PI-3K/Akt pathway, and regulation of PECAM-1 signalling are responsible for some of the phenotypic alterations resulting from raised intracellular SK activity (Chapter 5).

The endothelium is the gateway to inflammation and is finely balanced between anti-inflammatory and pro-inflammatory states. Hitherto, the balance has thought to be determined principally by the cytokine microenvironment. Here it has been demonstrated that expression of intracellular components within the cytokine signalling pathways can influence the responsiveness of the endothelium to ambient conditions, including cytokines. This provides a model for genetic variations in endothelial responsiveness to both trophic and inflammatory stimuli.

Although small but consistent changes in the expression of basal and TNFα-stimulated VCAM-1 and E Selectin expression were demonstrated (Fig 4.11, 4.12), by far the most impressive changes were in the sensitization of the endothelium to doses of TNFα below the threshold for direct stimulation (Table 4.2). These findings suggest that SK over-expression resets the threshold of endothelial responsiveness. Although such changes in adhesion molecule expression could be considered small, the effect when translated into neutrophil adhesion was substantial. An approximately five to ten-fold increase in numbers of

neutrophils adhering either to basal or TNFα-stimulated endothelium was seen in cells over-expressing SK (Fig 4.14, 4.15). This suggests that either the levels of adhesion molecules are synergistic in their regulation of neutrophil adhesion and/ or that SK alters other pathways which also act to influence neutrophil attachment to the endothelium. The role of SK as a key regulator of inflammation is substantiated by the effects of the dominant-negative G82D mutant, which has a single amino acid substitution in the catalytic domain of SK and which we have previously shown to block activation of SK (127;204). Over-expression of G82D inhibited neutrophil adhesion to TNFα-stimulated endothelial cells (Fig 4.14, 4.15).

In terms of the effects of raised intracellular levels/ activity of SK on endothelial activation, the distinction between intracellular actions of S1P and its actions on EDG receptors was complicated by the induction of adhesion molecules by pertussis toxin (Fig 4.16a,b). This is consistent with a previously reported inhibition mediated through GPCR which are pertussis toxin-sensitive (205). Pre-treatment of the cells with pertussis toxin did not however lead to a further increase in the cell surface expression of adhesion molecule in response to TNFα (Fig 4.16c,d), indicating that the induction of adhesion molecules by TNFα is not under inhibitory control through GPCR. It has been demonstrated here that the EDG receptor pathway is intact in cells over-expressing SK, as the induction of adhesion molecules in response to exogenous S1P was preserved (Fig 4.17a,b). In fact there was a suggestion that cells over-expressing SK intracellularly are primed to respond to extracellular S1P, as the magnitude of the E Selectin response to S1P was greater in these cells, and there was a shift in the E Selectin dose-response curve to S1P stimulation (Fig 4.17d).

These findings are consistent with the previous demonstration that SK is an obligatory enzyme in mediating the adhesion molecule up-regulation induced by TNFα. On the basis of these results, it is hypothesized that raised levels or activity of SK may play a role in the

pathogenesis of chronic inflammatory diseases such as RA. Thus, the determination of SK levels or activity in tissues will be important. The work documented here, showing a number of important and novel findings, extends our understanding of the function of SK in endothelial cells. Over-expression of SK per se in endothelial cells results in the induction of the inflammatory phenotype, as measured by changes in the basal levels of adhesion molecules and enhanced neutrophil adhesion. Furthermore, endothelial cells over-expressing SK display an augmented response to TNFα, both in terms of the levels and duration of expression of adhesion molecules and of neutrophil adhesion. Importantly, over-expression of SK sensitizes the endothelium to the effects of TNFα. The sensitization also appears to take place in terms of the angiogenic response as cells over-expressing SK respond more rapidly to an angiogenic milieu. Thus enhanced expression of SK predisposes the endothelium to an inflammatory and angiogenic phenotype and alters the threshold of response to pro-inflammatory cytokines such as TNFα. As TNFα, IL-1, and VEGF are all implicated in the pathogenesis of RA, clinical interest has been directed towards targeting these cytokines. However as each of these is known to activate SK (97;206)the possibility exists that SK represents an alternative downstream target for therapeutic manipulation.

It is concluded that moderately enhancing SK activity (three to fivefold above basal) by either retroviral or adenoviral-mediated gene delivery results in similar phenotypic alterations of enhanced angiogenesis and inflammatory potential. The complementary use of the two methods of transfection strengthens these data, and minimizes the possibility that the observed consequences of over-expression of SK are attributable to artifacts of transfection.

In essence, the phenotypic alterations arising from over-expression of SK are confirmatory of the known effects of exogenous stimulation with S1P. This lipid mediator is known to stimulate cell proliferation, cell survival, confer resistance to apoptosis, and enhance

endothelial cell morphogenesis, as well as causing endothelial activation. The question arising is whether the underlying mechanisms are identical or distinct to the cellular pathways engaged by endothelial cell stimulation with exogenous S1P.

	Virus 1		Virus 2	
	PFU/ml ($x10^{10}$)	GFP ($x10^{10}$)	PFU/ml ($x10^{10}$)	GFP ($x10^{10}$)
EV	3.16	7.94	3.51	7.04
SK	3.98	2.51	4.0	3.65
G82D	0.342	0.356		

(a)

PFU/ml	EV	SK	G82D
1	1.16	1.78	0.724
5	96.2	6.46	15.2
10	295.2	28.4	220.7
15	539.9	107.7	749.6
20	1010	312.9	1011
25	1012	405.5	639.9
30	1013	539.0	1012
35	1014	845.1	1013
40	1014	896.8	1012
45	1014	1012	1012
50	1014	1013	1012

(b)

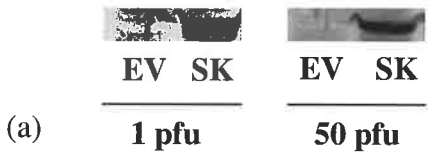
Table 4.1 shows the titre of the adenoviral supernatant carrying SK, G82D, and EV control, as determined on (a) HEK293 cells using plaque forming units /ml (PFU/ml) and GFP-related fluorescence and, (b) on HUVEC estimated from the median fluorescence intensity of GFP-related fluorescence 48 hours after infection. PFU/ml is the controlled variable and GFP fluorescence is the experimental variable. The data show fluorescence associated with different levels of pfu/ml, and define the range of pfu/ml for which fluorescence can be used as a measure of the strength of the correlation. The saturation limit of GFP-related fluorescence at approximately 1000 is a limitation of the assay.

	% apoptotic cells (mean ±SEM)		
Culture conditions	**EV**	**SK**	**p value**
Basal (20% FCS)	1.7 ±0.42	1.5 ±0.48	0.74
2% FCS	3.3 ±0.47	1.9 ±0.53	0.11
Serum free	22.6 ±1.11	3.5 ±0.50	<0.001

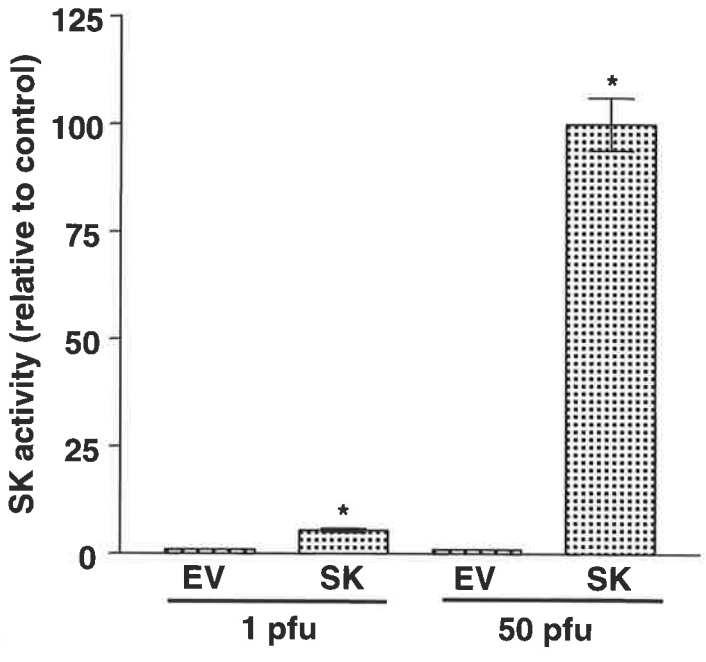
Table 4.2 shows the percentage of apoptotic endothelial cells over-expressing SK and cells infected with EV under culture conditions comprising different amounts of FCS, and as determined by DAPI staining. The data reflects the pooled data of ten observations derived from two separate experiments. Each observation comprises the number of apoptotic cells among a total count of 100 endothelial cells. The p value derived from the Student's t-Test is indicated.

Experiment Number	Treatment	VCAM-1 expression (MFI)		E Selectin expression (MFI)	
		EV	SK	EV	SK
1	nil	3.85	4.04	1.95	1.69
1	TNFα	3.88	19.6	6.51	11.2
2	nil	2.43	2.52	1.8	2.31
2	TNFα	2.23	2.4	1.69	4.69
3	nil	1.26	1.94		
3	TNFα	1.22	63.3		

Table 4.3 shows the adhesion molecule response of cells over-expressing SK and EV control cells, to stimulation with subliminal doses (4ng/μl) of TNFα for four hours. The effect on VCAM-1 expression is indicated in three separate endothelial cell lines, and E Selectin from two separate endothelial cell lines.



(a)



(b)

Fig 4.1 shows confirmation of SK expression in HUVEC by (a) Western blot and (b) by SK activity. Effects of HUVEC infection with 1pfu/cell or 50 pfu/cell of adenoviral supernatant carrying SK or control (EV) are shown. Western blot was performed with anti-flag antibody on whole cell lysates (20μg protein). (b) shows the composite analysis of two separate endothelial cell lines assayed in duplicate, and normalized to EV using Statistica Version 6.1 (Statsoft, Inc.). * $p<0.001$ SK compared with EV at equivalent pfu/cell. Bars represent 95% confidence intervals.

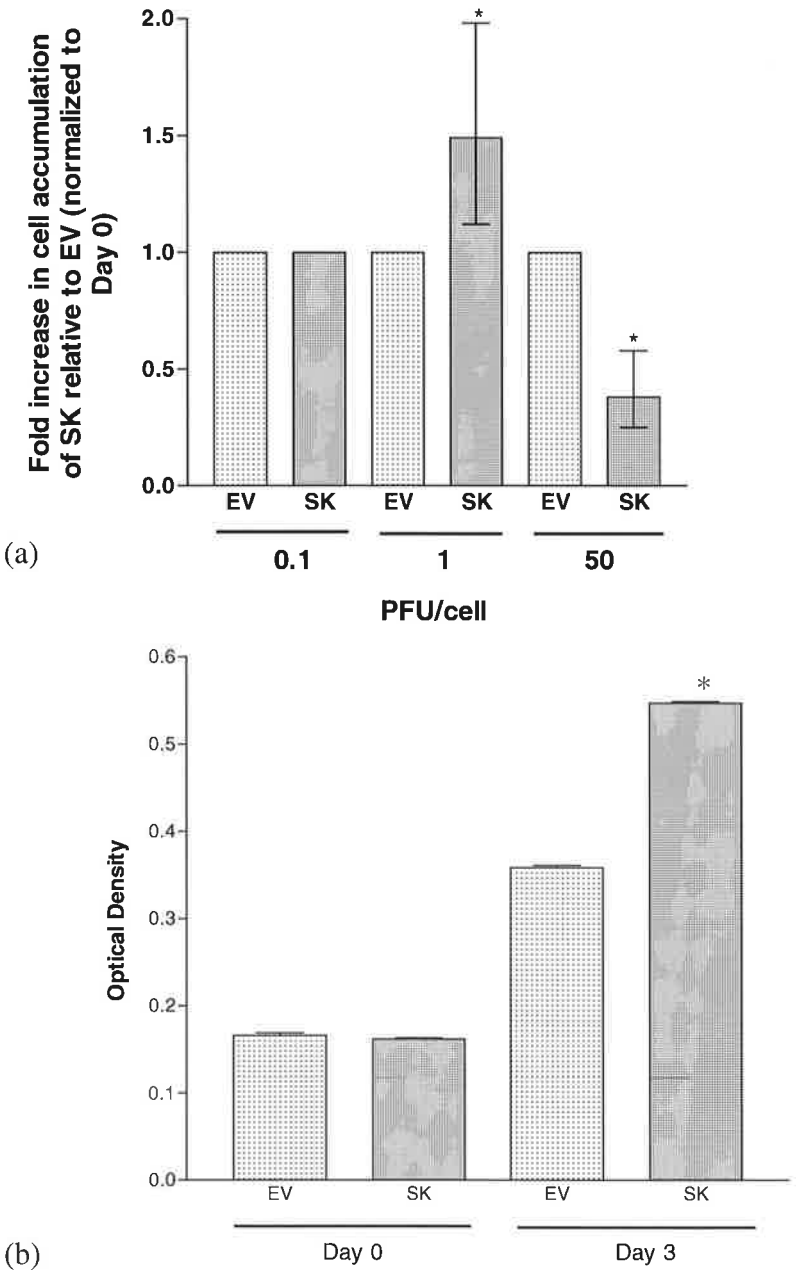


Fig 4.2 (a) shows the relative cell accumulation in medium containing 2% FCS for HUVEC infected with varying pfu/ cell of adenovirus carrying SK or EV. Results are normalized to the optical density at Day 0, and are adjusted to EV=1. The figure shows the composite analysis of six observations from two separate experiments (0.1pfu/cell), 31 observations derived from 7 separate experiments (1 pfu/cell) and 15 observations from 5 separate experiments (50 pfu/cell), using Statistica Version 6.1 (Statsoft, Inc.). *$p<0.001$ SK compared with EV at equivalent pfu/cell. Bars represent 95% confidence intervals. (b) shows the raw data from a single experiment performed in triplicate, in which HUVEC were infected with 1 pfu/cell. This experiment is representative of the other six such experiments depicted in (a). *$p<0.05$ SK compared with EV at Day 3. Bars represent SEM.

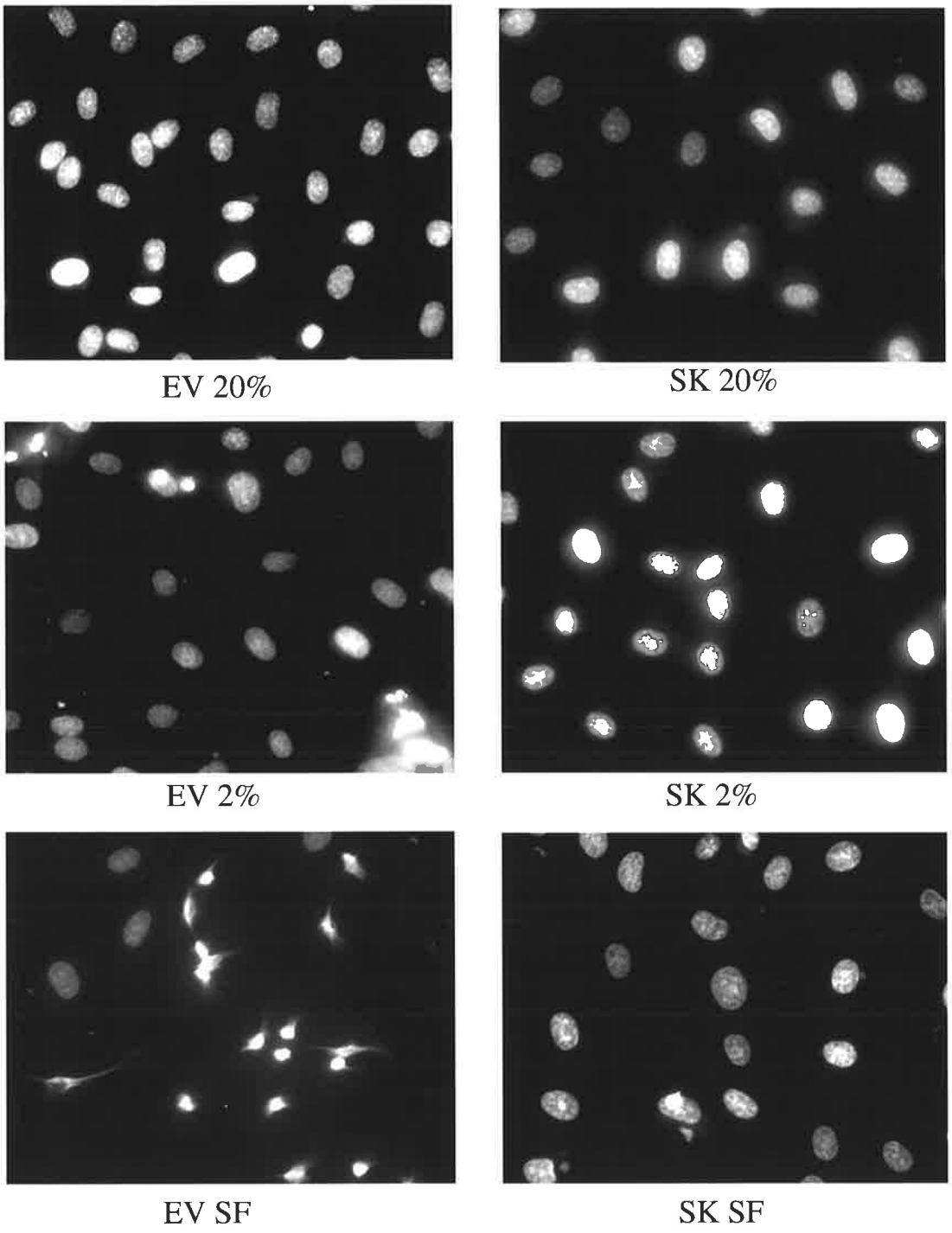


Fig 4.3 shows a DAPI stain performed on control cells (EV) and cells over-expressing SK in culture medium supplemented with 20% FCS, 2% FCS, or serum free (SF) medium. Apoptotic cells show intense nuclear staining of DAPI.

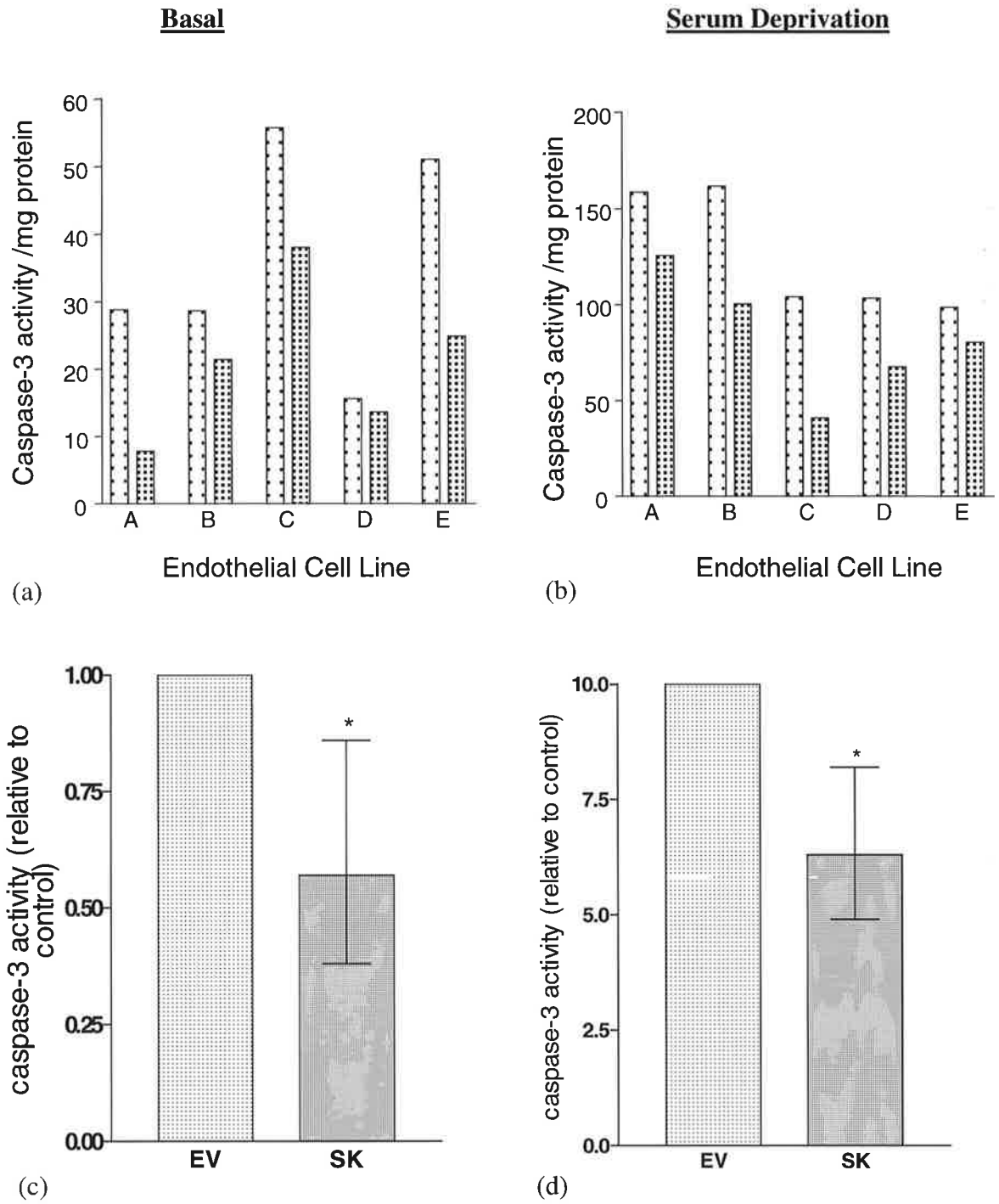


Fig 4.4 shows caspase-3 activity in five separate endothelial cell lines (A-E) over-expressing SK (dense dots) or EV control (sparse dots), measured under basal culture conditions in HUVEC Medium (a,c), or after 24 hours of serum deprivation (b,d). Fig (c), and (d) show the composite analysis of the same five endothelial cell lines indicated in (a) and (b) and normalized to EV=1 (c) or EV=10 (d), using Statistica Version 6.1 (Statsoft, Inc.). *$p<0.05$ compared with EV. Bars represent 95% confidence intervals.

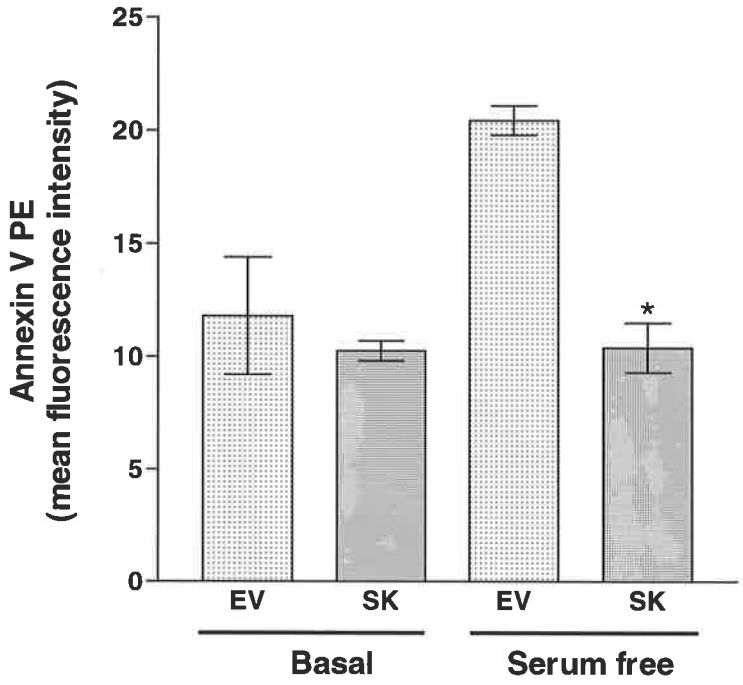


Fig 4.5 shows apoptotis in cells over-expressing SK and control (EV) as determined by staining with Annexin V-PE and reflected by the mean fluorescence intensity. The cells were taken under basal conditions (20% FCS) and after 24 hours of serum deprivation. *$p<0.05$ of SK compared with EV under serum free conditions by Student's t-Test. The figure shows the pooled data from two separate endothelial cell lines. Bars represent SEM.

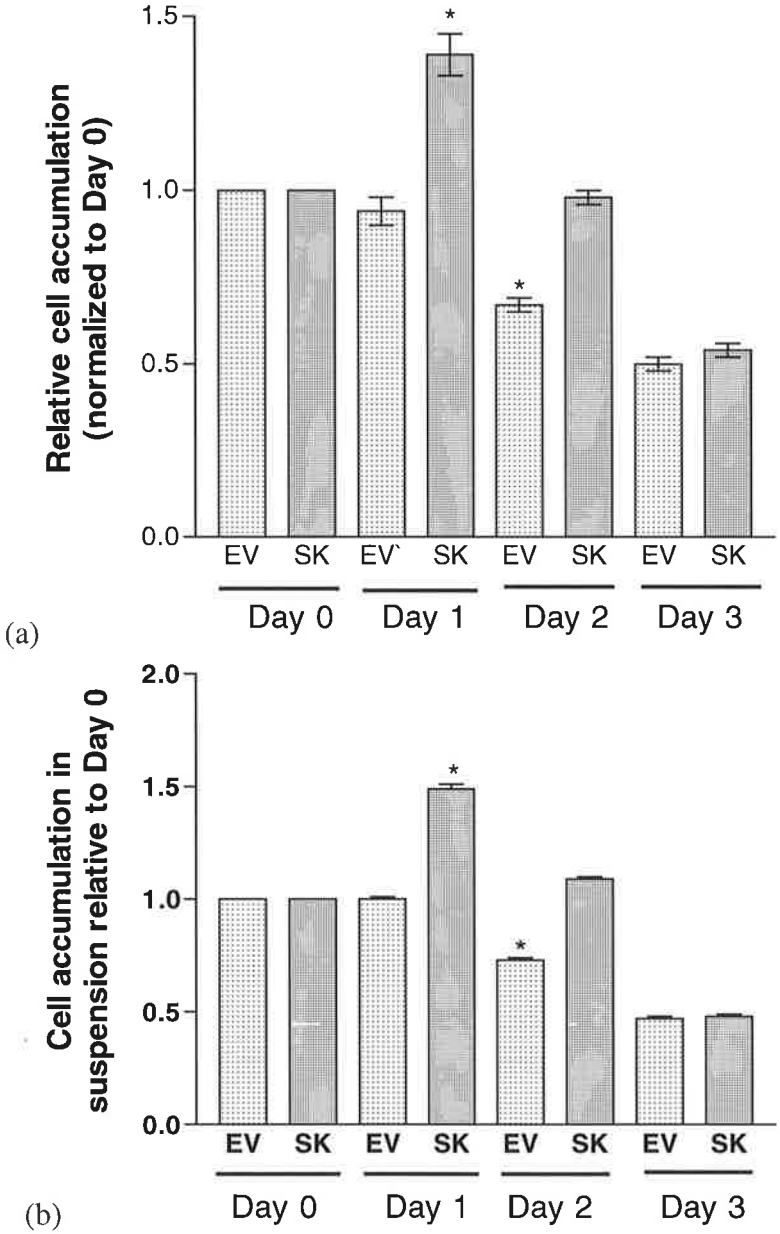


Fig 4.6 shows the survival of HUVEC over-expressing SK or EV as reflected by the optical density, (a) in the absence of FCS and (b) in the absence of both FCS and attachment to extracellular matrix. (a) shows the composite analysis of 43 observations derived from 9 separate experiments, (b) shows the composite analysis of 10 observations from two separate experiments, normalized to Day 0=1, using Statistica Version 6.1 (Statsoft, Inc.). *$p<0.001$ compared with corresponding vector at Day 0. Bars represent 95% confidence intervals.

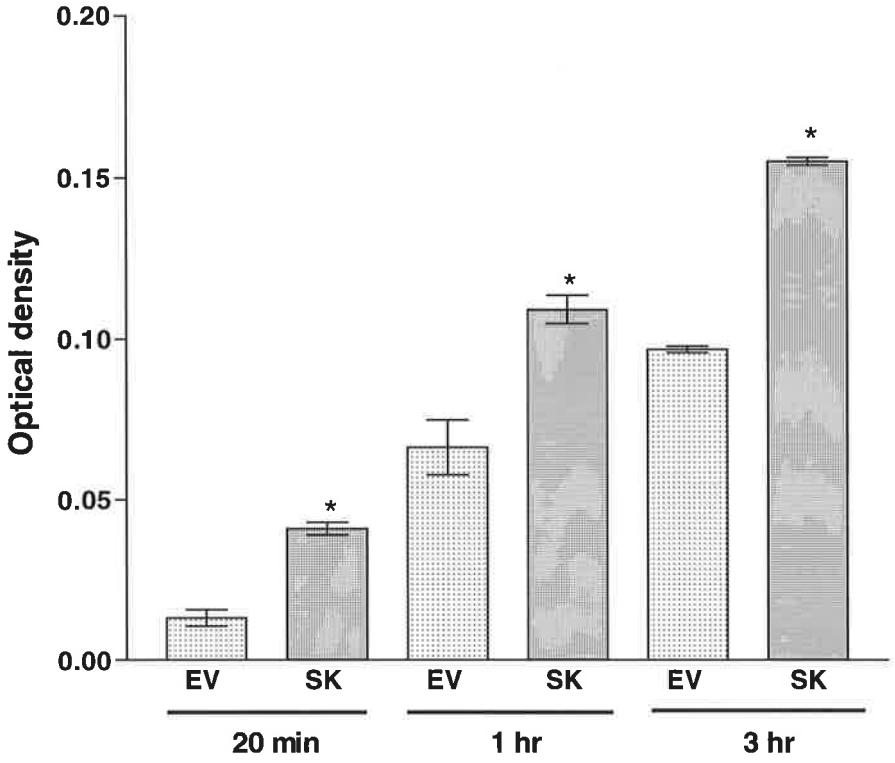


Fig 4.7 shows the attachment of cells over-expressing SK or EV control cells to fibronectin (50μg/ml) at three time points. The figure depicts the composite analysis of two separate experiments using different endothelial cell lines and each undertaken in replicates of three, using Statistica Version 6.1 (Statsoft, Inc.). *$p<0.05$ compared with EV at each time point. Bars represent SEM.

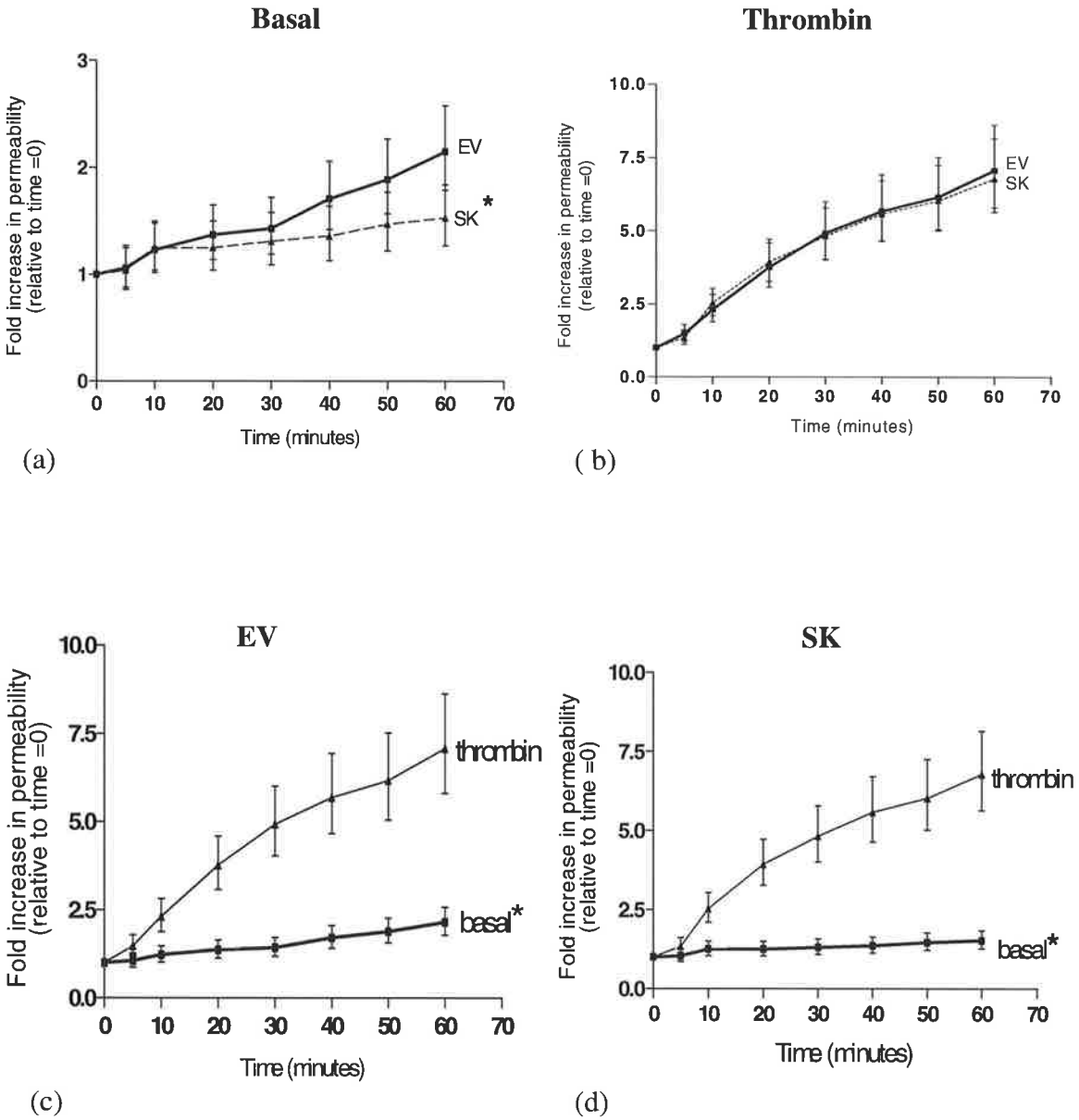


Fig 4.8 shows permeability (normalized to time =0) of EV control cells and cells over-expressing SK in monolayer culture, to FITC-dextran, across different time points under (a) basal conditions and (b,c,d) in response to thrombin stimulation (0.2 units/ml). (b) shows a comparison of permeability of EV and SK in response to treatment with thrombin, (c) and (d) show response of EV and SK cells to thrombin stimulation compared to their respective un-stimulated control preparations. *$p<0.001$ SK compared with EV under basal conditions across all time points (a) or untreated EV compared with thrombin treated vector across all time points (c) or SK compared with thrombin treated SK across all time points (d). The figure shows the composite analysis of 7 observations from 3 separate experiments using Statistica Version 6.1 (Statsoft, Inc.). Bars represent 95% confidence intervals.

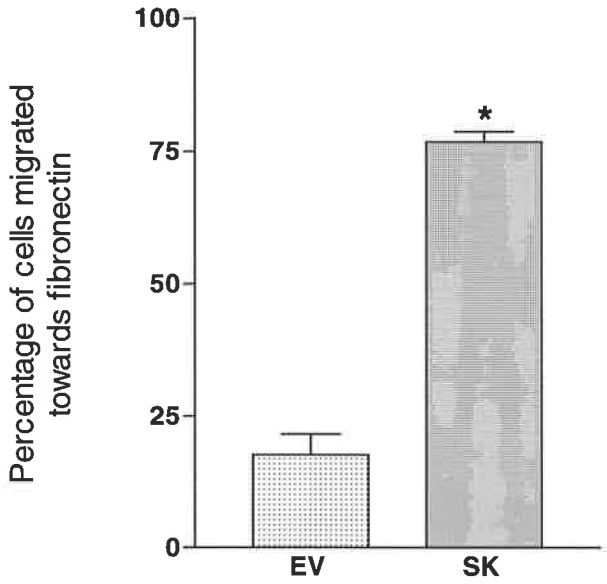


Fig 4.9 shows cell migration specifically towards fibronectin for cells over-expressing SK and control (EV) (migration towards uncoated wells was subtracted). * $p<0.001$ SK compared with EV by Student's t-Test. The figure shows the pooled data from two separate experiments performed in duplicate. Bars represent SEM.

Fig 4.10 shows tube formation by endothelial cells transfected with EV and cells over-expressing SK in the basement membrane matrix, Matrigel at various time points. Cells over-expressing SK are shown in the right panel, and control cells in the left panel. (a) reflects the time of seeding the cells, (b) =15 minutes, (c) =30 minutes, (d) =1 hour, (e) =24 hours.

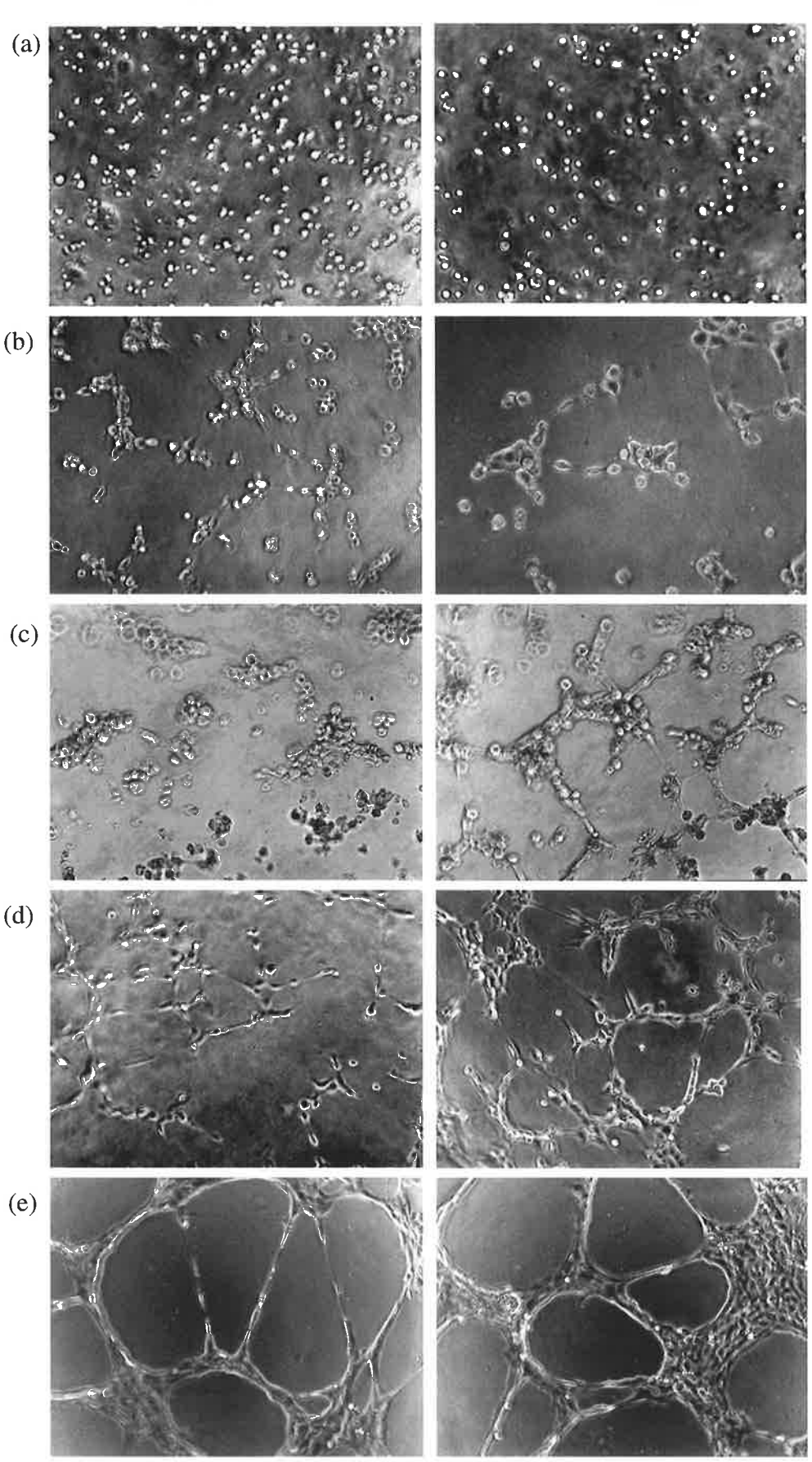


Fig 4.10

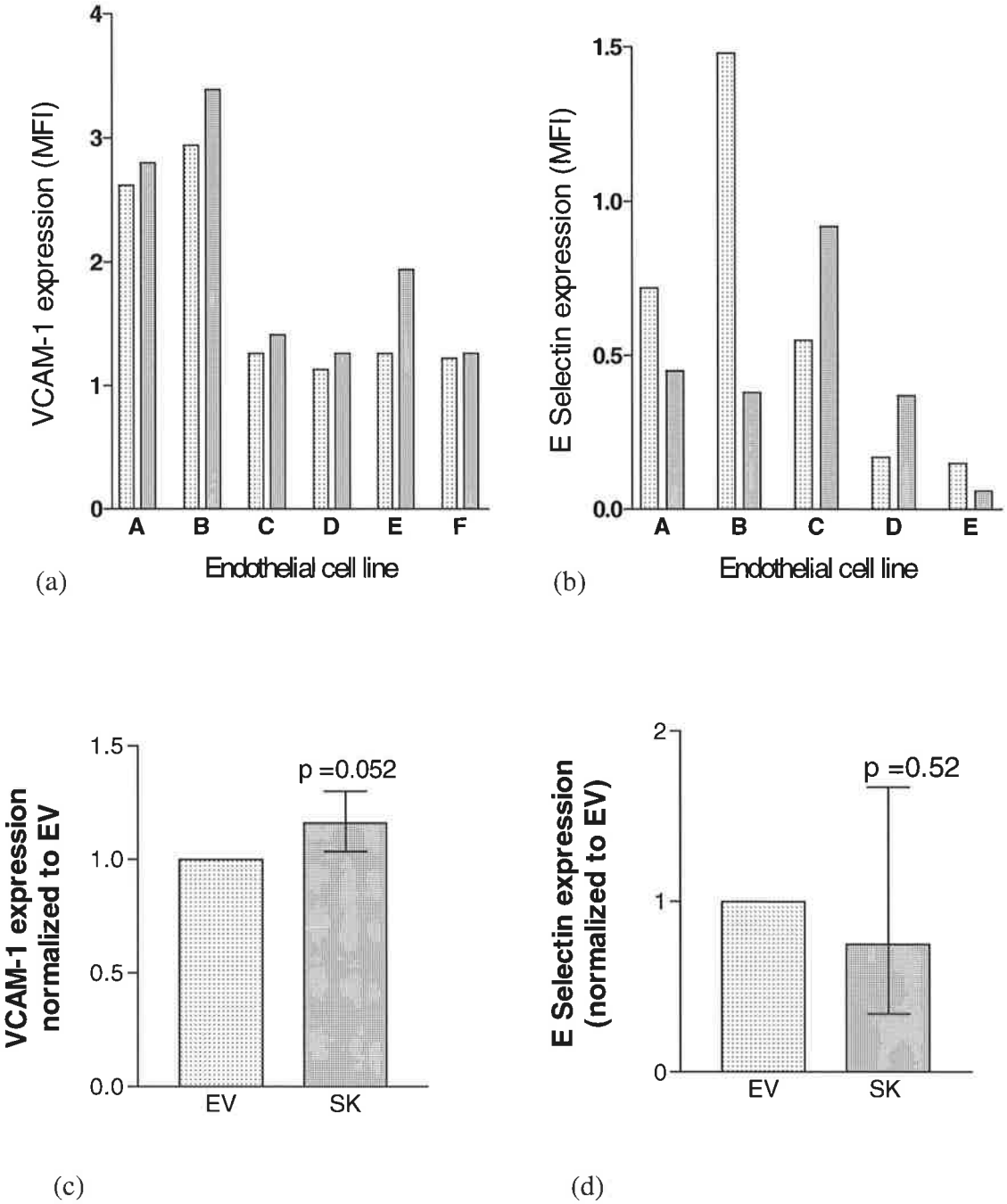


Fig 4.11 shows cell surface expression of VCAM-1 (a) and E Selectin (b) as indicated by the median fluorescence intensity (MFI) in EV control cells and cells over-expressing SK in six and five separate endothelial cell lines respectively. The composite analysis of (a) and (b), normalized to EV is shown in (c) and (d) respectively, using Statistica Version 6.1 (Statsoft, Inc.). p value of SK compared with EV is indicated. Bars represent 95% confidence intervals.

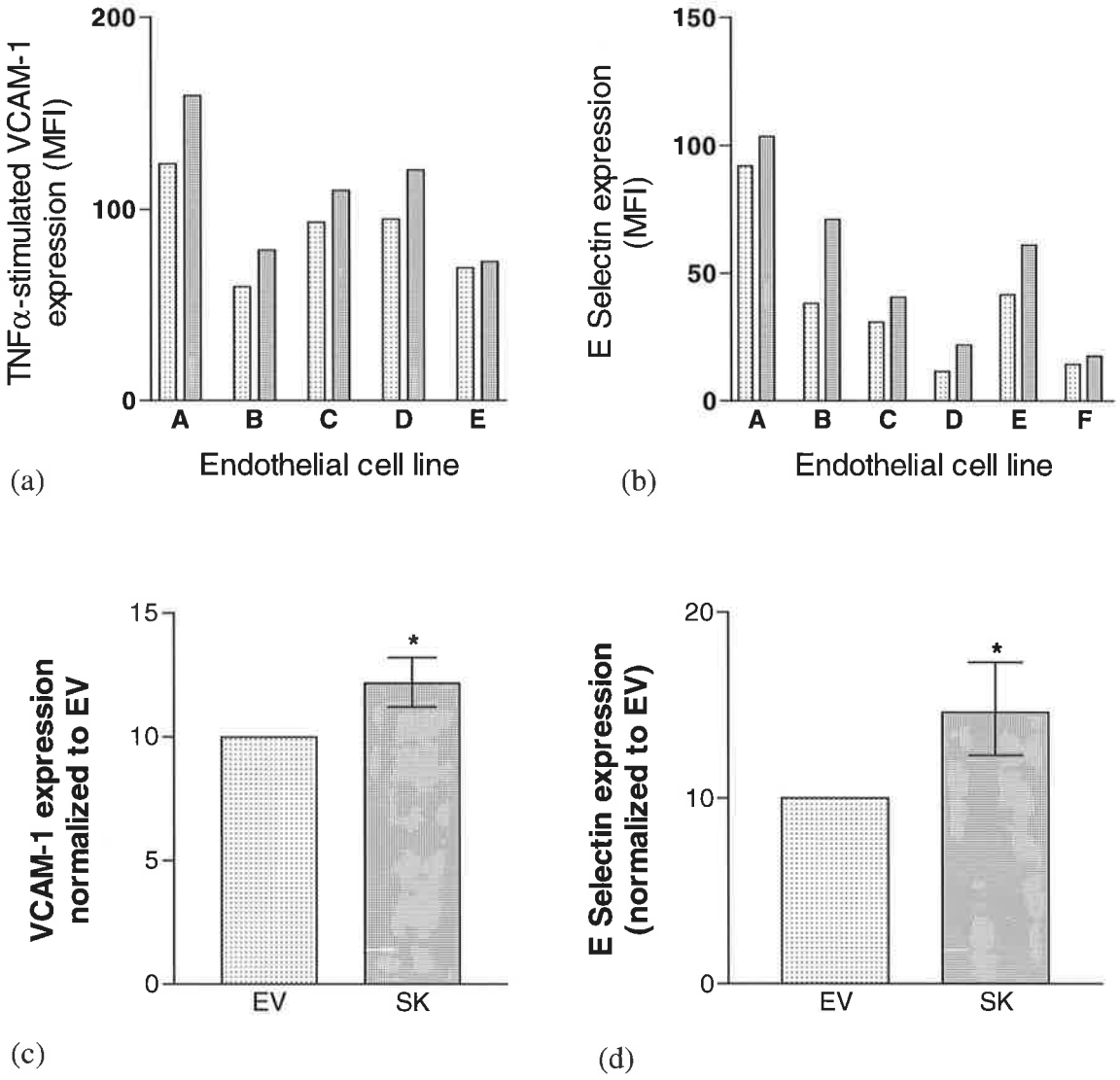
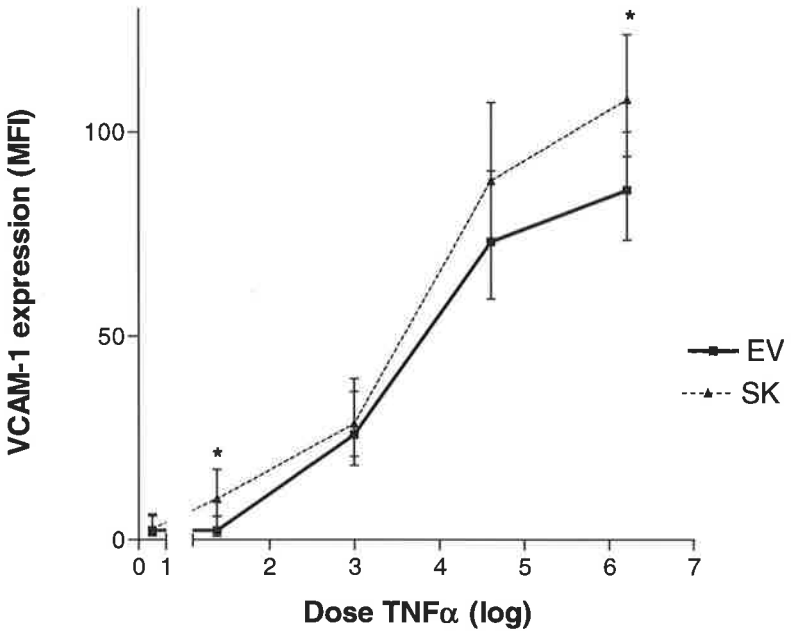


Fig 4.12 shows TNFα-induced VCAM-1 expression in five endothelial cell lines (a) and E Selectin in six endothelial cell lines (b), as indicated by the median fluorescence intensity (MFI), for cells over-expressing SK (dense dots) and EV control (sparse dots). Stimulation with TNFα was undertaken for four hours using 0.5ng/mL. Fluoresecnce resulting from staining with a non-relevant isotype matched negative control antibody (23-1F11) has been subtracted. The composite analysis (using Statistica Version 6.1 (Statsoft, Inc.) of the cell lines depicted in (a) and (b) showing the mean fold increase in VCAM-1 and E Selectin expression in cells over-expressing SK, normalized to EV is shown in (c) and (d) respectively. Bars represent 95% confidence intervals. *$p<0.01$ SK compared with EV.

(a)



(b)

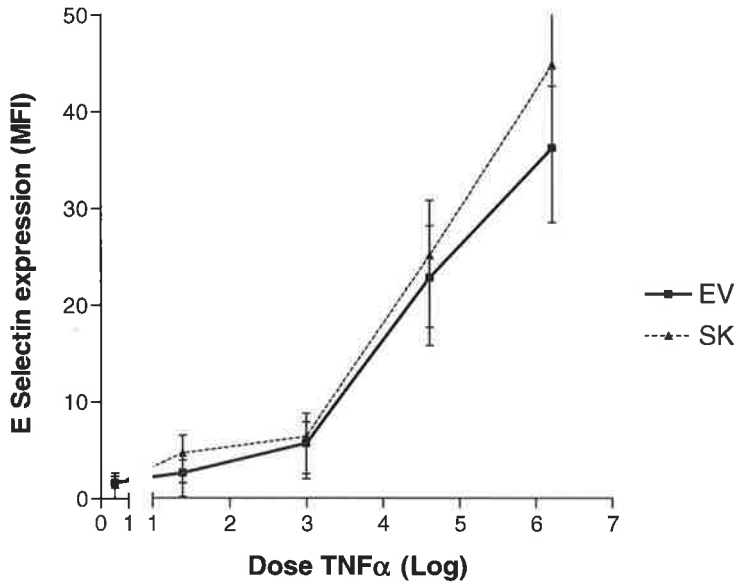


Fig 4.13 shows the effect of over-expression of SK on the dose response curve to TNFα. Endothelial cells transfected with EV and cells over-expressing SK were stimulated with varying doses of TNFα for four hours and cell surface expression of VCAM-1 (a) and E Selectin (b) expression were measured by flow cytometry and are indicated by the median fluorescence intensity (MFI). The figure shows the pooled data from three separate experiments. Bars represent 95% confidence intervals. *$p<0.05$ SK compared with EV at the indicated dose of TNFα.

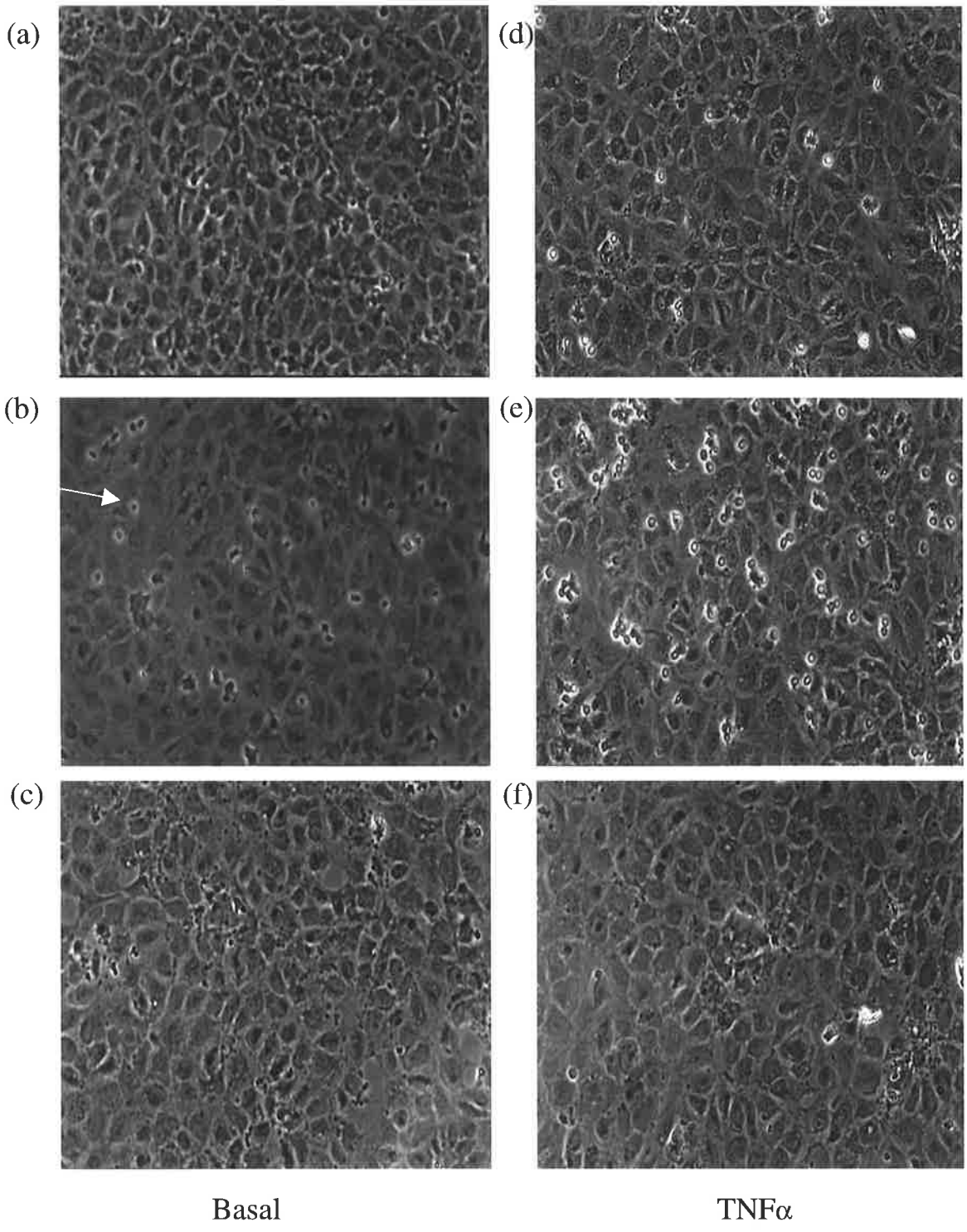


Fig 4.14 shows neutrophil adhesion to endothelial cells over-expressing EV control (a,d) SK (b,e), G82D (c,f) and in the basal state (a-c) and when stimulated for four hours with 0.04ng/mL TNFα (d-f). The white arrow indicates an adherent neutrophil. The figure shows results from one experiment which is representative of two separate experiments.

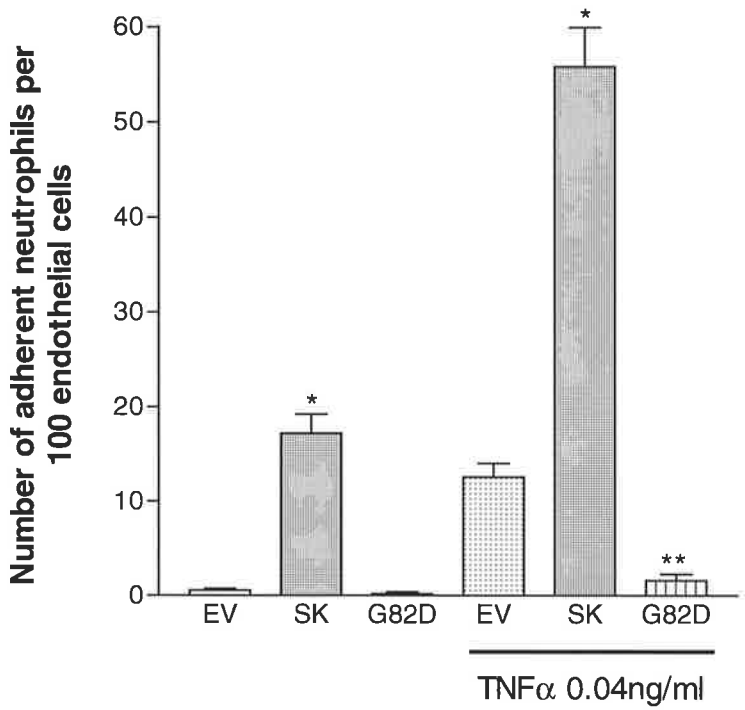


Fig 4.15 shows neutrophil adhesion per 100 endothelial cells over-expressing SK, G82D, or EV control under basal conditions, or in response to four hours of stimulation with TNFα 0.04ng/ml. The figure shows the pooled data of ten separate fields of view obtained from two separate experiments. *$p<0.001$ untreated SK compared with untreated EV, treated SK compared with treated EV, by Student's t-Test. **$p<0.05$ treated G82D compared with treated EV by Student's t-Test. Bars represent SEM.

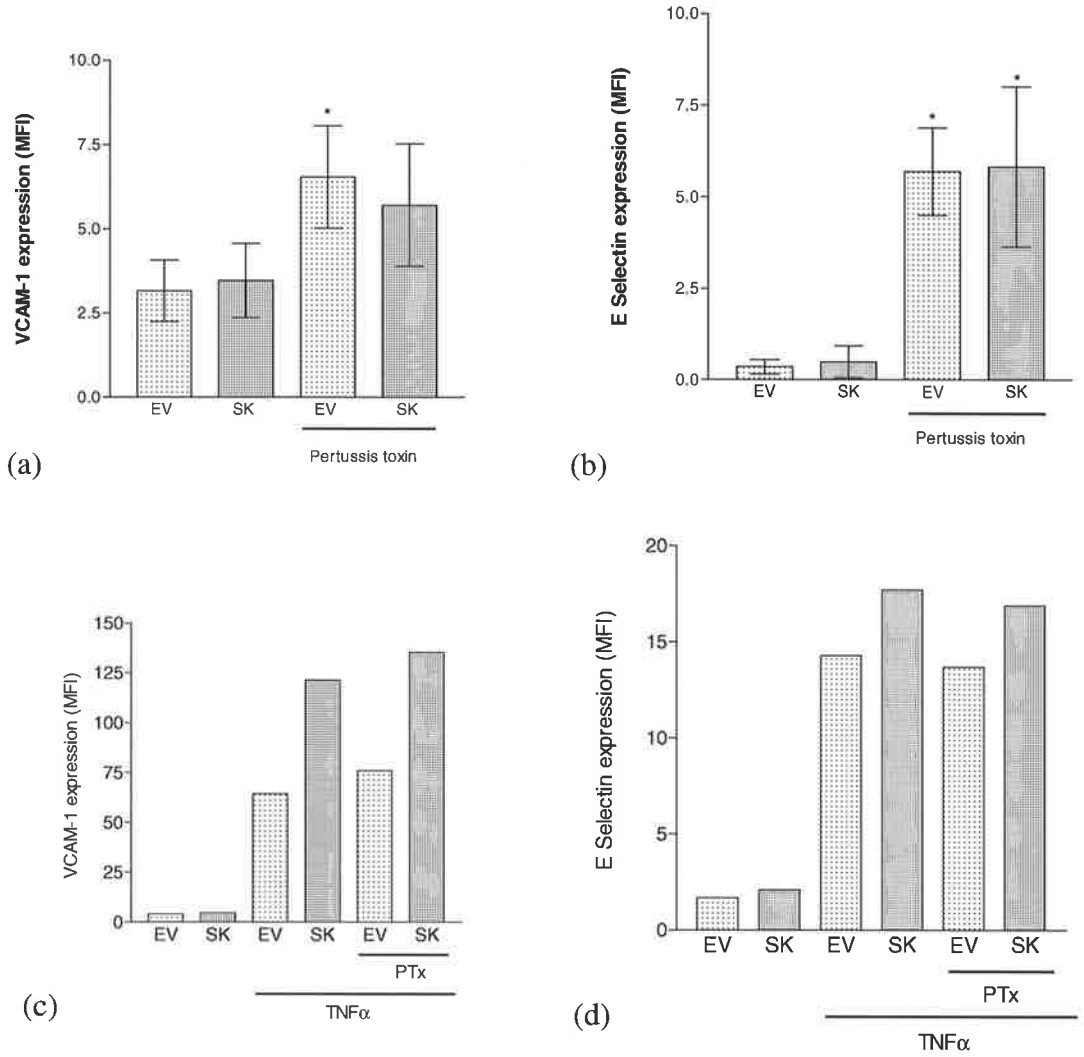


Fig 4.16 shows the effect of pertussis toxin (PTx) 50ng/ml on basal (a,b) and TNFα-stimulated (c,d) adhesion molecule expression in cells over-expressing SK and EV. (a) and (b) show the pooled data from three separate experiments, (c) and (d) show the results from a single experiment which is representative of three separate experiments. Bars represent SEM. *$p<0.05$ compared with corresponding untreated vector.

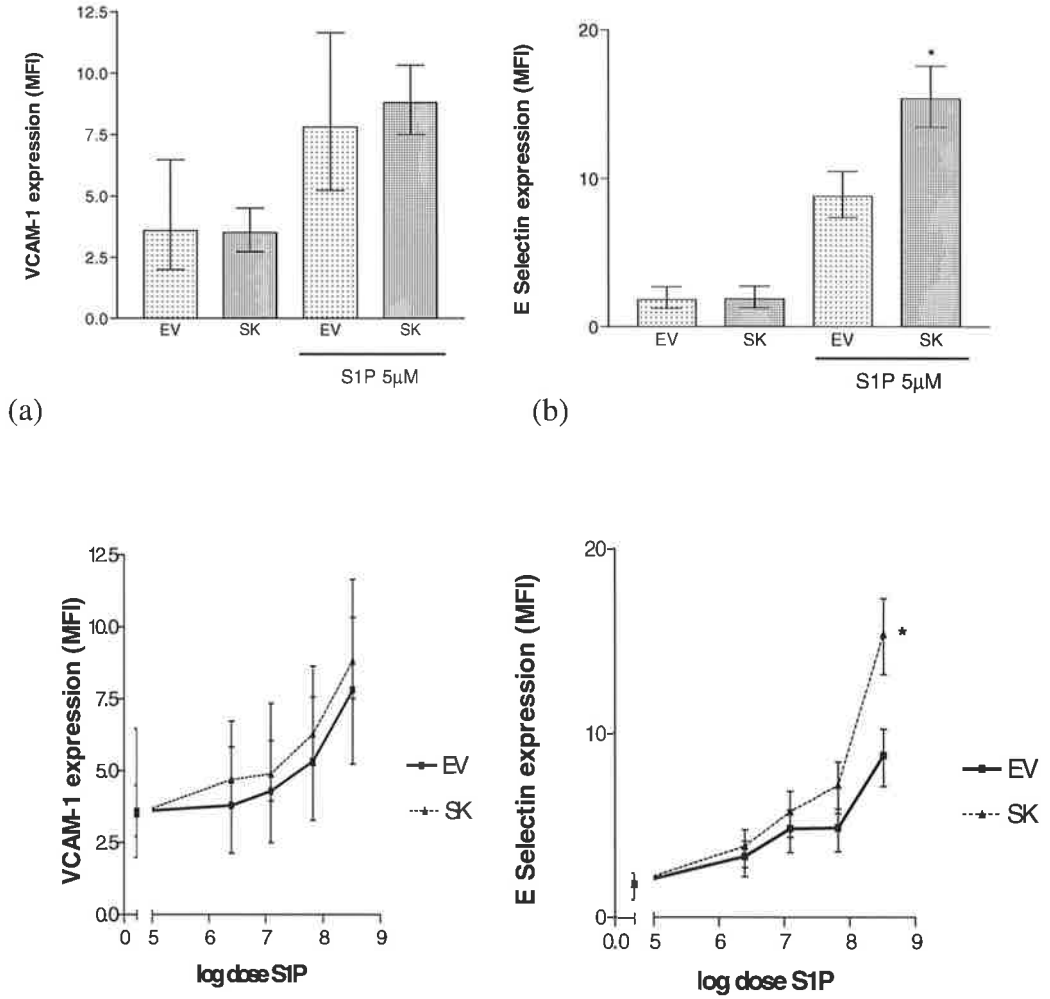


Fig 4.17 shows the adhesion molecule response as indicated by the median fluorescence intensity (MFI) to S1P stimulation in cells over-expressing SK or EV. The VCAM-1 (a) and E Selectin (b) response to stimulation with 5μM S1P for four hours is shown. *$p<0.001$ SK compared with EV in presence of S1P. (c) and (d) show the dose-response curves for VCAM-1 and E Selectin to S1P stimulation respectively. *$p<0.05$ SK compared with EV when analysed across all doses tested. The figure shows the composite analysis of three observations from three separate experiments using Statistica Version 6.1 (Statsoft, Inc.). Bars represent 95% confidence intervals.

CHAPTER 5

Mechanisms responsible for phenotypic alterations consequent upon moderately raised intracellular SK activity

INTRODUCTION

The phenotypic consequences of over-expression of SK in HUVEC have been described. Over-expression of SK achieved with retroviral or adenoviral-mediated gene delivery yielded similar phenotypic changes of enhanced angiogenesis and inflammatory potential. Many of these phenotypic changes are predictable from the known effects of stimulation of endothelial cells with exogenous S1P, yet it remains unclear whether the cellular pathways activated by intracellular over-expression of SK are identical or distinct to those triggered by stimulation of endothelial cells with S1P.

Endothelial cell survival is an essential mechanism in the intricately regulated process of angiogenesis and hence there has been much interest in elucidating the factors regulating the survival of endothelial cells. Cell survival is the net sum of those cells that die by the physiological process of programmed cell death, or apoptosis, and those which remain viable. Similarly, angiogenesis is dependent upon a dynamic balance between factors that promote angiogenesis and factors which inhibit angiogenesis. Indeed there is considerable evidence that suppression of apoptosis with subsequent cell survival is permissive to angiogenesis while induction of apoptosis prevents angiogenesis.

The role of growth factors in maintaining endothelial cell survival is well established. VEGF prevents endothelial cell apoptosis by induction of anti-apoptotic proteins Bcl-2 (207;208), A1 (207), and survivin (209). It also activates the phosphatidylinsoditol 3-kinase (PI3K)/Akt pathway (210;211), and the Raf-MEK-MAPK pathway both of which are critical pathways promoting cell survival. Similarly bFGF upregulates the expression of Bcl-2 (212)and

survivin (213), along with activating Akt to promote cell survival (214). Serum is also a recognized trophic factor for endothelial cells, and human umbilical vein endothelial cells (HUVEC) have been demonstrated to undergo apoptosis after 24 hours of serum deprivation, as indicated by enhanced caspase-3 activation, DNA fragmentation, cytochrome c release, and enhanced DEVDase activity(215).

Endothelial cell survival is further dependent upon adhesion to the extracellular matrix and also to surrounding cells. Cell-matrix attachments are mediated by the integrins and integrin ligation triggers downstream signalling events which inhibit apoptosis. Detachment of endothelial cells from extracellular matrix results in apoptosis(216). Cell-cell adhesion also plays a critical role in cell survival. In addition to the cell junctional molecule PECAM-1 (CD31), which clearly has anti-apoptotic effects (217), the major structural protein involved in adherens junctions, VE-cadherin is also involved in mediating the anti-apoptotic effects of VEGF (214).

In addition to the aforementioned survival factors for endothelial cells, S1P enhances cell survival. Addition of exogenous S1P to endothelial cells rescues the cells from serum deprivation-induced apoptosis, by virtue of its actions on the EDG-1 receptor to signal to the G(i) protein, resulting in activation of PI-3kinase, with subsequent Akt-mediated eNOS phosphorylation (215;218). Binding of S1P to the EDG receptor activates ERK1/2 resulting in stimulation of cell proliferation and cell survival (157;197). Hence involvement of these pathways in mediating the phenotypic alterations seen with over-expression of SK is envisaged.

With the acceptance that S1P may function both as an intracellular second messenger, and also as a specific ligand for the EDG receptors, a further question raised is whether the

cellular effects of raised intracellular levels of SK are mediated by an intracellular site of action of S1P, or whether they are a consequence of S1P binding the EDG receptor.

AIM

The aim is to elucidate the cellular mechanisms underlying the enhanced potential for inflammation and angiogenesis seen with raised intracellular levels of SK in endothelial cells.

RESULTS

5.1 Over-expression of SK alters the interaction of the cell with the extracellular matrix

5.1.1 Over-expression of sphingosine kinase up-regulates particular integrins

Integrins mediate the attachment of the cell to the extracellular matrix, and integrin ligation results in the transduction of signals from the exterior to the interior of the cell. These signals are known to regulate processes such as proliferation and migration and indeed attachment to the extracellular matrix is critical for endothelial cell viability. In light of the SK-mediated enhanced cell accumulation, attachment to fibronectin, and cell migration, cell surface expression of integrins expressed on endothelial cells was measured. This was undertaken by flow cytometry.

Over-expression of SK increased the cell surface expression of β1 integrin and αvβ3 integrin in HUVEC (Table 5.1). The increase in β1 integrin was also confirmed by Western blot analysis in two separate endothelial cell lines. The effect was specific to these integrins, as other integrins (α2β1) were not significantly altered (mean fold increase above EV control 2.06, 95% CI 1.1-3.85, n= 4, p=0.18).

5.1.2 SK-induced adhesion is mediated by β1 integrin

It has been established previously that over-expression of SK enhances adhesion to the extracellular matrix protein, fibronectin (Section 4.3.4). To determine whether the alterations in β1 integrin in cells over-expressing SK was responsible for this enhanced cell adhesion, the effect of blockade of this integrin on cell attachment to fibronectin was determined. Cells were pre-treated with a murine monoclonal antibody directed against β1 integrin which blocks adhesion (61-2C4, 20μg/ml). Attachment of the cells to fibronectin was measured in the presence and absence of 61-2C4.

In the presence of 61-2C4, both control cells and cells over-expressing SK showed significant reduction in cell adhesion (Fig 5.1(a)). Moreover, when adhesive interactions of β1 integrin were blocked, there was no difference in attachment of cells over-expressing SK compared with control ($p=0.96$). This indicates that SK-mediated enhanced cell adhesion to fibronectin is mediated largely by β1 integrin.

5.1.3 Over-expression of SK causes activation of β1 integrin.

The enhanced cell surface expression of β1 integrin seen with over-expression of SK raises the question of whether there is an accompanying qualitative change in β1 integrin (activation). Prior to measuring cell attachment to fibronectin, cells were treated for ten minutes with an IgG1 monoclonal antibody (8A2) (219), which induces a conformational change in β1 integrin, resulting in activation and therefore increased adhesiveness of β1 integrin to fibronectin. Cell adhesion was measured in the presence of 8A2.

Pre-treatment of cells with 8A2 significantly enhanced cell adhesion for control cells but did not alter adhesion of cells over-expressing SK ($p=0.66$) as shown in Fig 5.1(b,c). This suggests that β1 integrin is already activated in cells over-expressing SK.

5.2 The effect of over-expression of SK on cell cycling

The enhanced cell accumulation over three days seen in cells over-expressing low levels of SK (Chapter 4.2) prompted an enquiry into a role for SK in increasing cell cycling. The cyclin family of proteins regulates the cell's progression through the various stages of the cell cycle, and comprises two groups. Cyclins A and B regulate cell division and are known as mitotic cyclins, whilst the G1 cyclins (C, D and E) regulate the early stages of the cell cycle(220). Cyclins activate the cyclin-dependent kinases (cdk), a family of serine-threonine kinases, which when activated phosphorylate some oncogenes resulting in mitogenesis(220). It is the D-type cyclins which are essential for progression through the G1 phase of the cell cycle. Cyclin D binds and activates Cdk4 and Cdk6, and levels of cyclin D are known to rise in early G1 phase(221).

Cyclin D1 expression was measured under basal conditions as an indicator of cell cycling and it was shown that over-expression of SK does not alter cyclin D1 expression (Fig 5.2). Quantification using ImageQuant software confirmed there was no alteration in cyclin D1 expression in three separate endothelial cell lines over-expressing SK ($p=0.28$). Further strength to the lack of increase in cyclin D1 levels with over-expression of SK was obtained by measuring cyclin E expression after 24 hours of serum deprivation. There was no change in cyclin E expression in three separate endothelial cell lines (the same three cell lines in which cyclin D1 was measured) over-expressing SK compared with control ($p=0.82$).

To address the possibility that SK may alter stimulated cyclin D1 or cyclin E expression, (which may not have been detected in cells under basal conditions), the expression of these proteins was measured in cells taken at log phase (24 hours culture in reduced serum medium in the absence of mitogenic factors) and after stimulation of cell proliferation with ECGs for variable duration. Cyclin D1 and cyclin E expression were induced by stimulation for 24 hours with ECGs in both cells over-expressing SK and EV cells (Fig 5.2). There was however no difference in cyclin D1 or cyclin E expression between cells over-expressing SK compared with control.

Taken together, the lack of up-regulation of cyclin D1 and cyclin E in cells over-expressing SK suggests that SK-induced increase in cell accumulation is not due to increased cell cycling, but rather due to reduced cell death, consistent with the observed reduction in apoptosis seen in these cells.

5.3 The PI-3Kinase pathway plays a central role in mediating the phenotypic consequences of over-expression of SK

5.3.1 The PI-3Kinase/Akt pathway mediates SK-induced cell accumulation

The PI-3kinase/Akt pathway (PI-3K/Akt) and the Ras/ mitogen-activated protein kinase (MAPK) pathway are the major cellular pathways involved in mediating cell survival. The MAPK pathway is also involved in mediating cell proliferation. The reduction in caspase-3 activity in cells over-expressing SK (Chapter 4.3.1.1) together with the lack of up-regulation of cyclin D1 or cyclin E suggests that reduction in cell death may be the mechanism underlying the SK-mediated enhanced cell accumulation. Thus it was sought to determine whether cells over-expressing SK utilize the PI-3kinase/Akt pathway to enable enhanced cell accumulation or, whether there is a true increase in cell proliferation through the MAPK

pathway. To this end cell accumulation was measured by the colorimetric MTS assay and the effect of inhibiting the PI-3kinase/Akt pathway with 10 μM LY294002, and the MAPK pathway with 20μM of either of two inhibitors of mitogen-activated protein kinase kinase (UO126)(222) or PD98059(223) was determined.

Results are shown in Fig 5.3(a). The SK-mediated enhanced cell accumulation at Day 3 is shown. Control cells showed significant reduction in cell accumulation in response to LY294002, UO126, and PD98059 ($p<0.001$). When cells over-expressing SK were cultured in the presence of LY294002, the SK-mediated enhanced cell accumulation was abolished ($p=0.87$). However, cells over-expressing SK maintained enhanced cell numbers compared with control in the presence of either UO126 ($p<0.001$) or PD98059 ($p<0.001$).

Biochemical confirmation of inhibition of MAPK pathway by the inhibitors used was undertaken by Western blot to measure the phosphorylation of ERK1/2. Both UO126 and PD98059 inhibited ERK1/2 phosphorylation in cells over-expressing SK and EV as shown in Fig 5.3(b). These results confirm that the inhibitors used can inhibit the ERK1/2 pathway when this is activated. It also suggests that this pathway is not activated by over-expression of SK, and that SK-mediated enhanced cell accumulation is mediated through the PI-3K/Akt pathway.

5.3.2 SK provides resistance to caspase-3 activation through the PI-3 kinase/Akt pathway

As the PI-3K/Akt pathway is the major pathway mediating cell survival, a role for this pathway in mediating the anti-apoptotic effects conferred by over-expression of SK was sought. Caspase-3 activity was measured in whole cell lysates made under basal conditions

and after serum deprivation for 24 hours. The effect of inhibiting the PI-3kinase/Akt pathway with LY294002 was determined.

Cells over-expressing SK showed a statistically significant reduction in caspase-3 activation in response to 24 hours of serum deprivation compared with EV control ($p<0.05$), as indicated in Fig 5.4. In the presence of LY294002 (10μM), cells over-expressing SK responded with a larger increase in caspase-3 activity compared with EV control ($p<0.001$).

Thus, it was concluded that cells over-expressing SK utilize the PI-3kinase/Akt pathway to prevent caspase-3 activation in response to serum deprivation.

5.3.3 The PI-3kinase/Akt pathway mediates SK-induced cell survival

The observation that cells over-expressing SK engage the PI-3kinase/Akt pathway to prevent caspase-3 activation prompted an enquiry into whether this was the mechanism increasing cell survival in the absence of serum. Cell survival was measured by determining relative cell numbers using the colorimetric MTS assay after incubation in serum-free medium for two days. The effect on cell survival of inhibiting the PI-3 kinase/Akt pathway with LY294002, and the MAPK pathway with UO126 and PD98059 was investigated.

Whilst LY294002, UO126 and PD98059 all significantly reduced survival of control cells ($p<0.001$), cells over-expressing SK responded to LY294002 with reduced cell survival ($p<0.001$) but did not show a significant response to UO126 or PD98059 (Fig 5.5a). Cells over-expressing SK maintained significantly enhanced relative cell numbers compared with control in the presence of either UO126 ($p<0.001$) or PD98059 ($p<0.001$), but not in the

presence of LY294002. The presence of a vehicle control (DMSO) had no significant effect on either control cells or cells over-expressing SK.

The role of other pathways in mediating SK-induced cell survival, namely the JAK kinase and Src kinase pathways, which are also activated in response to growth factors, was also examined. Cells over-expressing SK and control (EV) were incubated in serum free medium, and the effect of 10μM AG490 (JAK-kinase inhibitor) and 10μM PP1 (Src-kinase inhibitor), (as well as LY294002 as a positive control) on cell survival was examined.

Results are shown in Fig 5.5(b). Incubation of cells with AG490 or PP1 reduced survival of control cells but not cells over-expressing SK. Cells over-expressing SK maintained survival advantage compared with control in the presence of AG490 ($p<0.001$) or PP1 ($p<0.001$), but not in the presence of LY294002 ($p=0.348$).

Thus I concluded that SK-mediated cell survival is mediated through the PI-3K pathway, and not through the MAPK pathway, or through JAK kinase or Src kinase pathways.

5.3.4 The PI-3Kinase/Akt pathway mediates SK-induced cell migration

Cells over-expressing SK show increased migratory capacity compared with control (Chapter 4.3.6). Exogenous stimulation with S1P is known to induce cell migration, and is dependent upon Akt-mediated phosphorylation of EDG-1(224). Hence a role for the PI-3kinase/Akt pathway in mediating the enhanced cell migration resulting from intracellular over-expression of SK was sought. To this end, cell migration towards fibronectin was measured in the presence and absence of an inhibitor of the PI-3kinase/Akt pathway (10μM LY294002).

Migration towards fibronectin at 20 hours was higher for cells over-expressing SK (76.8% ± 1.57) compared with control cells (17.6% ± 3.91), $p<0.001$ (Fig 5.6). Inhibition of the PI-3 kinase/Akt pathway significantly reduced migration of cells over-expressing SK ($p<0.001$), but did not alter migration of control cells ($p=0.71$). In the presence of LY294002 there was no significant difference in migration between cells over-expressing SK and control cells ($p=0.13$).

5.3.5. Over-expression of SK activates the PI-3 kinase/Akt pathway

Inhibitor studies shown above suggest that cells over-expressing SK utilize the PI-3kinase/Akt pathway to mediate many of the phenotypic changes, namely enhanced cell accumulation, cell survival, resistance to serum deprivation-induced apoptosis, and cell migration. Hence biochemical evidence for activation of this pathway by SK was sought. This was undertaken by quantifying proportionate staining of phosphorylated Akt (p-Akt) and total Akt on Western blot using ImageQuant software.

Under basal conditions there was no significant difference in the percentage of phosphorylated AKT (p-Akt) in cells over-expressing SK compared with control ($p=0.47$), as shown in Fig 5.7(a). The normal response as demonstrated in the control cells is a reduction in the phosphorylation of Akt in response to serum deprivation. Cells over-expressing SK however responded to the stress of serum deprivation by an increase in phosphorylation of Akt. Thus, in serum free conditions cells over-expressing SK had displayed significantly more phosphorylation of Akt than the EV control. This was confirmed by quantitative analysis of lanes using ImageQuant software in five separate endothelial cell lines (Fig 5.7(b)).

5.3.6 Conclusions

With the above evidence, it was concluded that cells over-expressing SK are able to activate the PI-3 kinase/Akt pathway in response to the stress of serum deprivation. This has functional consequences in terms of providing resistance to caspase-3 activation and enhancing cell survival in the absence of serum. The enhanced cell accumulation observed in cells over-expressing SK was also demonstrated to be mediated by the PI-3kinase/Akt pathway, and not by the MAPK pathway which is generally thought to be responsible for cell proliferation. Indeed this is consistent with the lack of up-regulation of cyclin D1 and cyclin E in cells over-expressing SK, and indicates that the enhanced cell accumulation conferred by SK is a result of increased cell survival (and reduction in cell death) rather than an increase in the rate of proliferation.

5.4 Over-expression of SK does not activate the MAPkinase pathway

Functional assays (cell accumulation and cell survival) did not support a role for the MAPK pathway in mediating the phenotypic changes resulting from intracellular over-expression of SK. It is known however that exogenous stimulation with S1P activates this pathway. Hence direct evidence for lack of activation of this pathway by SK over-expression was sought and furthermore, it was to be determined whether these cells maintained the ability to activate this pathway in response to exogenous stimulation with S1P. This was undertaken by quantitative analysis of Western blots stained for phosphorylated ERK 1/2 (p-ERK) and total ERK1/2 under basal conditions, in response to serum deprivation and in response to exogenous stimulation with S1P for six hours (5μM).

Over-expression of SK did not alter phosphorylation of ERK 1/2 under basal conditions Fig 5.8, lane 4 versus 1) or in response to serum deprivation (lane 5 versus 2) (mean fold increase above control 0.9 ±0.08, and 0.9 ±0.03 respectively, n=2). The normal response (as seen in the control, lane 3) of activation of the MAPK pathway by exogenous stimulation with S1P (5μM) for thirty minutes however was preserved in cells over-expressing SK (lane 6), indicating that the EDG receptor pathway is intact.

5.5. Is the activation and up-regulation of β1 integrin by SK involved in mediating the phenotypic changes of enhanced cell accumulation and cell survival?

The integrin β1 signals through the adaptor protein Shc to activate the MAPK pathway with resultant cell proliferation. It has however been shown that although over-expression of SK upregulates and activates β1 integrin (Chapter 5.1.1, 5.1.2), this does not lead to activation of ERK 1/2 or induction of cyclin D1 expression, two measures of cell proliferation. Hence it was to be determined whether activation of β1 integrin by SK has downstream effects relevant to the phenotype of these cells.

5.5.1.1 The effect of over-expression of SK on cellular levels of Shc

Integrins α1β1, α5β1, and αvβ3 signal through the adaptor protein Shc to engage the MAPK pathway. Shc comprises three isoforms; 46/52 kDa isoforms which when phosphorylated activate the MAPK pathway(225-227), and p66Shc which even when phosphorylated, fails to activate the MAPK pathway(228).

In view of the up-regulation and activation of β1 integrin by SK, associated with a lack of activation of ERK 1/2, it was to be determined whether SK signals through Shc. Total cellular levels of Shc were measured by Western blot.

Over-expression of SK did not alter the expression of 46/52 kDa Shc isoforms. However, in four separate endothelial cell lines over-expressing SK, there was a consistent increase in p66Shc expression, and indeed in two endothelial cell lines, p66Shc expression was detected in cells over-expressing SK, but not in control (Fig 5.9a).

5.5.1.2 The effect of over-expression of SK on activation of Shc

Shc is regulated by phosphorylation on tyrosine residues, and an enquiry into the tyrosine phosphorylation status of the various isoforms of Shc in cells over-expressing SK was made. This was done by Western blot using anti-phosphotyrosine antibody on whole cell lysates made after growth-factor stimulation, and re-blotting with anti-Shc antibody after stripping the membrane.

On blotting with anti-phosphotyrosine antibody a 66kDa band was present and somewhat more intense in cells over-expressing SK compared with control (Fig 5.9(b)). On re-blotting with anti-Shc antibody, this band overlay the 66kDa Shc band. This was confirmed in three separate cell lines and quantification is shown in Fig 5.9(c). Analysis of the pooled data revealed over-expression of SK enhanced phosphorylation of p66Shc, although this did not reach statistical significance ($p=0.13$) in the small sample studied.

Thus it was concluded that over-expression of SK may increase phosphorylation of p66Shc. The possibility however that this band is a non-specific background band which

coincidentally overlays with the 66kDa Shc band has not been excluded, and this issue could be resolved by measuring phosphorylation in immunoprecipitated Shc. It was thought not to be necessary to proceed to this step, as the chances of consistently, coincidentally detecting a more intense band in cells over-expressing SK (with equal protein loading) are remote.

As p66 Shc is a known negative regulator of the MAPK pathway, enhanced phosphorylation of this isoform of Shc in cells over-expressing SK is consistent with the lack of activation of the MAPK pathway seen in these cells. It is noteworthy that over-expression of SK has no effect on the reduction in phosphorylation of ERK1/2 in response to serum deprivation (Fig 5.8 lane 1,2 compared with lane 4,5). No phosphorylation of the 46/52 kDa isoforms of Shc could be detected in either cells over-expressing SK or control cells, even after growth-factor stimulation.

5.5.2 SK-induced enhanced cell accumulation is independent of β1 integrin

As over-expression of SK activates β1 integrin but does not lead to enhanced phosphorylation of 46/52 kDa Shc or ERK1/2 activation, the question was raised as to the whether the activation of β1 integrin by SK is inconsequential in terms of cell proliferation. Hence it was sought to determine whether the increase in relative cell number in cells over-expressing SK is maintained when β1 integrin interactions are blocked by the non-signalling monoclonal antibody, 61-2C4.

Cell accumulation in medium supplemented with 2% FCS was measured over three days by the colorimetric MTS assay. Anti-β1 integrin (61-2C4) was added to the cells two hours after seeding the cells in order to allow attachment to the extracellular matrix, and each day the medium was replaced with medium containing fresh 61-2C4.

It was found that blocking β1 integrin using 20μg/ml of 61-2C4 did not inhibit cell accumulation in either cells over-expressing SK or control. To ensure blockage of β1 integrin, the effect on cell proliferation of a higher dose of 61-2C4 (100μg/ml) was assessed. Results are shown in Fig 5.10(a). There was no difference in the numbers of cells seeded at Day 0 ($p=0.34$), however there were significantly more cells over-expressing SK compared with control at Day 3 ($p<0.05$). Blocking β1 integrin with 61-2C4 resulted in an increase in cell number for control cells ($p=0.01$), and a non-significant increase in relative cell number of cells over-expressing SK ($p=0.195$). It is shown that cells over-expressing SK maintained an increase in relative cell number at Day 3 in the presence of 61-2C4, suggesting that the SK-mediated increase in cell accumulation is not dependent on β1 integrin.

The experimental design used is recognised not to be optimal, as it is possible that β1 integrin is already activated at the time of cell attachment to the extracellular matrix, and that 61-2C4 may not be accessible to the β1 antigen once the cells have attached. Furthermore, an isotype-matched control antibody was not used. The small but statistically significant increase in relative cell number of control cells in the presence of 61-2C4 is interesting, and one possible explanation is that the antibody is enabling these cells to detach and "round up" to facilitate cell proliferation. In cells over-expressing SK, the SK-induced activation of β1 integrin may mediate firmer adhesion to the extracellular matrix, and thus detachment and a further increase in cell proliferation is not seen. As over-expression of SK may increase cell number in other ways, cell survival ay be less dependent on β1 integrin related events.

5.5.3 β1 integrin does not mediate SK-mediated cell survival

As SK-mediated enhanced cell accumulation was shown not to be largely mediated by β1 integrin, the effect of inhibiting signalling by this integrin (with 61-2C4) on cell survival in serum free medium was assessed. This was done using the MTS assay with the addition of 61-2C4 (100μg/ml) two hours after seeding the cells. The medium was replaced daily with fresh 61-2C4.

Results are shown in Fig 5.10(b). There was no difference in the number of cells over-expressing SK compared with control cells seeded at Day 0 ($p=0.56$). At Day 2 there were significantly more viable cells among those over-expressing SK compared to the EV control ($p<0.05$). Cells over-expressing SK increased this survival advantage in the presence of 61-2C4 ($p<0.05$).

It is noted that it cannot be conclusively stated that SK-mediated cell survival is independent of β1 integrin, as it is possible that 61-2C4 has negligible effect once β1 integrin activation has occurred at the time of cell attachment to extracellular matrix and an isotype-matched control antibody was not used. It is however noteworthy that although blocking β1 integrin did not reduce survival of control cells ($p=0.13$), cells over-expressing SK showed a statistically significant increase in cell survival in the presence of 61-2C4 ($p<0.05$). The interpretation and relevance of this is uncertain, however it at least indicates that 61-2C4 is having some effect in the experimental system, and is again consistent with SK-mediated survival not being mediated positively through β1 integrin signalling.

5.5.4 SK-induced β1 integrin activation is independent of the PI-3 kinase/Akt pathway

The involvement of the PI-3K/Akt pathway in mediating many of the functional changes resulting from over-expression of SK prompted an enquiry into whether this pathway is involved in activating β1 integrin in these cells. Attachment of cells to fibronectin was measured in the presence and absence of an antibody which activates β1 integrin (8A2), and the effect of inhibiting the PI-3K pathway with LY294002 was determined.

In two separate endothelial cell lines assayed in triplicate, it was again confirmed that pre-treatment of cells with 8A2 resulted in an increase in attachment of control cells ($p<0.001$) but did not alter attachment of cells over-expressing SK ($p=0.53$), confirming that β1 integrin is already activated in cells over-expressing SK (Fig 5.11). Pre-incubation of the cells with LY294002 (10μM) for six hours did not alter attachment of control cells ($p=0.90$) or cells over-expressing SK ($p=0.66$), and cells over-expressing SK maintained enhanced cell attachment in the presence of LY294002. Furthermore, inhibition of PI-3K pathway did not influence the response to 8A2 in control cells ($p=0.27$) or cells over-expressing SK ($p=0.89$).

Thus, I concluded that the PI-3 kinase/Akt pathway does not regulate activation of β1 integrin in control cells or in cells over-expressing SK.

5.5.5 Conclusions

With the above evidence I concluded that although over-expression of SK activates β1 integrin, this does not enhance 46/52kDa Shc phosphorylation and thus does not lead to ERK 1 /2 activation. It has previously been demonstrated that the increase in cell accumulation

conferred by SK is not attributable to enhanced cell cycling (no up-regulation of cyclinD1 or cyclin E) or proliferation (blocking MAPK pathway does not abolish the effect), but rather due to a reduction in cell death. This is consistent with the demonstration here that β1 integrin activation by SK is not required to enhance cell accumulation or cell survival.

5.6 The Effect of Over-expression of SK on the Cell Junction

5.6.1 Over-expression of SK alters cell junctional proteins and up-regulates PECAM-1

Both PECAM-1 and VE cadherin have been linked to regulation of endothelial cell permeability and to survival. As over-expression of SK reduced basal permeability (Chapter 4.3.5.1) and enhanced cell survival (Chapter 4.3.2), it was sought to determine whether over-expression of SK could regulate the levels or function of either of these junctional molecules.

Over-expression of SK significantly increased cell surface expression of PECAM-1 compared to EV control as measured by flow cytometry and shown in Fig 5.12(a). The mean fold increase in PECAM-1 expression in cells over-expressing SK compared with control was 1.7 (1.65-1.73) in three separate endothelial cell lines ($p<0.001$). Western blot analysis confirmed that over-expression of SK increased total PECAM-1 expression (Fig 5.12(b)). Quantitative analysis of Western blots for PECAM-1 expression performed on five separate endothelial cell lines revealed that over-expression of SK resulted in 2.1 (95% CI 1.74-2.49)-fold increase in PECAM-1 expression compared with control ($p<0.05$). In contrast to this, there was a small decrease in the cell surface expression of VE-cadherin in cells over-expressing SK (mean fold increase above control 0.82 (0.79-0.84)), $p<0.001$ (data not shown). There was no change in β-catenin, another protein involved in the regulation of cell junctions (Fig 5.12(b)).

Thus, I concluded that over-expression of SK specifically up-regulates expression of PECAM-1 among several junctional proteins tested.

5.6.2 SK reduces the phosphorylation of PECAM-1

In endothelial cells PECAM-1 is phosphorylated on tyrosine residues, and phosphorylation is one method of regulation of PECAM-1 activity. Hence phosphorylation of PECAM-1 in cells over-expressing SK was measured. This was done by probing whole cell lysates with anti-phosphotyrosine antibody, and re-blotting (after stripping the membrane) with anti-PECAM-1 antibody. To ensure that the 130kDa band visualized on the anti-phosphotyrosine blot which overlay the PECAM-1 band was the appropriate band, the results were confirmed measuring phosphorylation in immunoprecipitated PECAM-1.

Over-expression of SK reduced the phosphorylation of PECAM-1, as indicated in Fig 5.12(c). Quantification of PECAM-1 phosphorylation from three separate experiments showed that over-expression of SK resulted in a 0.42 (95% CI 0.28-0.63)-fold change in the proportion of PECAM-1 which was phosphorylated compared with EV control ($p=0.054$). This marginal level of significance needs to be taken in the context of a very small sample size ($n=3$) and a method of quantification which lacks precision. These considerations make the chance of β error (acceptance of the null hypothesis when a difference exists) very substantial.

Thus I concluded that over-expression of SK may result in reduction in phosphorylation of PECAM-1. This would be consistent with the observed reduction in basal permeability (Chapter 4.3.5.1).

5.6.3 Immunolocalisation of PECAM-1

The SK-induced changes in PECAM-1 regulation, in conjunction with the reduction in basal permeability in cells over-expressing SK prompted a determination of whether SK alters the time course of PECAM-1 localisation to cell junctions. The localisation of PECAM-1 at cell junctions was assessed by immunostaining HUVEC over-expressing SK (and EV control HUVEC) at 15 minutes and one hour after seeding the cells.

No consistent differences in localisation of PECAM-1 to the cell junctions was observed between cells over-expressing SK and control (Fig 5.13). At 15 minutes the cells over-expressing SK were noted to have a flatter appearance than control cells, which is consistent with the greater adhesion of these cells to extracellular matrix (Chapter 4.3.4).

5.6.4 SK-induced survival in suspension is mediated by PECAM-1

In view of the importance of cell-cell contacts in the survival of endothelial cells, together with the regulation of PECAM-1 (shown above), the role of cell junctional proteins in mediating SK-induced ability to survive in suspension was investigated. This was done by measuring cell accumulation in suspension in the presence and absence of antibodies directed to PECAM-1 and VE cadherin over two days.

Results are shown in Fig 5.14(a). There was no difference in cell numbers seeded at Day 0 however at Day 2, cells over-expressing SK showed significantly greater numbers of viable cells than control. Rabbit polyclonal anti-PECAM-1 antibody significantly reduced the survival of cells over- expressing SK and control cells ($p<0.001$) and in the presence of this antibody, there was no significant difference in viable cell numbers of cells over-expressing

SK compared with control (p=0.30). The effect was specifically mediated by PECAM-1 signalling, as normal rabbit serum had no effect on either cells over-expressing SK (p=0.9) or control cells (p=0.7). Interestingly, a murine monoclonal antibody directed to PECAM-1 (51-6F6) significantly enhanced the ability of EV control cells to survive in suspension but had no effect on cells over-expressing SK. A murine monoclonal antibody directed to VE-cadherin (55-7H1) had no effect in altering survival of cells over-expressing SK (p=0.61) or control (p=0.69).

Hence I concluded that SK-induced ability to survive in suspension was mediated by PECAM-1 signalling. This may be mediated by homophilic PECAM-1-PECAM-1 interactions between the cells, as indeed in suspension, cells over-expressing SK showed a greater tendency to adhere to neighbouring cells and form spheroidal aggregates, than control cells.

The differential effects of the monoclonal and polyclonal antibodies directed to PECAM-1 on the ability of EV control cells and cells over-expressing SK to survive are intriguing. It was hypothesized that incubation of cells with the monoclonal antibody under serum free conditions enhances PECAM-1 signalling, whereas incubation with the polyclonal antibody reduces PECAM-1 signalling through recognition and therefore blockade of multiple epitopes, or alternatively through internalisation of PECAM-1.

In an attempt to delineate the effects on the cell surface expression of PECAM-1 by pre-treatment with 51-6F6 and rabbit polyclonal anti-PECAM-1, normal HUVEC were subjected to six hours of serum deprivation and treatment with 20μg/ml of 51-6F6, an isotype matched murine monoclonal antibody directed to VE Cadherin (55-7H1), rabbit polyclonal anti-

PECAM-1, or normal rabbit serum. Cell surface expression of PECAM-1 was measured by flow cytometry.

As shown in Fig 5.14(b), in two isolates of HUVEC tested, pre-treatment of cells with rabbit polyclonal anti-PECAM-1 resulted in a reduction in the cell surface expression of PECAM-1. The effect was specifically mediated by anti-PECAM-1 antibody, as normal rabbit serum had no demonstrable effect. In contrast with the polyclonal anti-PECAM-1 antibody, 51-6F6 did not alter the cell surface expression of PECAM-1, and similarly an isotype-matched antibody directed to VE Cadherin (55-7H1) had no effect.

Thus it was concluded that rabbit polyclonal anti-PECAM-1 results in reduction in the cell surface expression of PECAM-1, possibly through internalisation of the protein. This is consistent with the reduction in cell survival of both EV control cells and cells over-expressing SK resulting from incubation with this antibody.

The effects of rabbit polyclonal anti-PECAM-1 antibody and 51-6F6 on the phosphorylation of PECAM-1 was investigated to further the understanding of the effect of these antibodies on PECAM-1 signalling. Normal HUVEC were treated for six hours in serum free conditions with rabbit polyclonal anti-PECAM-1 antibody and with 51-6F6 each at 20μg/ml. The cells were lysed and phosphorylation of PECAM-1 was measured by Western blot analysis by probing with anti-phosphotyrosine antibody. Blotting with 51-6F6 served as a loading control.

Results are shown in Fig 5.14(c). Pre-treatment of HUVEC with rabbit polyclonal antibody enhanced the phosphorylation of PECAM-1, while pre-treatment with 51-6F6 resulted in a reduction in the phosphorylation of PECAM-1. Together, the results suggest that both antibodies cross-link PECAM-1. As monoclonal antibodies recognize a single antigenic

epitope, this is likely to lead to less dimerization, less aggregation of PECAM-1, and thus no effect on the cell surface expression of PECAM-1. The polyclonal antibody through induction of multiple divalent interactions, may lead to substantial aggregation of PECAM-1 and thus result in internalization of the protein. The enhanced phosphorylation of PECAM-1 resulting with pre-treatment with the polyclonal antibody is consistent with breakage of cell-cell junctions resulting from internalization of PECAM-1.

5.6.5 SK-mediated survival in serum free conditions is mediated by PECAM-1

To determine whether PECAM-1 also plays a role in mediating SK-induced survival in serum-free conditions, the effect of antibody blockade of PECAM-1 on the ability of cells over-expressing SK to survive in serum-free conditions was investigated. In this assay the cells are seeded at lower cell number than the suspension assay, and attach to the gelatin coated wells as isolated cells, without evidence of cell-cell contact as visualized by light microscopy. Cells were seeded at Day 0 in serum-free medium supplemented with ECGs. Rabbit polyclonal anti-PECAM-1 antibody, normal rabbit serum, or a monoclonal antibody directed to VE cadherin were added to the wells, each at 20μg/ml. The absorbance at Day 0 and Day 2 was determined, and results were adjusted to Day 0=1. The cells were visualized by light microscopy at Day 0 and at Day 2 to ensure they remained as individual, isolated cells without contact with neighbouring cells.

The enhanced ability of cells over-expressing SK (compared with control cells) to survive in the absence of serum at Day 2 was abolished in the presence of rabbit polyclonal anti-PECAM-1 antibody (Fig 5.15). Incubation with normal rabbit serum or an antibody against VE cadherin had no effect on either control cells or cells over-expressing SK. These results suggest a specific effect of the anti-PECAM-1 antibody in abrogating the increase survival of

SK over-expressing cells relative to EV control cells. The basis for this effect is not clear. Candidate mechanisms include inhibition of signalling due to heterotypic interactions of PECAM-1 or a negative signal arising from engagement of PECAM-1 by the antibody, that normalized survival of SK cells relative to the controls. It is possible that the antibody against PECAM-1 could exert its effects through or after internalisation of PECAM-1.

This indicates that PECAM-1 mediates SK-induced ability to survive in serum-free conditions, in the absence of demonstrable cell-cell contact. This suggests that the consequences of altered PECAM-1 signalling induced by SK and responsible for enhanced cell survival may not be mediated by homophilic PECAM-1-PECAM-1 interactions between cells.

5.6.6 SK signals through PECAM-1 to activate the PI-3K/Akt pathway

Both PECAM-1 and PI-3K/Akt pathways have documented roles in cell survival and prevention of apoptosis. Both of these signalling pathways have been shown here to be involved in mediating the phenotypic consequences of resistance to apoptosis and enhanced cell survival seen with over-expression of SK. The suggestion that the effects of PECAM-1 to promote cell survival may be mediated intracellularly, prompted an enquiry into whether it is activation of the PI-3K/Akt pathway which is responsible for this effect. Thus it was sought to determine whether the engagement of the PI-3K/Akt pathway by SK is dependent upon PECAM-1 signalling.

Phosphorylation of Akt was measured by Western blot under basal conditions and in response to six hours of serum deprivation. The effect of inhibiting PECAM-1 signalling by incubating the cells with rabbit polyclonal anti-PECAM-1 antibody (20μg/ml) was investigated.

Results of representative blots are shown in Fig 5.16(a), and results of quantitative analysis shown in Fig 5.16(b). The SK-mediated increase in phosphorylation of Akt in response to serum deprivation is demonstrated (lane 6 versus lane 5). By contrast, in control cells phosphorylation of Akt is seen in response to serum deprivation was somewhat diminished. Rabbit polyclonal anti-PECAM-1 antibody significantly reduced the stress-induced-increase in phosphorylation of Akt for cells over-expressing SK (lane 7 versus 6), ($p<0.05$), but had no significant effect in control cells (lane 2 versus 3), ($p=0.44$). Although under serum free conditions cells over-expressing SK had greater phosphorylation of Akt than control ($p<0.05$), in the presence of anti-PECAM-1 antibody, there was no significant difference in the percentage of phosphorylated Akt between cells over-expressing SK and control (lane 7 versus 3), ($p=0.69$). The effect was specifically mediated by anti-PECAM-1 antibody, as incubation of the cells under serum free conditions with normal rabbit serum (20μg/ml) did not alter the phosphorylation of Akt in cells over-expressing SK (lane 8 versus 6) or control (lane 4 versus 2).

Thus I concluded that cells over-expressing SK signal through PECAM-1 to engage the PI-3K/Akt pathway.

5.6.7 SK signals through PECAM-1 to enable activation of BCL-2

The Bcl-2 protein is a 26kDa integral membrane protein associated with smooth endoplasmic reticulum, mitochondria, and perinuclear membrane, and is an important factor in the suppression of apoptotic signals(229). One of the critical functions of Bcl-2 is to bind and neutralize various proapoptotic proteins, such as Bax and Bim which normally reside in the

cytoplasm, but in response to cellular stress, undergo translocation to the mitochondria (230;231).

It was sought to determine by Western blot analysis, whether raised intracellular SK activity alters the expression of Bcl-2, Bax, and Bim under basal conditions and in response to the stress of serum deprivation.

Cells over-expressing SK showed less Bim expression, but no difference in Bax expression under basal conditions compared with EV control cells (Fig 5.17a,b). In response to serum deprivation, control cells showed an increase in Bim expression, but cells over-expressing SK did not. In two lines tested, neither control cells nor cells over-expressing SK showed an increase in Bax expression in response to serum deprivation.

Although there was no difference in Bcl-2 expression between control cells or cells over-expressing SK under basal conditions (Fig 5.17c, lane 1 versus 5), in response to serum deprivation, cells over-expressing SK upregulated Bcl-2 expression (lane 6 versus 5) while control cells did not (lane 2 versus 1). In view of the role of PECAM-1 in mediating SK-induced cell survival (5.6.4, 5.6.5) and Akt activation (5.6.6), the role of PECAM-1 signalling in activation of Bcl-2 by SK was determined. Treatment with a rabbit polyclonal antibody against to PECAM-1 inhibited Bcl-2 expression induced in SK cells in response to serum deprivation (lane 7 versus 6), whereas normal rabbit serum had no effect (lane 8 versus 6). This indicates that the induction of the antiapoptotic protein Bcl-2, mediated by SK is, at least in part, PECAM-1 dependent.

5.6.8 A physical association of SK with PECAM-1 was not evident

The above evidence for the junctional molecule PECAM-1 in mediating many of the phenotypic consequences seen with over-expression of SK prompted an enquiry into a possible physical association of PECAM-1 with SK. There is recent evidence to support a physical association of PECAM-1 with SK in transfected HEK293 cells over-expressing PECAM-1 and SK(232). A similar association in HUVEC over-expressing SK was sought by immunoprecipitating SK using anti-flag antibody, and performing a Western blot on the SK immunoprecipitate, and blotting with a murine monoclonal antibody directed to PECAM-1 (51-6F6). Under the conditions employed, no PECAM-1 expression was detected in either the control or SK immunoprecipitate (data not shown).

To further investigate this, PECAM-1 was immunoprecipitated from cells over-expressing SK and EV control, and the immunoprecipitate was run on a gel and blotted with anti-flag antibody. Again, under the conditions employed, no flag band was detected in the immunoprecipitate (data not shown).

It is concluded that under the conditions employed, no physical association between SK and PECAM-1 was demonstrated, however it is noted that this evidence does not exclude such an association.

5.6.9 Conclusions

The above evidence indicates that over-expression of SK specifically up-regulates and dephosphorylates the junctional molecule PECAM-1. This results in enhanced cell survival

which is mediated by a PECAM-1-dependent activation of the PI-3K/Akt pathway, and induction of Bcl-2 expression. Thus I concluded that over-expression of SK alters PECAM-1 signalling to enable activation of the major cell survival pathway, the PI-3K/Akt pathway.

5.7 Extracellular versus intracellular effects of S1P

There has been considerable controversy regarding the predominant site of action of S1P, however it is now accepted that S1P can act both upon the EDG receptors and also intracellularly on as yet undefined targets. Hence it was investigated whether the phenotypic changes resulting from intracellular over-expression of SK are explained by S1P acting back on the EDG receptor, or as an intracellular second messenger. The effects of SK on cell survival and migration were ascertained and it was these phenotypic changes to which an enquiry regarding intracellular versus receptor-mediated actions was directed.

The EDG receptor being a G protein-coupled receptor (GPCR) is pertussis toxin sensitive and hence pertussis toxin was used as a tool to inhibit signalling from GPCRs.

5.7.1 Cell Survival

The effect of pertussis toxin on SK-induced cell survival in the absence of serum was measured (Fig 5.18(a)). SK-induced cell survival at Day 2 was preserved in the presence of pertussis toxin. Hence I concluded that enhanced survival in serum free medium associated with SK over-expression is not mediated by S1P signalling through GPCR.

5.7.2 Cell migration

Cell migration towards fibronectin was measured in the presence and absence of pertussis toxin (50ng/ml). Pertussis toxin significantly inhibited migration of cells over-expressing SK (by 37.3% ±4.07 %) but not control cells (p=0.115) (Fig 5.18(b)). However, in the presence of pertussis toxin, cells over-expressing SK still showed enhanced migration towards fibronectin compared with control (48.0 % ±2.20% versus 30.8% ± 3.69% respectively, $p<0.05$). Thus pertussis toxin partially inhibited SK-induced migration.

I concluded that SK-induced cell migration was mediated at least in part through a GPCR, as pertussis toxin significantly reduced migration conferred by SK but did not alter migration of control cells. However, as pertussis toxin acts on all GPCR and not on the EDG receptors specifically, it was not possible to determine whether this reduction was due to an alternate GPCR which is activated by the over-expression of SK, but is not the EDG receptor.

5.7.3 Enhanced expression of β1 integrin and PECAM-1 is mediated intracellularly by SK

In order to delineate whether the SK-induced up-regulation in β1 integrin and PECAM-1 was mediated by an intracellular S1P action, or whether it is attributable to secreted S1P acting on EDG receptors, the ability of exogenously added S1P to induce these proteins was determined. Normal HUVEC were stimulated for variable duration with exogenously added S1P (5μM), and cell surface expression of β1 integrin and PECAM-1 was assessed by flow cytometry.

Stimulation of HUVEC with exogenous S1P failed to up-regulate either β1 integrin (p=0.17) or PECAM-1 expression (p=0.14) (Fig 5.19) suggesting that EDG receptor activation does not regulate β1 integrin or PECAM-1 expression. Thus it further suggests that regulation of both

β1 integrin and PECAM-1 by intracellular over-expression of SK is likely to be mediated intracellularly.

5.7.4 Activation of MAPK and PI-3K/Akt pathways by exogenous S1P in normal HUVEC

To further delineate whether the observed consequences of intracellular over-expression of SK were mediated by S1P binding the EDG receptor, the ability of variable doses of S1P to activate the PI-3K/Akt and MAPK pathways in normal HUVEC was determined. General consensus is that the binding affinity of the EDG receptors is 4-8 nM. Whereas nanomolar concentrations of S1P can activate PI-3K/Akt pathway by EDG receptor ligation, higher concentrations are required for activation of the MAPK pathway. It was sought to determine the dose-response of S1P in activating each of these pathways in endothelial cells.

Endothelial cells were cultured in EBM supplemented with 0.5% FCS without ECGs overnight, and then stimulated for 30 minutes with S1P at the following concentrations: 5μM, 1μM, 100 nM, and 10 nM. Activation of the PI-3K/Akt and MAPK pathways was measured by Western blot analysis under each of these conditions.

Activation of the PI-3K/Akt pathway occurred even in response to the lowest dose of S1P (10 nM), as shown in Fig 5.20(a,b). Activation of the MAPK pathway as assessed by ERK1/2 phosphorylation was evident in response to stimulation with 5μM, but not in response to the lower doses of S1P (Fig 5.20c,d). Both results were confirmed using two different endothelial cell lines.

The above evidence confirms that in the experimental system employed, very low doses of S1P can activate the PI-3K/Akt pathway, but that higher doses are required to activate the MAPK pathway. In cells over-expressing only low levels of SK, extracellular concentrations of S1P are likely to be minimal, if not negligible, and insufficient to activate the MAPK pathway through the EDG receptor. Whether the concentrations are sufficient even for activation of the PI-3K/Akt pathway remains unknown, as measurement of S1P in the conditioned medium was not undertaken (the assay was unavailable in the laboratory at the time of writing this thesis). The above does however demonstrate that very low doses of exogenous S1P can activate the PI-3K/Akt pathway, and thus EDG-receptor mediated activation of this pathway has not been excluded definitively in cells over-expressing SK intracellularly.

5.7.5 Conclusions

I concluded that SK-mediated cell survival is independent of GPCR and is potentially mediated intracellularly by S1P. Similarly both PECAM-1 and β1 integrin are regulated by intracellular actions of S1P as exogenous S1P failed to regulate these proteins. The engagement of the intracellular signalling pathway, the PI-3K/Akt pathway through PECAM-1 signalling (Section 5.6.6) is also consistent with an intracellular site of action of SK/ S1P, although the precise mechanism for this has not been elucidated. Furthermore, the adhesion molecule response to intracellular over-expression of SK has been shown not to be GPCR-dependent (Section 4.5). It is of interest that SK-induced cell migration is mediated in large part by S1P action on GPCR. This provides confirmation that pertussis toxin is inhibiting signalling fro GPCR at the doses employed and adds strength to the above data.

5.8 Does over-expression of SK induce a stem-cell phenotype?

Stem cells are undifferentiated cells which have the ability of self-renewal and differentiation into specialized mature cells. They are multipotent, and have proliferative capacity(233). Haematopoietic stem cells are the best characterised, and offer great promise for autologous transplantation in the treatment of haematological malignancies. Ability to survive in the absence of serum, or in suspension, are properties consistent with a primitive cell or a circulating endothelial cell, and hence a role for SK in inducing a stem-cell phenotype was sought.

5.8.1 The effect of over-expression of SK on CD34 expression

One of the characteristic cell surface markers of progenitor cells is CD34. CD34 is a glycosylated transmembrane protein which is present on haematopoietic progenitor cells(234)but is down-regulated with differentiation into more mature cells(235;236). Human CD34 is expressed on stem cells during haematopoiesis(237), but is also found on vascular endothelial cells(238;239). The function of the CD34 antigen is largely unknown, but there is some evidence to support a role in the adhesion of cells to the stromal microenvironment(240). Indeed selection of CD34+ stem cells from the bone marrow for autologous stem cell transplantation in haematological malignancies is promising(241).

CD34 expression was measured in cells over-expressing SK and EV controls by flow cytometry. Control cells showed reduction in CD34 expression over time. Cells over-expressing SK showed a tendency to less down-regulation of CD34 expression with time (Fig 5.21(a)).

By Day 4-6, cells over-expressing SK from four separate endothelial cell lines showed consistently higher expression of CD34 than control cells (Fig 5.21(b)). Analysis of the raw data by Student's t-Test showed that this was a statistically significant difference ($p=0.02$).

The increase in CD34 expression in cells over-expressing SK was not mediated by S1P binding EDG receptors, as stimulation of endothelial cells from two separate endothelial cell lines did not affect CD34 expression ($p=1$), as shown in Fig 5.21(c).

5.8.2 VEGF-Receptor Expression

The VEGF-RII (KDR) is another cell surface marker used for the isolation of stem cells(242), and thus cell surface expression of VEGF-RI and VEGF-RII was assessed by flow cytometry in cells over-expressing SK and EV control cells. There was no difference in cell surface expression of VEGF-RI ($p=1$) or VEGF-RII ($p=0.81$) in cells over-expressing SK compared with control (Fig 5.22).

DISCUSSION

The results presented here reveal a novel mechanism of SK-mediated cell survival, which is of interest as it raises new therapeutic possibilities.

The Regulation of PECAM-1 by over-expression of SK

Moderately raised intracellular levels of SK have been shown here to target the cell junction to specifically induce the expression of PECAM-1 (Fig 5.12), an otherwise stable and long-lived protein. In contrast, there was no effect on the levels of expression of β-catenin and no major change in VE cadherin, the other junctional molecule that is specific to endothelial cells and which by mediating the anti-apoptotic effects of VEGF, is also implicated in cell survival

(214). The results suggest the regulation of PECAM-1 by over-expression of SK was mediated by intracellular SK/ S1P, as exogenous S1P was not able to alter PECAM-1 expression (Fig 5.19).

PECAM-1 (CD31), a 130 kDa member of the immunoglobulin superfamily is constitutively and abundantly expressed on normal endothelium (243) and plays a role in cell-cell attachments, suppression of apoptosis (189), and leukocyte trans-endothelial migration (188). PECAM-1 can form homophilic PECAM-1-PECAM-1 interactions between adjacent cells (186;187)to mediate cell adhesion, and can also bind glycosaminoglycans (244) and CD38 (245). Although integrin $\alpha v\beta 3$ is a heterophilic ligand for PECAM-1 (246), this has been shown not to be an operational mechanism in endothelial cells (217). It is the amino-terminal 2 of the extracellular domain of PECAM-1 (composed of 6 extracellular Ig-like homology units (243)) which possesses homophilic binding properties (187;247)and has a role in leukocyte transmigration (188) and in angiogenesis.

The other interesting finding in cells over-expressing SK was the reduction in tyrosine phosphorylation of PECAM-1 (Fig 5.12c). The cytoplasmic domain of PECAM-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) (248) which when tyrosine phosphorylated results in the recruitment of the src homology 2 (SH2) domain-containing protein tyrosine phosphatase, SHP-2 in bovine aortic vascular endothelial cells (249). On this basis, PECAM-1 is classified among a family of transmembrane receptors known as the Ig-ITIM family of inhibitory receptors (250).

The interaction of PECAM-1 with the SK/S1P system is in keeping with the recent findings relating cell motility with PECAM-1. PECAM-1 is known to undergo dephosphorylation during endothelial cell migration both in vitro, and during the process of vasculogenesis in the

murine embryo (251). Cell lines lacking PECAM-1, but not their PECAM-1 reconstituted counterparts, show reduced motility and deficient S1P responses in wound healing assays(203). Enhanced cell motility has been shown to be associated with dephosphorylation of PECAM-1(191;203), and the finding that over-expression of SK enhanced endothelial cell motility (Chapter 4.3.6) and inhibited tyrosine phosphorylation of PECAM-1 (Fig 5.12c) is thus in keeping with its functional activation.

The regulation of PECAM-1 by SK is intriguing and the underlying mechanism remains to be elucidated. Although a physical association of SK and PECAM-1 was not demonstrable in the experimental system utilized, recent evidence indicates that in transfected HEK293 cells over-expressing PECAM-1 and SK, such a physical co-association is present (232). Moreover, the association was dependent upon the phosphorylation status of PECAM-1, with greater phosphorylation resulting in a weaker association with SK. It would be of interest to determine whether endothelial cells over-expressing both SK and PECAM-1 show a co-association of these two proteins, and further to determine whether alterations in tyrosine phosphorylation of PECAM-1 are involved in regulating SK activity.

PECAM-1: A critical player in SK-mediated cell survival

The changes in PECAM-1 regulation were intriguing, as PECAM-1 has been linked to cell survival, and thus the hypothesis that PECAM-1 mediates SK-induced cell survival was tested. Polyclonal anti-PECAM-1 antibody was very effective in totally reversing the phenotype of enhanced survival either under serum free or suspension conditions suggesting that PECAM-1 engagement was an important and perhaps even dominant factor. PECAM-1 was shown to mediate survival in suspension when the cells were in contact with each other (Fig 5.14a), suggesting that the protection from apoptosis may be conferred by homophilic PECAM-1 interactions between adjacent cells, as is recognized to occur in endothelial cells

(186;187;190). However PECAM-1 was also shown to mediate SK-induced survival in serum-free conditions, when cells were relatively sparsely plated (Fig 5.15).

Antibodies have been very useful in studying PECAM-1 function, and are able to inhibit or mimic PECAM-1 interactions (189;252). It is acknowledged that the use of rabbit polyclonal antibodies can give aberrant results due to the complex mixture of IgGs present. Such issues may be overcome by the use of affinity purified, monovalent Fab fragments, or by the use of murine monoclonal antibodies directed against PECAM-1. It has been shown here that a murine monoclonal antibody directed against PECAM-1 (51-6F6) enhanced survival of EV control cells in suspension, without altering the survival of cells over-expressing SK (Fig 5.14a). This was in contrast to the inhibition of cell survival of both EV control cells and cells over-expressing SK seen with rabbit polyclonal anti-PECAM-1 (Fig 5.14a). One possible explanation for this discrepancy resides in the demonstration that the polyclonal anti-PECAM-1 antibody reduced the cell surface expression of PECAM-1 (Fig 5.14b), which was not regulated by treatment of cells with 51-6F6. Differential effects on the phosphorylation status of PECAM-1 (Fig 5.14c) are likely to play an important role, as the induction of PECAM-1 phosphorylation by the polyclonal antibody may lead to substantial PECAM-1-PECAM-1 interactions with resultant aggregation and clearance of PECAM-1 from the cell surface. In contrast, it is not surprising that the more limited extent of PECAM-1-PECAM-1 interactions resulting from treatment with the monoclonal antibody, 51-6F6, do not result in internalization of the protein. Thus it is likely that polyclonal anti-PECAM-1 results in inhibition of PECAM-1 signalling through internalisation of the protein. Furthermore, as it can often be difficult to determine whether antibodies exert their effects by activating or inhibiting cellular responses, the use of complementary techniques such as wild-type versus PECAM-1-deficient cell lines (HEK293, endothelial cells, or Chinese Hamster Ovary cells) would add strength to this data.

The findings documented here are consistent with the known role of PECAM-1 in promoting cell survival and suppressing apoptosis. In endothelial cells, homophilic PECAM-1 interactions between cells have been shown to abrogate the extent of serum deprivation-induced apoptosis (217). Endothelial cells from PECAM-1-deficient mice are more susceptible than their PECAM-1-replete counterparts to the apoptotic stimuli of irradiation as well as to the cytotoxic effects of staurosporine (253). Furthermore, in Jurkat T lymphocytes lacking PECAM-1, restoration of PECAM-1 expression was protective against UV-induced apoptosis (253). Thus PECAM-1 is recognised to provide resistance to a broad range of apoptotic stimuli in various naturally occurring cells in which it is expressed. Treatment of endothelial cells with anti-PECAM-1 monoclonal antibody (LCI-4) which cross links PECAM-1 through domain 5, has led to reduction in apoptosis induced by serum deprivation (189). Associated with this protection against apoptosis conferred by PECAM-1 were increased message levels of the anti-apoptotic proteins A1 and A20 (189). However another study showed that the protection against Bax-mediated apoptosis provided by PECAM-1 is not mediated by induction of A1 or A20 in HEK 293 T cells or Jurkat T cells but was rather mediated through the mitochondrial-dependent pathway of apoptosis (253). In neutrophils, PECAM-1 has been shown to be associated functionally with the major cell survival pathway, the PI-3Kinase pathway, as PI-3kinase activity was detected in immuno-precipitated PECAM-1 (254). The same laboratory has demonstrated that antibody-mediated cross-linking of PECAM-1 can lead to activation of the PI-3K/Akt pathway in a subset of CD14+, CD34+ haematopoietic precursors (255). In endothelial cells thus far, nothing has been known of any interaction between PECAM-1 and the PI-3kinase/Akt pathway. The demonstration here that SK signals through PECAM-1 to activate the PI-3K/Akt pathway is novel, and the possibility exists that SK may induce a co-association of PECAM-1 and PI-3K/Akt. This specific

question was not addressed here, but would best be investigated by measuring PI-3kinase activity in immunoprecipitated PECAM-1.

In the experimental system utilized here, intracellular consequences of PECAM-1 signalling have been identified and include activation of the PI-3K/Akt pathway (Fig 5.16) and upregulation of the antiapoptotic protein Bcl-2 (Fig 5.17). Although PECAM-1 is known to suppress Bax-mediated apoptosis (253), in the present investigation, serum deprivation resulted in induction of Bim but not Bax expression in control cells (Fig 5.17a,b). It was further demonstrated that raised intracellular activity of SK enables induction of Bcl-2 expression in a PECAM-1- dependent manner and indeed, PI-3K/Akt activity is recognised to modulate Bcl-2 expression (256).

The PI-3K/Akt pathway and SK-mediated cell survival

The PI-3 kinase/Akt cascade is the central component of survival signalling. PI-3kinase catalyzes the phosphorylation of phosphatidylinositol lipids at the inositol ring, thus producing phosphatidylinsoitol 3,4-biphosphate and phosphatidylinositol 3,4,5-triphosphate (201). Akt is a serine/threonine kinase, which in unstimulated cells is in the cytoplasm. In response to trophic stimulation and receptor activation, Akt binds lipid products of PI-3kinase via its pleckstrin-homology domain and is recruited to the plasma membrane. Phosphorylation of Akt at T308 by 3-phosphoinositide-dependent protein kinase (PDK1) and S473 (by PDK2) results in full activation of the kinase (257;258).

Downstream effects of Akt activation include factors involved in cell survival, cell cycle regulation (259), glucose metabolism (260), and protein synthesis (258). Best defined are the factors which influence cell survival. Akt is known to modulate BAD (261), the forkhead family of transcription factors (262), as well as IKKα, a kinase which regulates the

transcription factor NFκB (263). The factors downstream of Akt which mediate survival in endothelial cells are poorly defined at present, but possible roles for survivin (264), MEKK3 (265), and FLICE-inhibitory protein (FLIP) (266)have been suggested. Among numerous factors which may activate the PI-3K/Akt pathway in endothelial cells (267), is S1P(218;268) as stimulation of cells with exogenous S1P activates the PI-3K/Akt pathway as well as the MAPK pathway. The complexity of the system is reflected in the recent observation that Akt may also act upstream of S1P, by phosphorylating EDG-1, to induce activation of Rac, and subsequent stimulation of endothelial cell migration (224). The demonstration here that SK-induced cell migration is mediated through the PI-3K/Akt pathway (Fig 5.6) is consistent with this.

A role for the PI-3kinase/Akt pathway in mediating SK-induced resistance to serum deprivation-induced caspase-3 activation (Fig 5.4), and in serum free cell survival (Fig 5.5) is shown here. Furthermore, evidence for activation of this pathway by SK has been confirmed, with an increase in phosphorylation of Akt in response to the stress of serum deprivation in cells over-expressing SK (Fig 5.7). This is in contrast with control cells, which responded to these conditions with a reduction in phosphorylation of Akt. Indeed S1P is known to activate Akt through a PI-3kinase pathway in endothelial cells (201) and the results presented here are in keeping with this. Furthermore in human hepatocytes, S1P (induced by TNFα stimulation of SK) is also known to inhibit apoptosis through the PI-3kinase/Akt pathway (269).

SK and Shc

I postulate the preferential activation of the PI-3K/Akt pathway over the MAPK pathway by intracellular over-expression of SK (although the capacity to activate the MAPK pathway in response to exogenous stimulation with S1P is preserved, Fig 5.3) is partly attributable to the increase in phosphorylation of p66 Shc (Fig 5.9). Shc is an adaptor protein which couples

α1β1, αvβ3, α5β1, and α6β4 integrins to the Ras MAPK pathway, thereby promoting cell proliferation. There are three isoforms (66, 52, 46 kDa), which are functionally non-redundant. Growth factor-stimulation leads to the binding of 46/52 Shc to phosphorylated receptors, and the tyrosine phosphorylation of these two smaller isoforms of Shc. The phosphotyrosine residues then interact with Grb-2 SH2 domain, with subsequent Ras activation. In this manner, the 46/52 Shc isoforms link activated receptor kinases to Ras, thus mediating mitogenic signals(225-227). On the other hand, p66Shc, although it undergoes tyrosine phosphorylation and binds Grb-2 after growth factor stimulation, it fails to activate Ras and is thus a negative regulator of the MAPK pathway (228;270). Furthermore, although it is well established that Shc signalling activates the MAPK pathway, there is recent evidence that Shc may also signal through the PI-3K/Akt pathway (271). At present it remains unknown whether this is mediated by any specific isoform of Shc.

How is the PI-3K/Akt pathway activated by raised intracellular levels of SK?

It is plausible that there are several mechanisms whereby raised intracellular levels of SK result in activation of the PI-3K/Akt pathway. Firstly, it may be postulated that enhanced phosphorylation of p66Shc while a negative regulator of the MAPK pathway, may perhaps by default, lead to activation of the PI-3K/Akt pathway. This issue would best be addressed by demonstrating a failure to activate the PI-3K/Akt pathway with over-expression of dominant-negative p66Shc. The dominant-negative Shc which is available is not directed to this isoform. Time constraints made it impossible for a p66 dominant-negative Shc to be generated in order to test this hypothesis. A second possibility that extracellular export of SK to generate S1P activates Akt through EDG receptors is not discounted, and indeed, this work has shown that in the experimental system utilized, very low extracellular levels of S1P which fail to activate the MAPK pathway may still activate the PI-3K/Akt pathway (Fig 5.20). A third possibility is that although SK is a constitutively active enzyme, it is able to become

further activated by a mechanism that involves phosphorylation on serine 225, altering its membrane localisation and increasing its Vmax by 14 fold (120). The activation of SK is clearly linked to its phosphorylation by ERK1/2 and in turn an increased ERK1/2 phosphorylation in the cytosol (120). Thus the possibility arises that over-expression of unactivated SK may be preferentially linked to Akt activation, with ERK1/2 (as well as Akt) being engaged upon more acute stimulation, such as is provided by TNFα or PMA (97). A fourth mechanism is the newly identified pathway described here, of signalling through PECAM-1, induced by SK, to activate the PI-3K/Akt pathway. This is believed to be the major mechanism of activation of the PI-3K/Akt pathway in cells over-expressing SK, as activation of the PI-3K/Akt pathway was completely abolished when PECAM-1 was subjected to antibody blockade (Fig 5.16).

Slow and steady wins the race

The enhanced cell accumulation (above control) of cells over-expressing SK after three days in culture was attributable entirely to increased cell survival, as there was no up-regulation of cyclin D1 or cyclin E (Fig 5.2), no evidence of activation of the MAPK pathway (Fig 5.8), and furthermore the increase in relative cell number of SK cells at Day 3 was abolished by inhibiting the PI-3K pathway and unaffected by inhibition of the MAPK pathway (Fig 5.3). The enhanced cell survival resulting from over-expression of SK is in accordance with the known biological effects of SK/S1P, as indeed, S1P is recognized to induce cell survival in endothelial cells (272) and in various other cell types. The mechanism of enhanced survival mediated by SK/S1P appears cell-type dependent and is mediated by the PI-3K/Akt or MAPK pathways. For example, in human melanocytes MAPK activation by S1P (273) plays a dominant role in protecting from UV irradiation, whereas in osteoblasts, activation of PI-3K protects from serum withdrawal (274). The reasons for differential usage of the PI-3K/Akt pathway and the MAPK pathway are not clear. In endothelial cells over-expressing SK the

selective use of the PI-3K/Akt pathway over the MAPK pathway under conditions of deprivation is intriguing, and indicates a strategy for survival more suited to limiting conditions than cell proliferation. Indeed this appears to be an example of "slow and steady wins the race", as by enhancing survival, this achieved ultimately, an increase in cell number, akin at first glance to proliferation.

SK and circulating endothelial cells

In recent years there has been considerable interest in the therapeutic use of stem cells in organ transplantation, augmentation of vessel growth in ischemic tissue, and cell-mediated gene therapy. Hence research has been directed to strategies for identifying and culturing these cells. Cell surface markers utilized for isolation of haematopoietic stem cells include the sialomucin CD34, and VEGF-RII (233). CD133 is also recognised to be a marker of stem cells, however it is not known whether this marker provides any additional benefit over CD34 (233). The enhanced ability of cells over-expressing SK to survive in the absence of serum and in the absence of attachment to extracellular matrix suggested these cells have a primordial morphology and resemble stem cells or perhaps a circulating progenitor-like endothelial cell. The demonstration that these cells have an increase in CD34 expression (Fig 5.21) is novel, and although an increase in the other stem cell marker VEGF-RII was not found (Fig 5.22), this work suggests that raised intracellular levels of SK confer upon the cells a more primitive phenotype. The question is raised whether a similar increase in SK activity is present in circulating endothelial cells. Agonist-induced activation of SK is however transient, and therefore unlikely to result in such alterations in the physiological state. The possibility exists that targeting SK to enhance its activity in a sustained fashion will provide a novel source of stem cells for therapeutic use.

Summary

Although the phenotypic consequences of raised intracellular levels of SK are consistent with those resulting from exogenous stimulation of endothelial cells with S1P, evidence provided here indicates that the underlying mechanisms are clearly distinct. Whereas stimulation of endothelial cells with S1P activates ERK 1 /2 by virtue of EDG receptor activation to induce a mitogenic response, raised intracellular levels of SK/ S1P result in a PECAM-1-dependent activation of the PI-3K/ Akt pathway, to enhance cell survival. Activation of this pathway plays a critical role in mediating many of the phenotypic alterations described, including resistance to apoptosis and augmented cell accumulation. The demonstration that intracellular SK/ S1P regulates PECAM-1 is novel, and indeed exogenous S1P was not able to regulate PECAM-1 expression (Fig 5.19). PECAM-1 signalling was responsible for SK-induced cell survival through activation of the PI-3K/Akt pathway since phosphorylation of Akt was dependent on PECAM-1 signalling. This exposes a hitherto un-recognized pathway of SK-induced endothelial cell survival. Whereas regulation of PECAM-1 and engagement of the PI-3K/Akt pathway by SK resulted in major phenotypic alterations, the induction of β1 integrin was shown to be largely inconsequential in terms of effects on downstream cellular targets, with failure to enhance 46/52kDa Shc signalling, failure to active the MAPK pathway, and the lack of induction of G-phase cyclins. The persistence of the stem cell marker CD34 on cells over-expressing SK is consistent with a more primordial morphology, and raises the question whether a similar increase in SK activity is present in stem cells. This work also substantiates a role for S1P as an intracellular second messenger, and further defines three downstream targets of S1P, namely PECAM-1, β1 integrin, as well as CD34. The results presented here confirm a role for SK in regulation of blood vessel growth, and identify some potentially useful therapeutic targets in the SK signalling pathway.

The proposed model of signal transduction pathways engaged by raised intracellular SK activity

Endothelial cell survival requires integrin activation (provided by attachment with extracellular matrix), cell-cell contacts (mediated by cell junctional proteins PECAM-1, VE Cadherin, and catenins), and growth factors. As would be expected of a critical and dynamic process such as cell survival, there is interplay between these factors and it is of interest that β1 integrins are known to influence not only growth factor signalling (210;275), but also the signalling function of PECAM-1. PECAM-1 ligation by antibody enhances β1 integrin-induced adhesion in both CD34+ haematopoietic progenitor cells (276) and CD8+ T lymphocytes (277). Furthermore, β1 integrin signalling can result in dephosphorylation of PECAM-1, and thus regulation of PECAM-1 activity which has been implicated as a signalling pathway during cell migration (191). It is conceivable that in endothelial cells over-expressing SK, upregulation of β1 integrin expression is the primary event with subsequent induction and dephosphorylation of PECAM-1. This hypothesis has not been specifically tested here. The possibility also exists that in cells over-expressing SK, p66Shc is linked to PECAM-1, and that PECAM-1 is functioning as a growth factor, which in concert with p66Shc and β1 integrin, leads to activation of the PI-3K/Akt pathway. The signal transduction pathway postulated to be engaged by raised intracellular levels of SK in HUVEC along with hypothesized interactions is summarized in Fig 5.23.

Concluding remarks

The functional and gene expression heterogeneity of endothelial cells (fro various organs or disease states) is just being recognised. The work reported here shows that alteration of the intracellular levels of SK in endothelial cells results in distinct phenotypic changes, with resetting of signalling pathways and very likely associated alterations in gene expression. In understanding mechanisms which disrupt the fine balance between factors that promote and

those that prevent angiogenesis, much attention has focused on factors exterior to the cell, namely cytokine growth factors, inflammatory cytokines, as well as cell surface expression of integrins. In view of the known angiogenic effects of S1P, it was sought to determine whether intracellular signalling components in the sphingomyelin pathway play a role in resetting the angiogenic rheostat. The capacity of endothelial cells over-expressing SK to survive in suspension suggests that they may have increased capacity to survive in the circulation or in situations such as during angiogenesis where cell-matrix attachments are limited. Raised intracellular levels of SK were shown to enhance angiogenesis (Chapter 3,4) and the mechanisms whereby this is mediated have been partially elucidated here. Whereas endothelial cell stimulation with exogenous S1P activates ERK 1/2 resulting in stimulation of proliferation, raised intracellular levels of SK activate the PI-3K/Akt pathway through induction of PECAM-1, resulting in enhancement of cell survival. These results demonstrate a hitherto unexplored pathway of SK-induced endothelial cell survival, which is distinct from that seen with exogenous stimulation with S1P. Furthermore raised intracellular levels of SK confer upon the cell a phenotype resembling in some respects a circulating endothelial progenitor cell. Taken together, this work substantiates the role of S1P as an intracellular second messenger, identifies PECAM-1 and CD34 as important cellular targets, and raises the possibility of therapeutic manipulation not only of intracellular levels of SK but also of its downstream targets in diseases of aberrant angiogenesis.

	MFI	
β1	1.3 (1.21-1.42)	n=5, p<0.001
αvβ3	1.3 (1.07-1.44)	n=5, p<0.05
α5β1	1.2 (1.09-1.23)	n=4, p<0.05

Table 5.1 shows the mean fold increase (MFI) in integrin expression for endothelial cells over-expressing SK compared with control, as determined by the median fluorescence intensity. Fluorescence resulting from staining with a non-relevant isotype-matched negative control antibody (23-1F11) has been subtracted. The associated 95% confidence intervals, number of separate experiments (n) and corresponding p values are indicated.

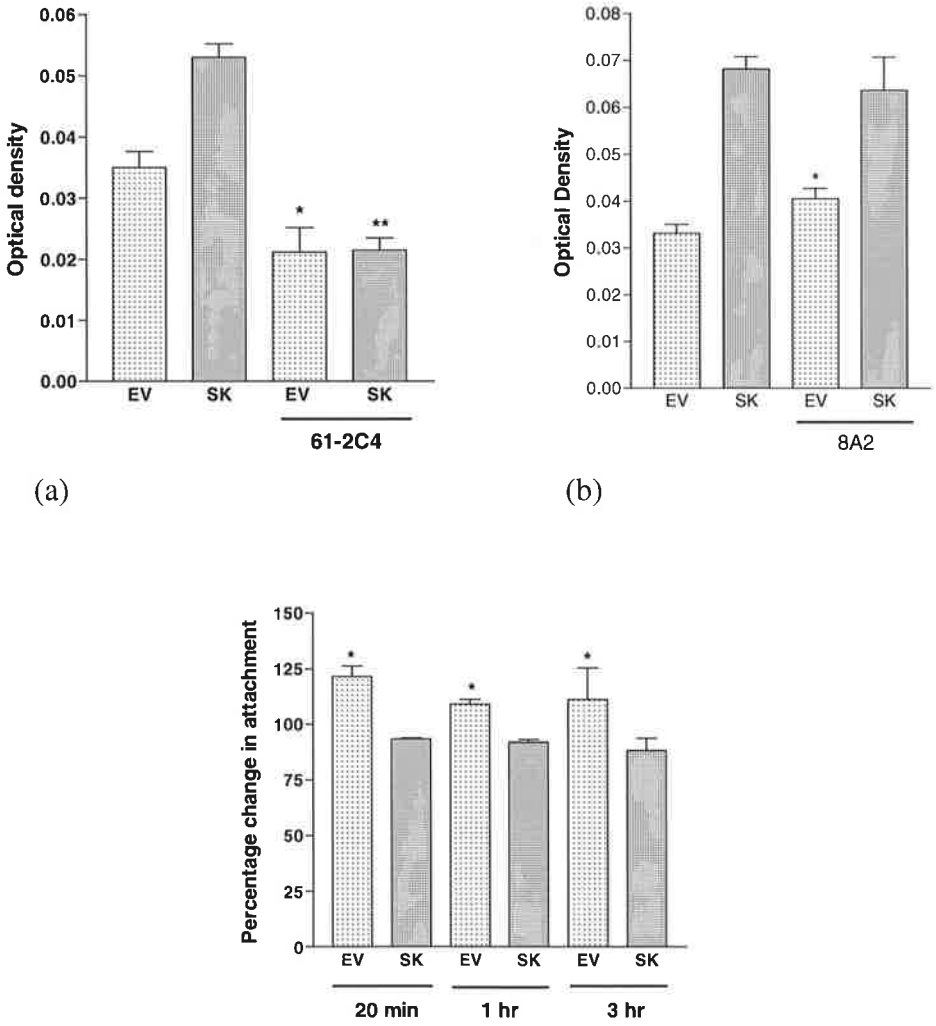


Fig 5.1 shows the role of β1 integrin in attachment of cells over-expressing SK and control (EV) to fibronectin (50μg/ml). (a) shows the effect of ten minutes of pre-treatment of cells with a murine monoclonal antibody directed against β1 integrin (61-2C4, 20 μg/ml). (b) shows the effect of pre-treatment of cells with 10 μg/ml 8A2, a β1 integrin activating antibody on cell adhesion at 20 minutes (c) shows the percentage change in attachment (above time =0) induced by pre-treatment of cells with 10 μg/ml 8A2, across 3 different time points. * $p<0.05$ of EV compared with time=0. (b) * $p<0.05$ compared with untreated EV, ** $p<0.001$ compared with untreated SK. The figure shows the pooled data of six observations derived from two separate experiments. Bars represent SEM.

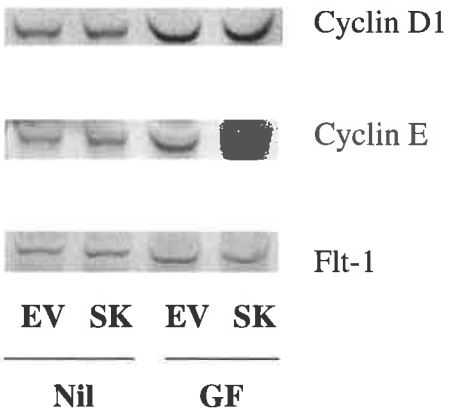
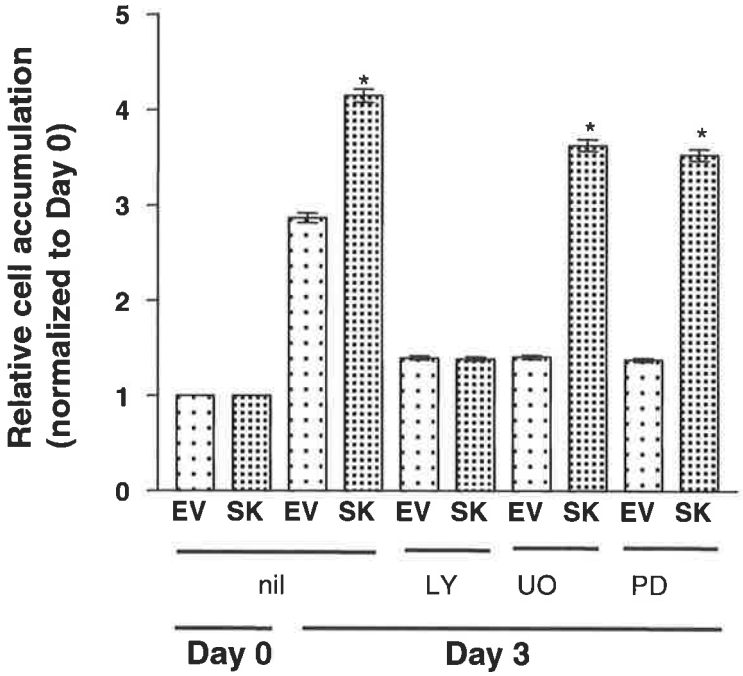
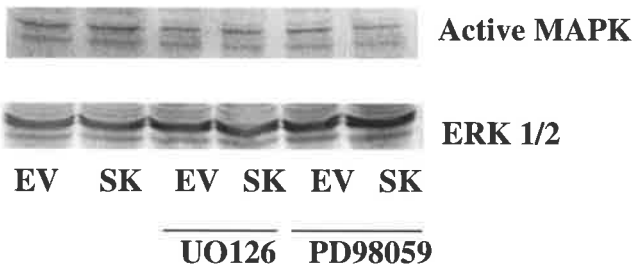


Fig 5.2 shows (by Western blot) cyclin D1 and cyclin E expression in cells over-expressing SK and control (EV) under basal conditions (24 hours in EBM supplemented with 0.5% FCS without ECGs) and in response to 24 hours of stimulation with ECGs). The membrane was probed with murine antibodies to cyclin D1 and cyclin E, and the same membrane was blotted with a murine antibody to Flt-1 (VEGF-RI) to confirm equivalent protein load between the lanes.



(a)



(b)

Fig 5.3 shows the effect of inhibiting the PI-3 kinase pathway (10μM LY294002) and the MAPK pathway with 20μM UO126 or 20μM PD98059, on cell accumulation of cells over-expressing SK and EV (control). (a) shows relative cell accumulation over 3 days in HUVE medium supplemented with 2% FCS. The figure shows the composite analysis of ten observations derived from two separate experiments using Statistica Version 6.1 (Statsoft, Inc.), and results are normalized to Day 0=1. *$p<0.001$ SK compared with corresponding EV. Bars represent 95% confidence intervals. (b) shows a Western blot for active (phosphorylated) MAPK and total ERK1/2 in cell lysates of cells cultured in HUVE medium supplemented with 2% FCS in the presence and absence of UO126 and PD98059 for 24 hours.

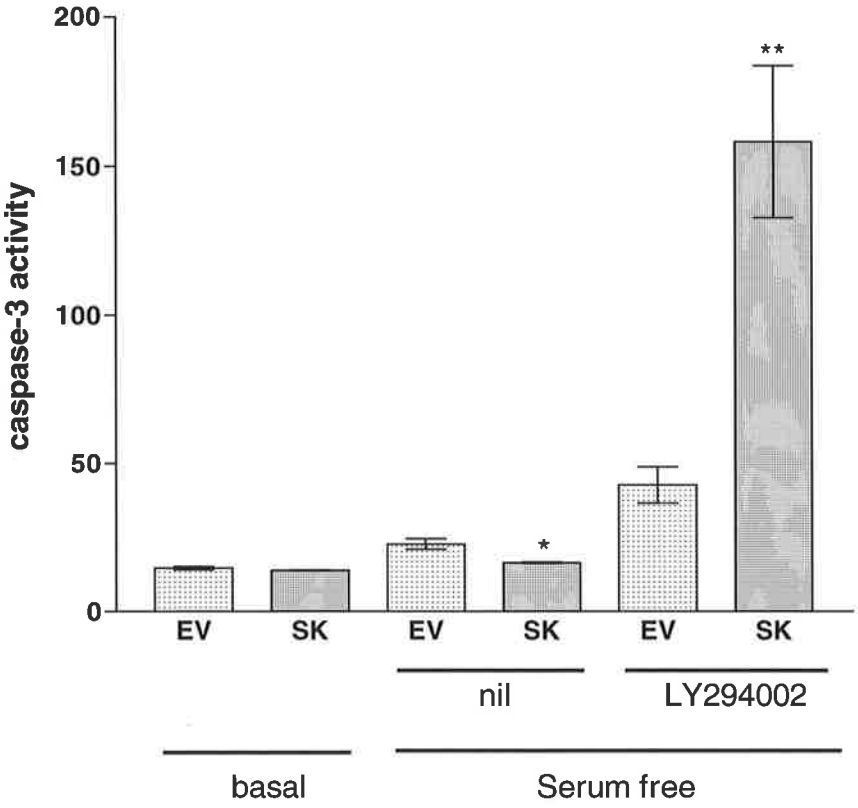
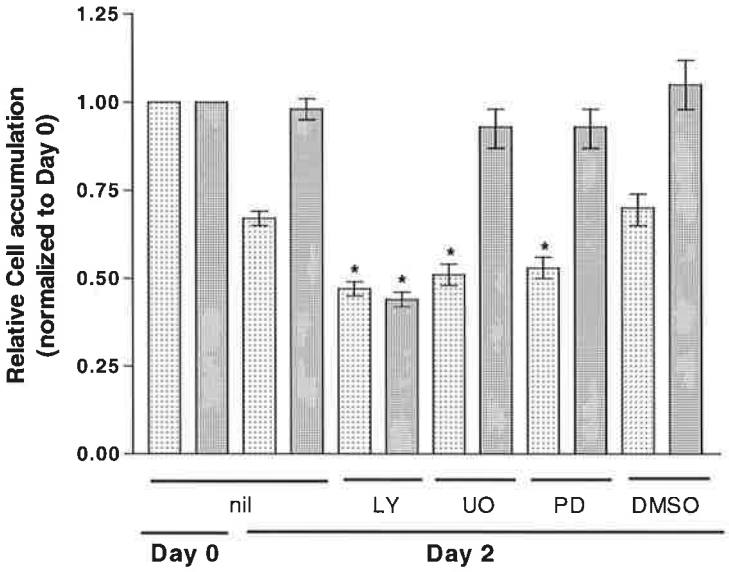
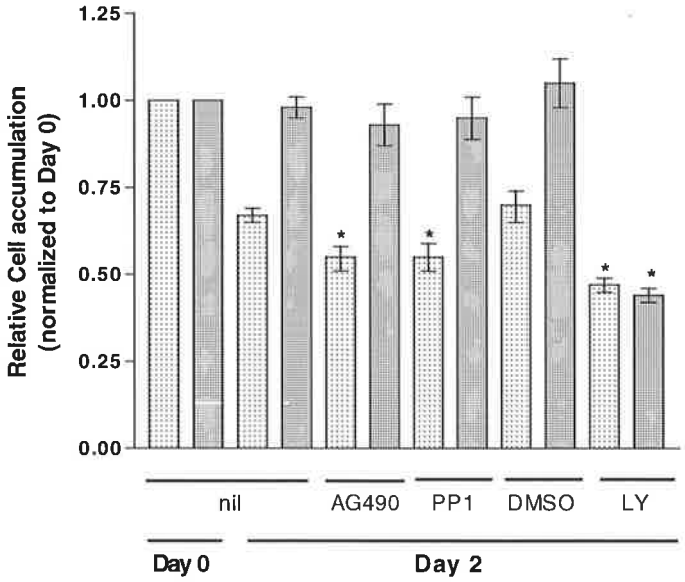


Fig 5.4 shows caspase-3 activity under basal conditions, serum free (SF) conditions and in the presence of LY294002 (10μM) for 24 hours for cells over-expressing SK and EV control. * $p<0.05$ of SK compared with EV in untreated SF conditions, **$p<0.001$ of SK compared with EV in the presence of LY294002. The figure shows the pooled results from two separate experiments done in duplicate. Bars represent SEM.



(a)



(b)

Fig 5.5 shows the effect of inhibiting various growth-factor activated cellular pathways on cell survival of HUVEC over-expressing SK (dark columns) or EV (light columns). (a) shows the effect of inhibiting the PI-3K pathway with 10μM LY294002 (LY), or the MAPK pathway with 20μM UO126 (UO) or 20μM PD98059 (PD). The effect of 20μM PP1 (Src-family tyrosine kinase inhibitor) and 20μM AG-490 (JAK-2 tyrosine kinase inhibitor) is shown in (b). A vehicle control of equivalent concentration of DMSO is indicated in both (a) and (b). (a) shows the composite analysis of 8 observations from two separate experiments using Statistica Version 6.1 (Statsoft, Inc.), (b) shows the composite analysis of 8 observations from a separate two experiments (using two different endothelial cell isolates compared with (a)). All results are adjusted to Day 0=1. Bars represent 95% confidence intervals. *$p<0.001$ compared with corresponding untreated control cells containing the same vector at Day 2.

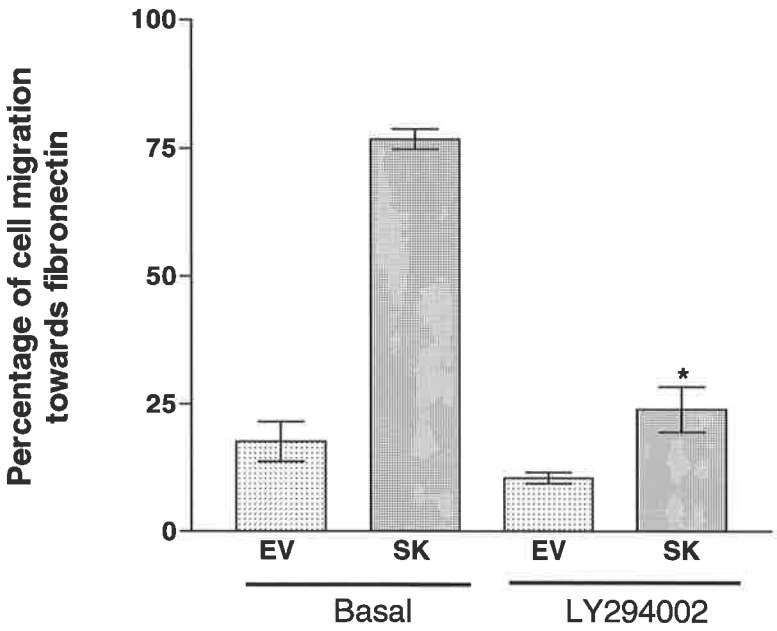
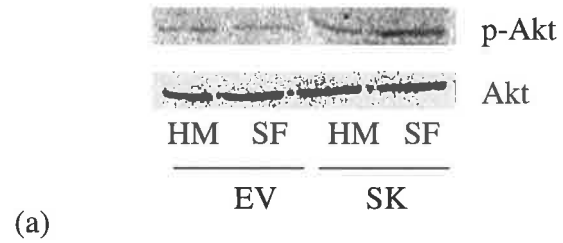
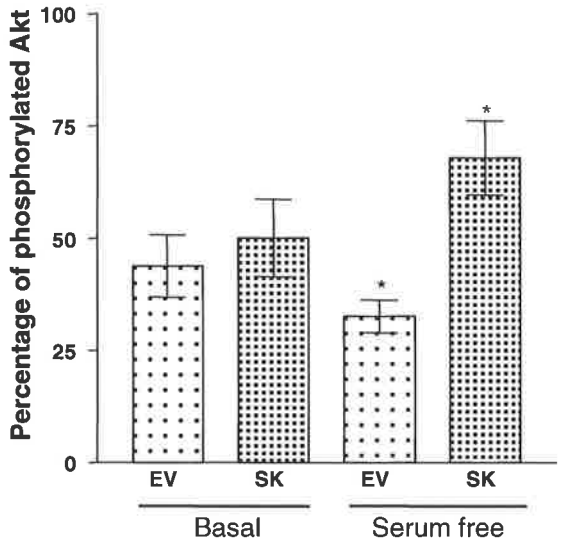


Fig 5.6 shows cell migration towards fibronectin (50μg/ml) under basal conditions, and in the presence of LY294002 (10μM) for cells over-expressing SK and control (EV). *p<0.001 SK treated with LY294002 compared with untreated SK using Student's t-Test. The figure shows the pooled data of two separate experiments each done in duplicate, and values reflect mean ±SEM.



(a)



(b)

Fig 5.7 (a) shows by Western blot the phosphorylation of Akt (p-Akt) in cells over-expressing SK and EV control, under basal conditions (culture in HUVE medium, HM) and in response to six hours of incubation in serum free (SF) medium. Fig 5.7(b) shows the pooled data from five separate endothelial cell lines, *$p<0.05$ SK compared with EV in serum free conditions, and EV in serum free conditions compared with EV in basal conditions by Student's t-Test. Bars represent SEM.

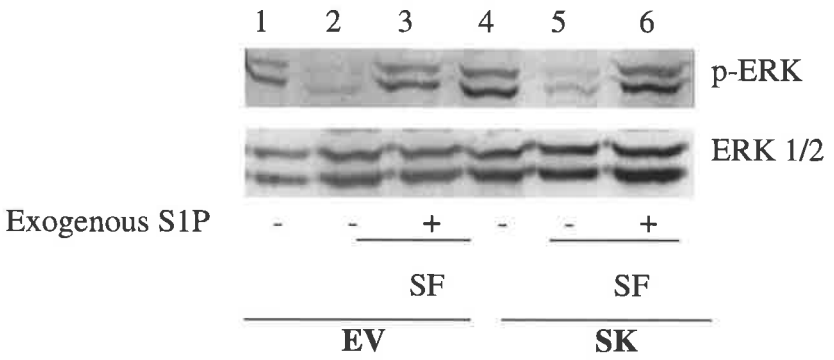


Fig 5.8 shows a Western blot for phosphorylated ERK 1/2 (p-ERK) and total ERK1/2 in cells over-expressing SK and control (EV) under basal conditions (culture in HUVE medium), incubation in serum free (SF) medium for 6 hours, and in response to exogenous S1P (5μM) stimulation (+) for 30 minutes.

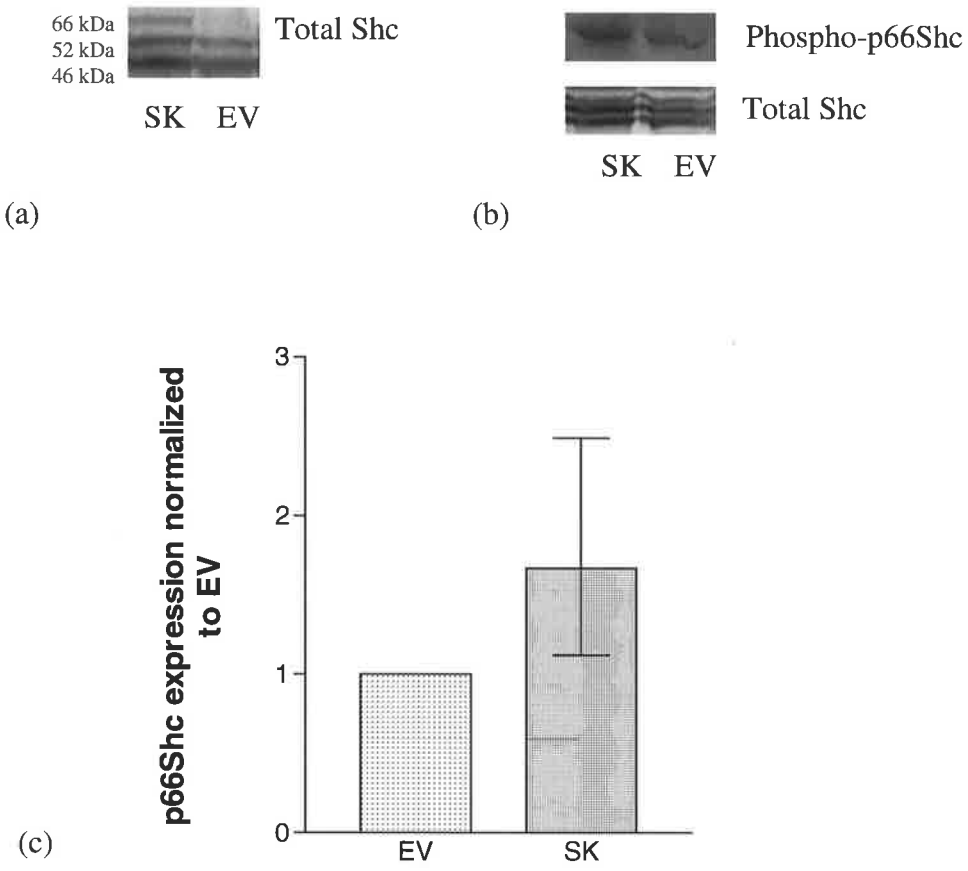


Fig 5.9 shows Shc expression and activation in cells over-expressing SK and control (EV). Total Shc expression as determined by Western blot using polyclonal anti-Shc antibody is shown in (a), and phosphorylation of 66kDa Shc as determined by Western blot using anti-phosphotyrosine antibody, and re-blotting with anti-Shc antibody is shown in (b). (c) shows the composite analysis derived from quantitative analysis of p66Shc phosphorylation from three separate experiments normalised to the EV, using Statistica Version 6.1 (Statsoft, Inc.). p=0.13 SK compared with EV. Bars represent 95% confidence intervals.

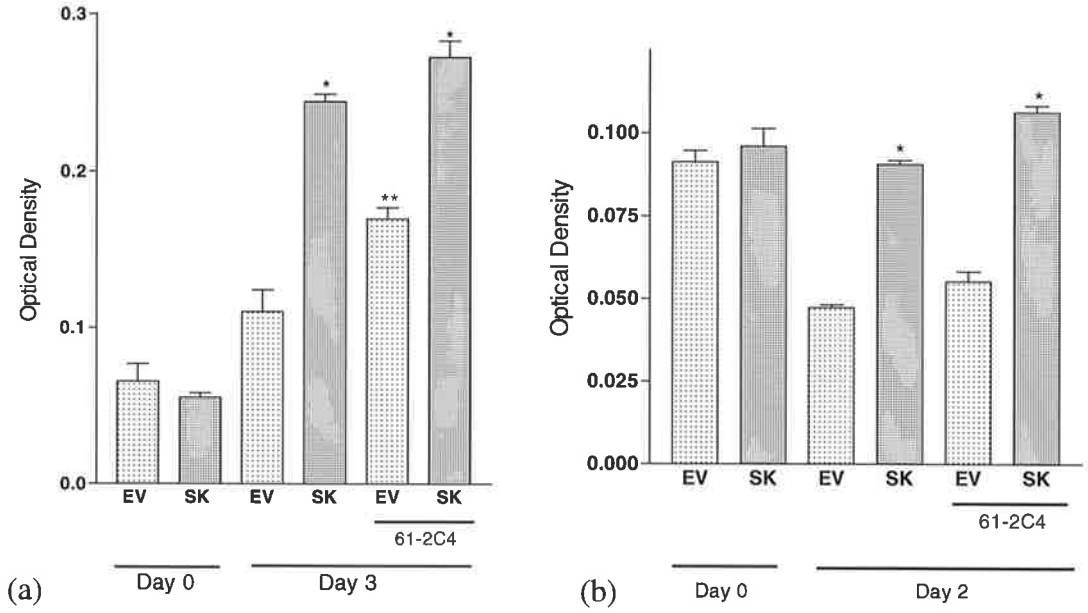


Fig 5.10 shows the effect of β1 integrin on the ability of HUVEC over-expressing SK or EV control to increase in relative cell number or survive in serum free conditions, as reflected by the optical density. (a) shows cell accumulation in 2% FCS over three days and the effect of daily addition of an antibody that blocks β1 integrin, 61-2C4 (100μg/ml). *$p<0.05$ SK compared with EV at Day 3, in the presence or absence of 61-2C4. **$p=0.01$ EV in the presence of 61-2C4 compared with untreated EV at Day 3. (b) shows cell survival in serum-free medium supplemented with ECGs and the effect of 61-2C4. *$p<0.05$ of SK compared with EV at Day 2, SK compared with EV in the presence of 61-2C4, and SK in the presence of 61-2C4 compared with untreated SK at Day 2. The figure shows the data from a single experiment undertaken in triplicate, which is representative of three separate experiments. Bars represent SEM.

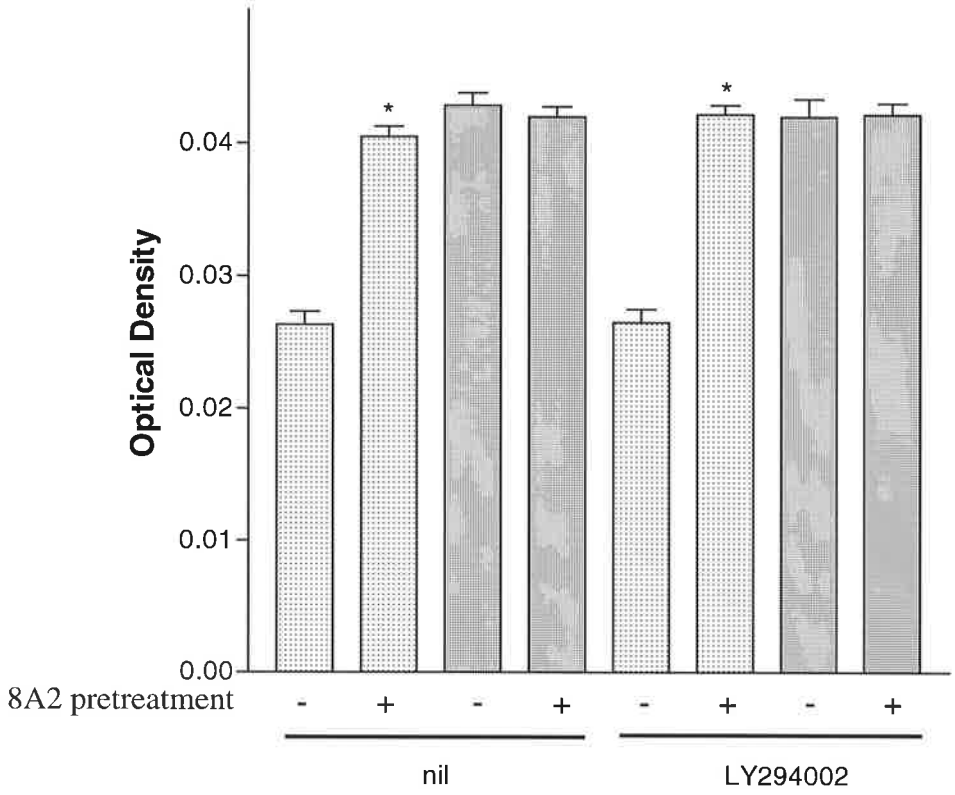
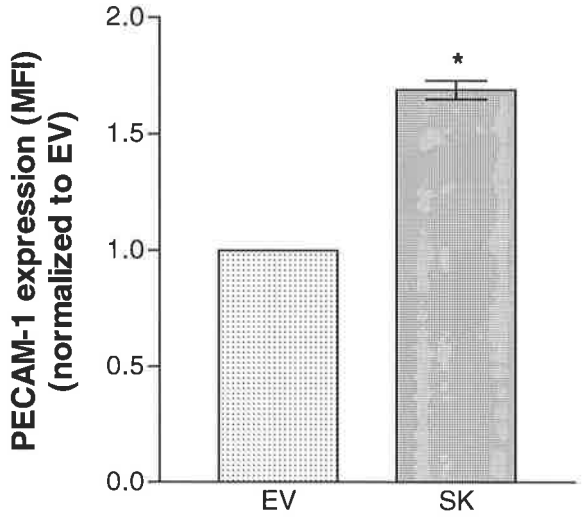
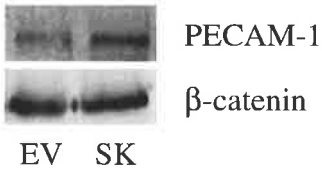


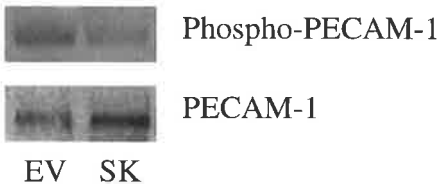
Fig 5.11 shows the attachment (as reflected by the optical density) of cells over-expressing SK (dark columns) and control (light columns) to fibronectin (50μg/ml) at 20 minutes. + indicates pre-treatment with 8A2 (20μg/ml for ten inutes); – represents the absence of 8A2. The effect of inhibiting the PI-3K pathway with 10μM LY294002 is shown. *$p<0.001$ of EV with 8A2 compared with untreated EV, and EV with 8A2 in the presence of LY294002 compared with EV without 8A2 treatment in the presence of LY294002, by Student's t-Test. The figure represents the pooled data of six observations derived from two separate endothelial cell lines. Bars represent SEM.



(a)



(b)



(c)

Fig 5.12 (a) shows the effect of over-expression of SK on PECAM-1 expression. (a) Cell surface expression of PECAM-1 as measured by flow cytometry using indirect fluorescence staining with 20μg/ml mouse monoclonal anti-PECAM-1 antibody (51-6F6) and indicated by the median fluorescence intensity (MFI) in cells over-expressing SK and EV control cells. Fluorescence resulting from staining with a non-relevant isotype-matched negative control antibody (23-1F11) has been subtracted. The figure shows the composite analysis of three separate experiments, normalized to EV using Statistica Version 6.1 (Statsoft, Inc.). *$p<0.001$ SK compared with EV. Bars represent 95% confidence intervals. (b) shows a Western blot for PECAM-1 and β-catenin expression in these cells. (c) shows PECAM-1 phosphorylation by Western blot in cells over-expressing SK and control (EV).

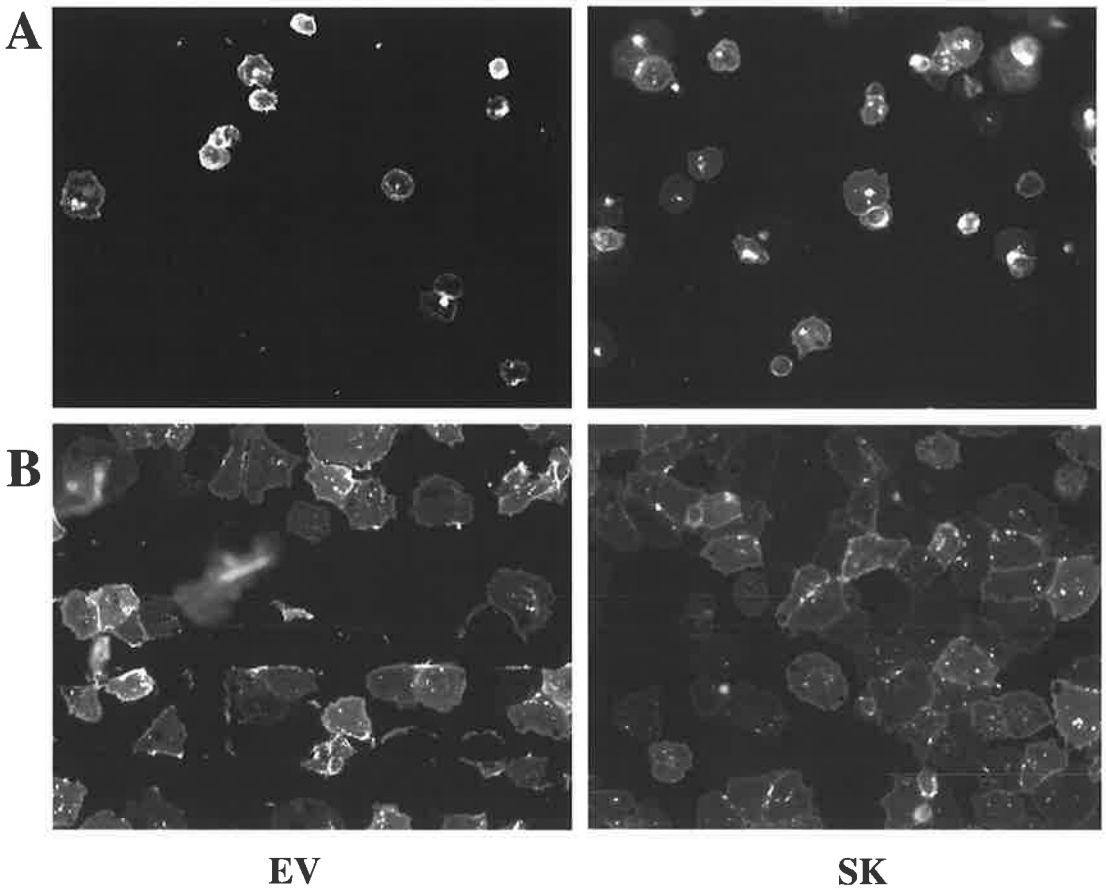
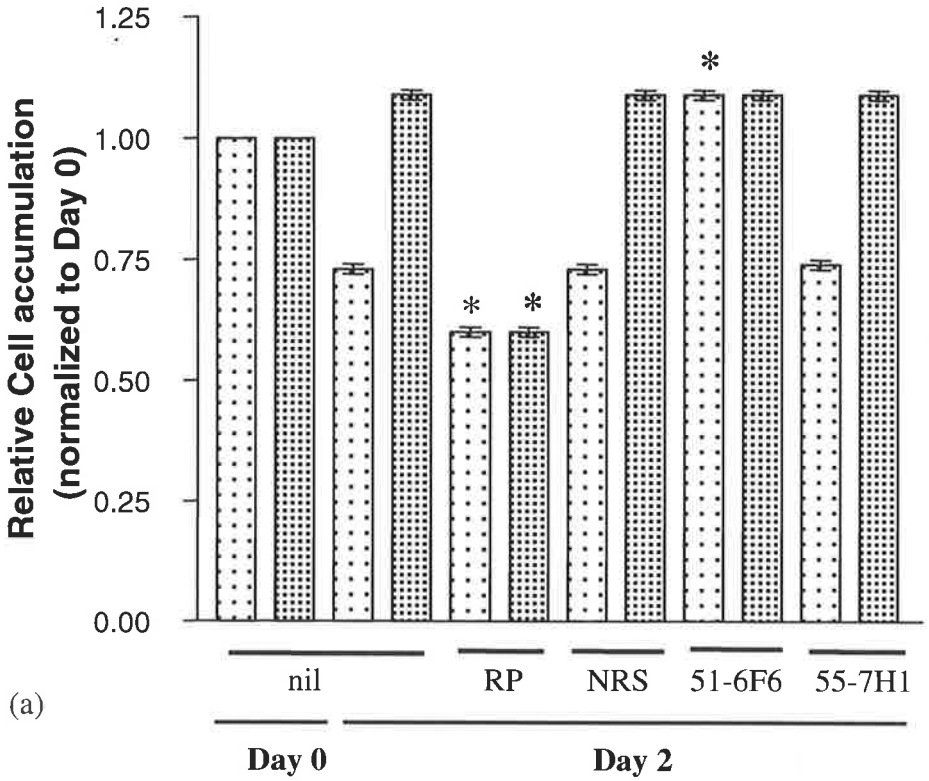
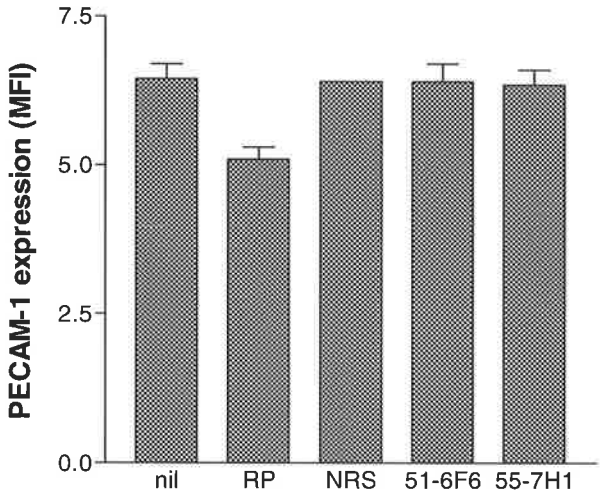


Fig 5.13 shows PECAM-1 immunostaining in cells over-expressing SK and control (EV) at 15 minutes (A) and 1 hour (B) after seeding the cells.

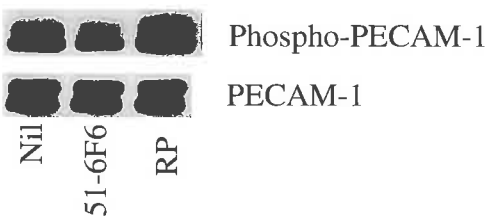
Fig 5.14 (a) shows the effect of antibody blockade of PECAM-1 on the survival of cells over-expressing SK (dense dots) and EV control (sparse dots) in suspension (unattached in serum free conditions). The figure shows the composite analysis of two separate experiments each performed in replicates of 5, and normalized to Day 0=1 using Statistica Version 6.1 (Statsoft, Inc.). Bars represent 95% confidence intervals. The effect of 20μg/ml rabbit polyclonal anti-PECAM-1 (RP), 20μg/ml of normal rabbit serum (NRS), 20μg/ml of a monoclonal antibody to PECAM-1 (51-6F6) and 20μg/ml of a monoclonal antibody to VE cadherin (55-7H1) is shown. *$p<0.001$ EV treated with RP compared with untreated EV at Day 2, and SK treated with RP compared with untreated SK at Day 2, and EV treated with 51-6F6 compared with untreated EV at Day 2. (b) shows the effect of pre-treatment of normal HUVEC with RP, NRS, 51-6F6 and 55-7H1 (20 μg/ml) for six hours in serum free conditions on the cell surface expression of PECAM-1 as measured by flow cytometry and indicated by the mean fluorescence intensity (MFI). The figure shows the pooled data from two separate endothelial isolates. (c) shows the effect of pre-treatment of normal HUVEC with RP and 51-6F6 (20 μg/ml) for six hours in serum free conditions, on the phosphorylation of PECAM-1. The membranes were blotted with phosphotyrosine antibody and anti-PECAM-1 antibody (51-6F6).


Relative Cell accumulation
(normalized to Day 0)
1.25
1.00
0.75
0.50
0.25
0.00
*
*
*
nil
RP
NRS
51-6F6
55-7H1
Day 0
Day 2

(a)


PECAM-1 expression (MFI)
7.5
5.0
2.5
0.0
nil
RP
NRS
51-6F6
55-7H1

(b)


Phospho-PECAM-1
PECAM-1
Nil
51-6F6
RP

(c)

Fig 5.14

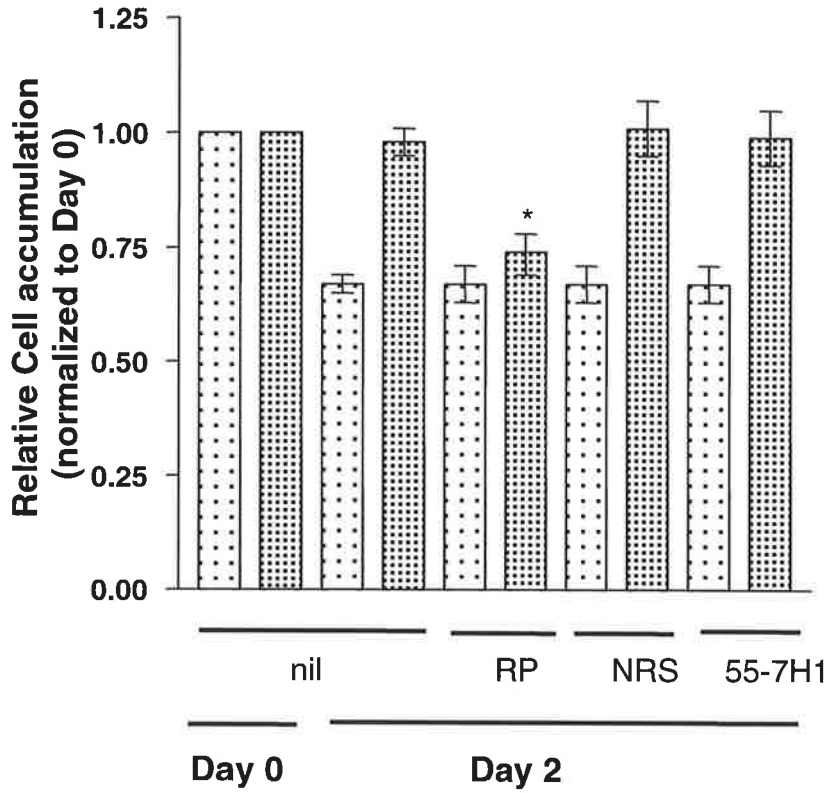


Fig 5.15 shows the role of cell junctional molecule PECAM-1 in mediating SK-induced cell survival in HUVEC over-expressing SK (dense dots) or EV control (sparse dots). Cells were seeded into gelatin-coated wells at a concentration of $8X10^3$ cells per well. At this concentration, cell-cell contact was not observed during the time course of the experiment. The effect on cell survival of 20 μg/ml rabbit polyclonal anti-PECAM-1 antibody (RP), 20 μg/ml normal rabbit serum (NRS), and a monoclonal antibody directed to VE cadherin (55-7H1) at 20 μg/ml is shown. The figure shows the composite analysis of ten observations from two separate experiments, normalized to Day 0=1 using Statistica Version 6.1 (Statsoft, Inc.). Bars represent 95% confidence intervals. * $p<0.001$ compared with untreated SK at Day 2.

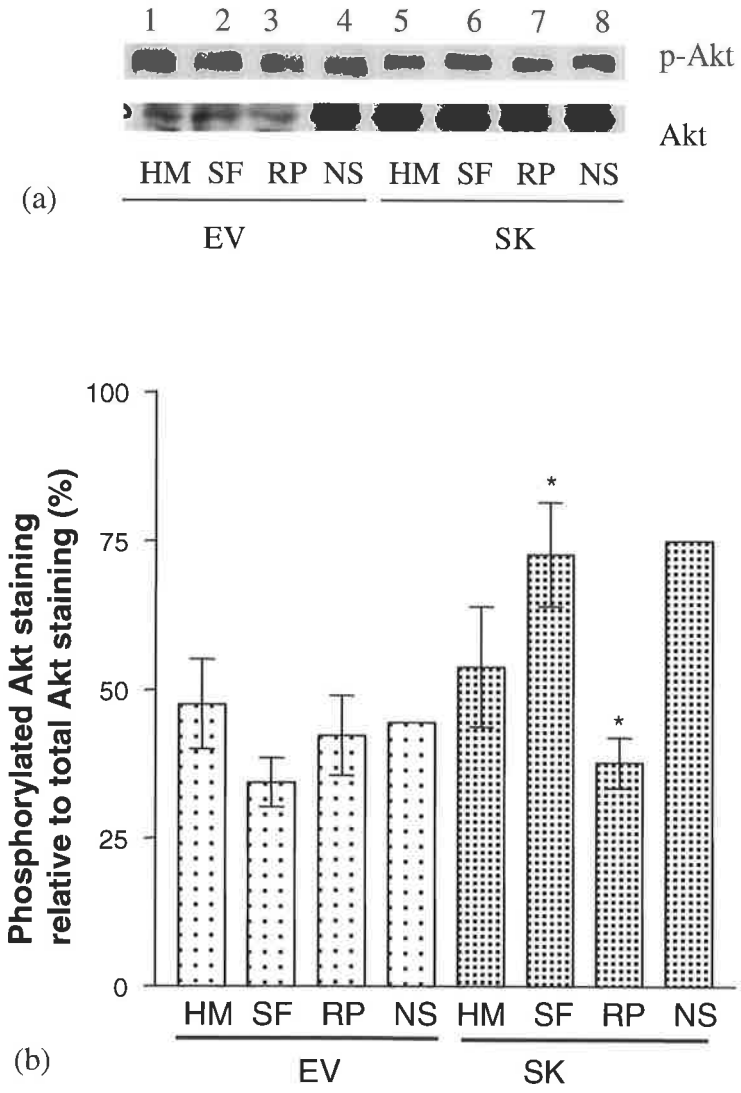


Fig 5.16 shows the effect of PECAM-1 signalling on the activation of the PI-3K/Akt pathway in cells over-expressing SK (dark columns) and EV (light columns).
(a) shows a Western blot measuring phosphorylated Akt (p-Akt) and total Akt in cells taken from basal culture conditions (culture in HUVE medium, HM) and after 6 hours of serum incubation under serum free (SF) conditions. The effect of 20μg/mL of rabbit polyclonal anti-PECAM antibody (RP), and 20μg/mL normal rabbit serum (NS) on cells incubated under SF conditions is shown. (b) shows the pooled data from quantitative analysis of the proportionate intensities of staining of p-Akt and Akt from four separate experiments performed as in (a). Bars represent SEM. *$p<0.05$ of untreated SK versus untreated EV in serum free conditions, and SK treated with RP compared with untreated SK in serum free conditions by Student's t-Test.

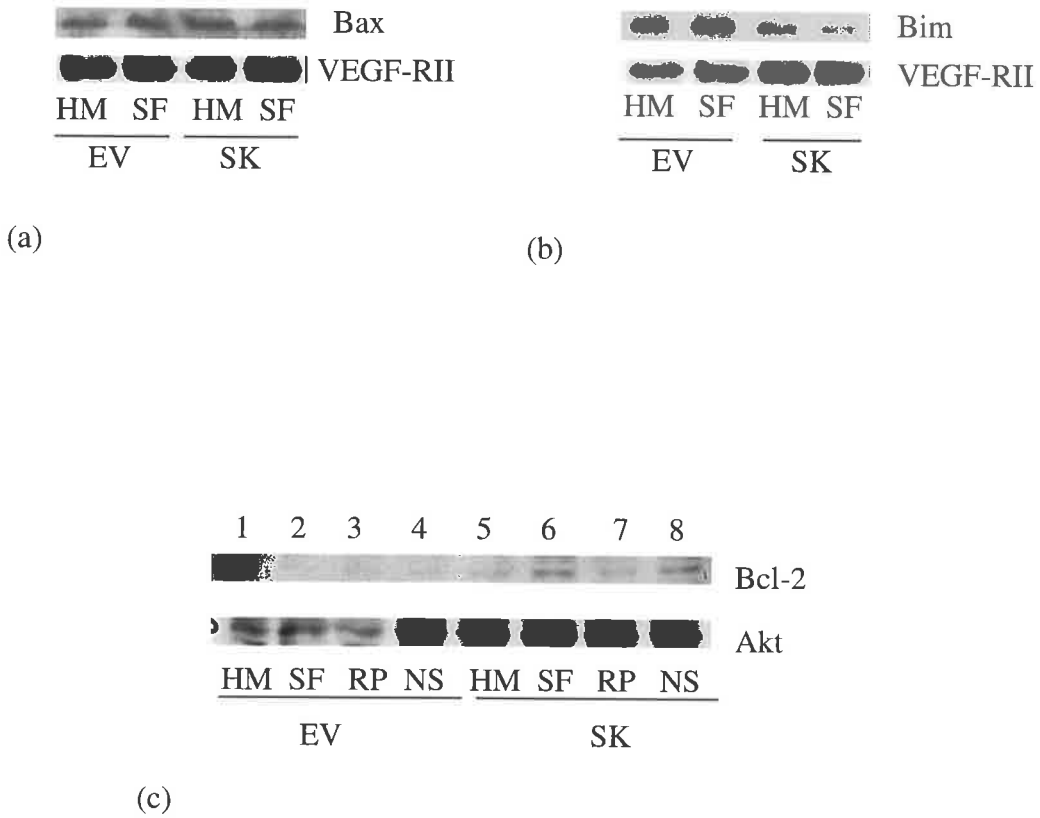


Fig 5.17 shows Bax (a), Bim (b) and Bcl-2 expression (c) in cells over-expressing SK and EV (control) under basal culture conditions in HUVE medium (HM) and after 6 hours of incubation under serum free (SF) conditions. The effect of 20µg/mL of rabbit polyclonal anti-PECAM antibody (RP), and 20µg/mL normal rabbit serum (NS) on Bcl-2 expression is shown. The loading control of VEGF-RII expression in (a,b) and Akt (c) is shown. The same membrane used in Fig 5.16 was stripped and re-blotted with rabbit anti-Bcl-2 antibody.

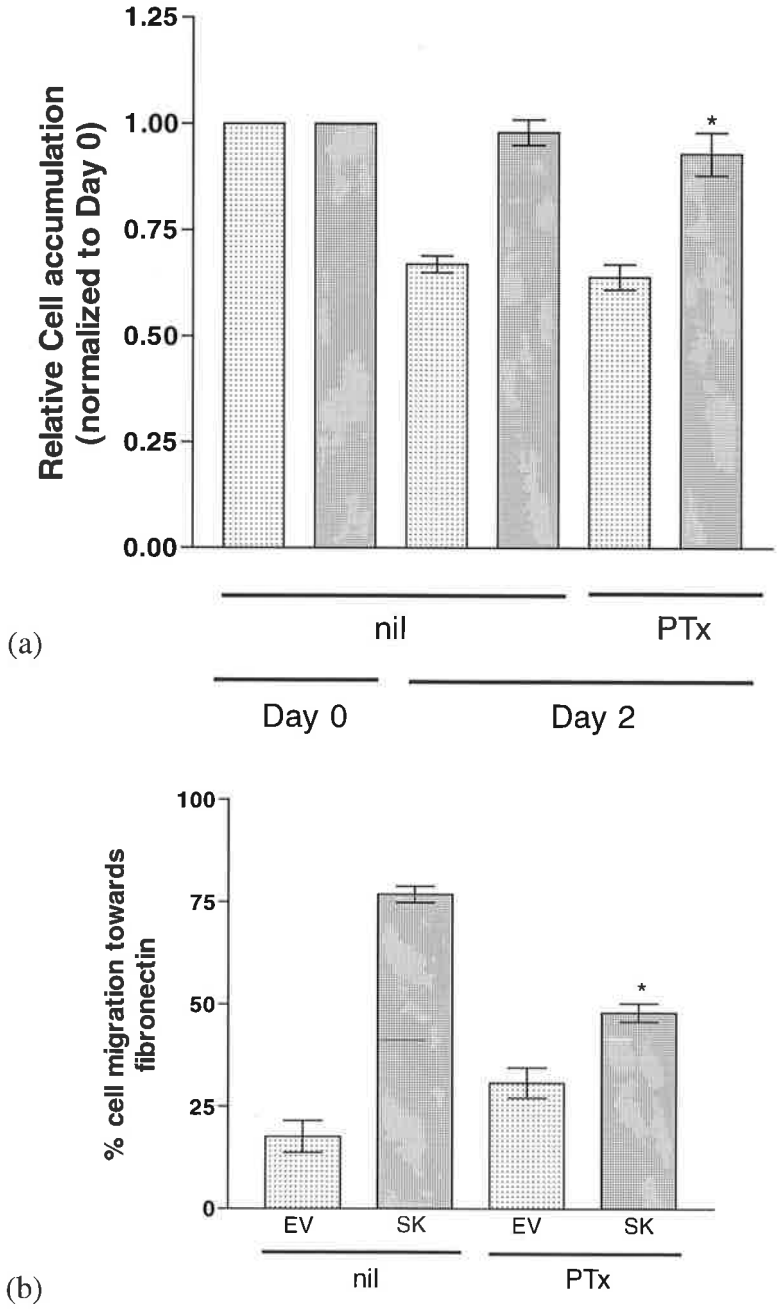
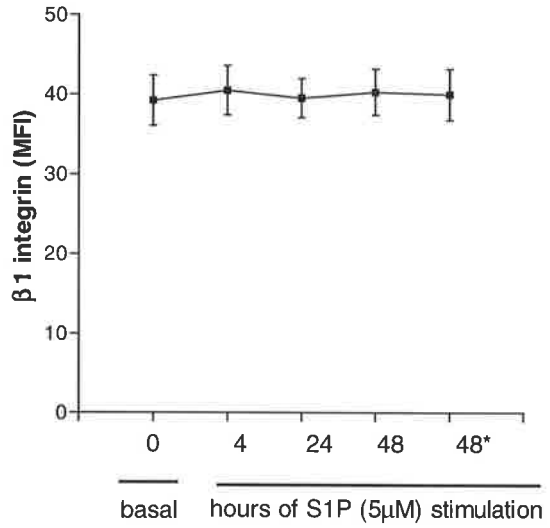
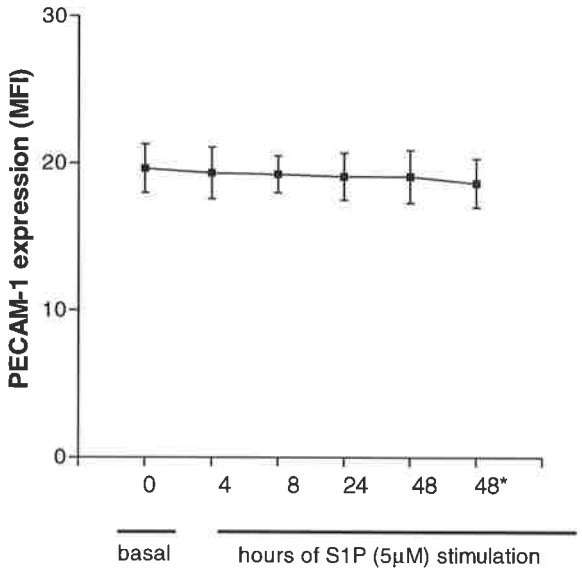


Fig 5.18 shows the effect of inhibiting GPCR with pertussis toxin (50 ng/ml) on cell survival (a) and cell migration (b) in HUVEC over-expressing SK (dark columns) or EV (light columns). (a) shows the composite analysis of eight observations from two separate experiments, normalized to Day 0=1 using Statistica Version 6.1 (Statsoft, Inc.). *$p<0.05$ compared with untreated SK at Day 2. Bars represent 95% confidence intervals. (b) shows the pooled data of six observations from two separate experiments, bars represent SEM. *$p<0.05$ compared with untreated SK.



(a)



(b)

Fig 5.19 shows the effect of stimulation of HUVEC with S1P (5μM) for variable duration on β1 integrin (a) and PECAM-1 (b) expression as reflected by the median fluorescence intensity (MFI). * indicates that the medium was replaced each 24 hours with medium containing fresh S1P. The figure shows the pooled data of two observations from two different endothelial cell lines, and bars represent SEM.

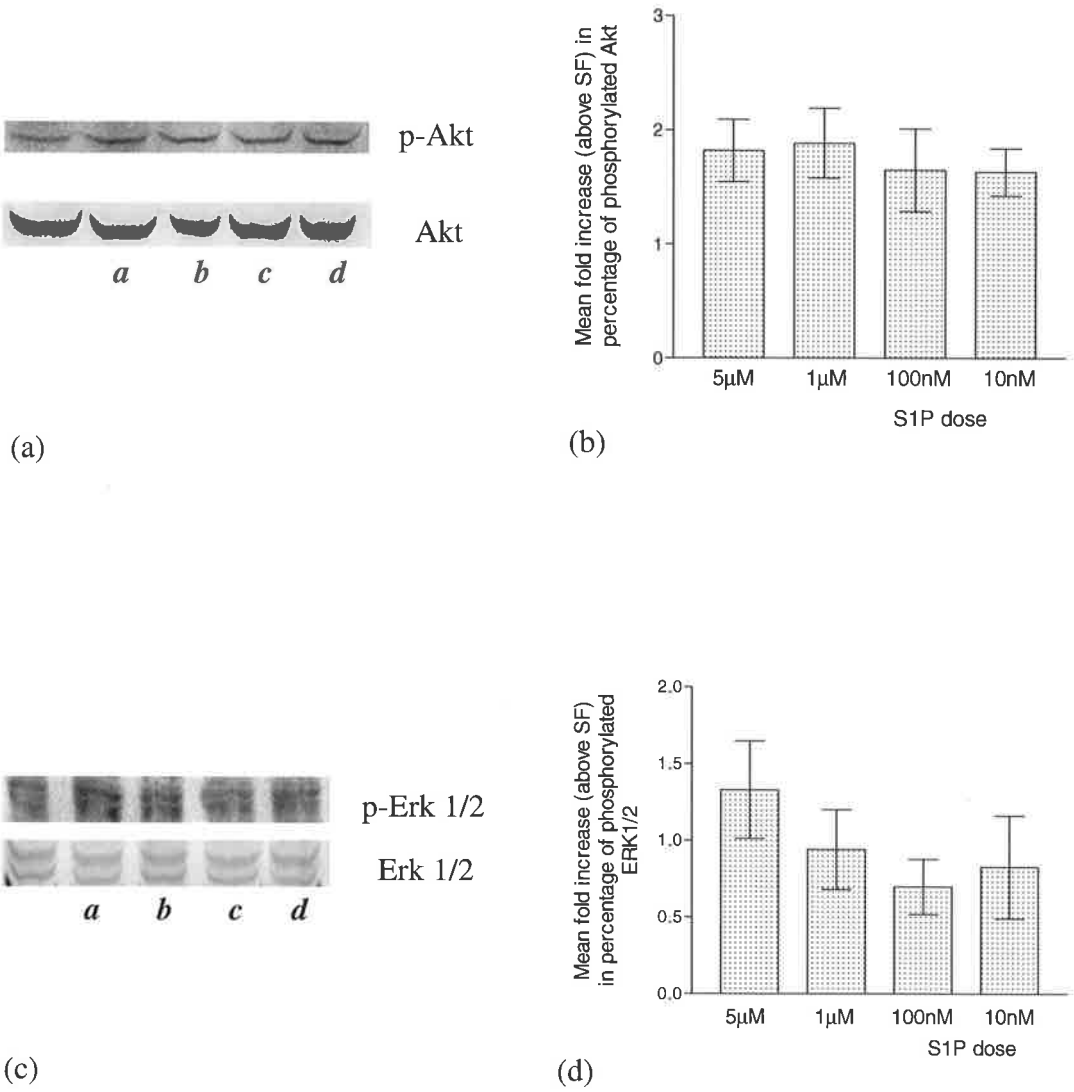


Fig 5.20 shows by Western blot the effect of different concentrations of S1P on the activation of (a) the PI-3K/Akt pathway and (c) the MAPK pathway in normal endothelial cells in EBM. S1P was used at the following doses: *a*=5μM, *b*=1 μM, *c*=100nM, *d*=10nM for 30 minutes. (b) and (d) show the mean fold change in the percentage of phosphorylated Akt and phosphorylated ERK1/2 respectively (above basal). (b) shows the pooled results from three separate endothelial cell lines and (d) shows the pooled data from two separate endothelial cell lines. Bars represent SEM.

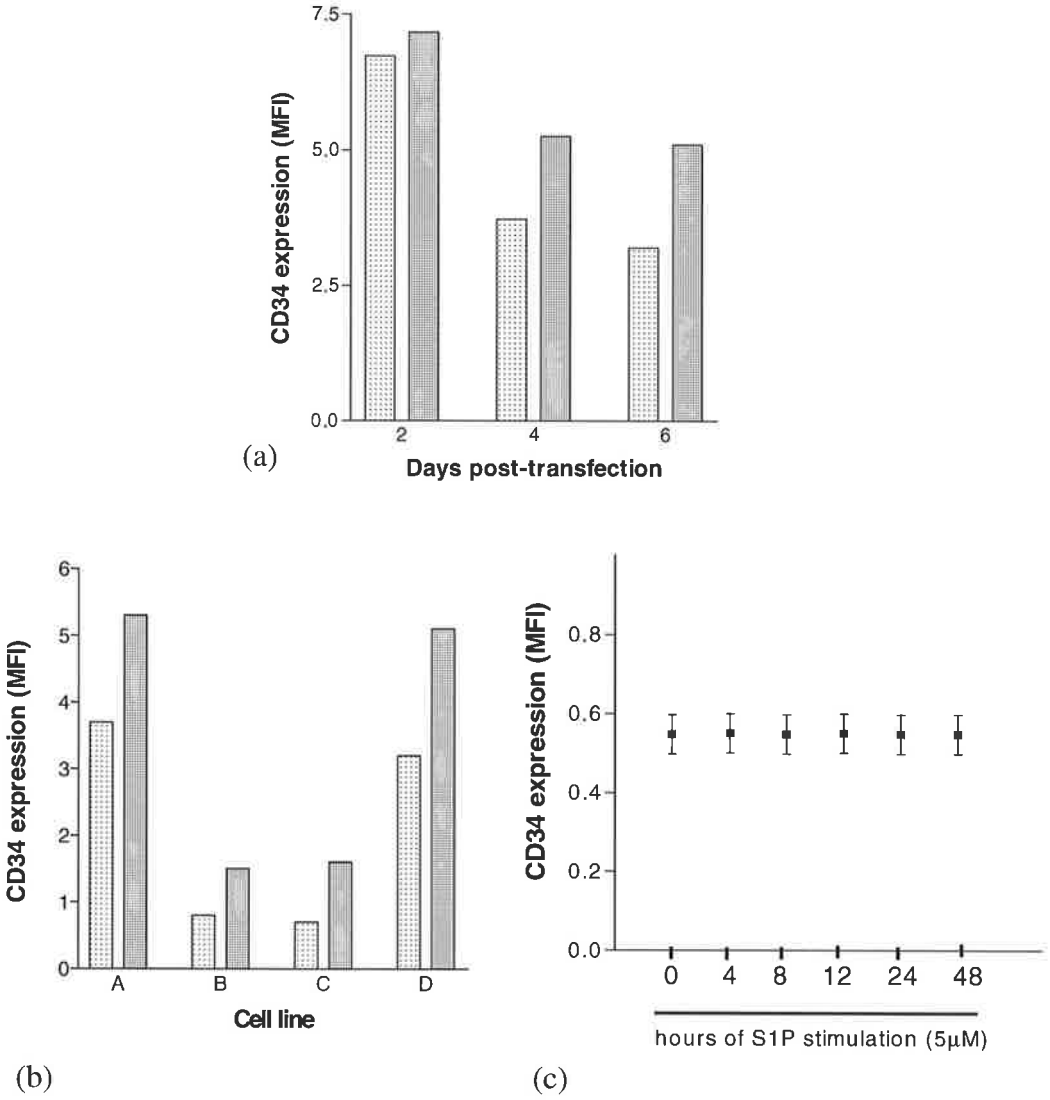
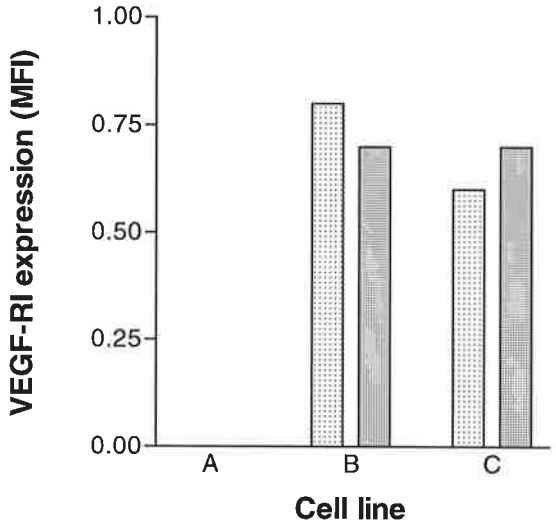
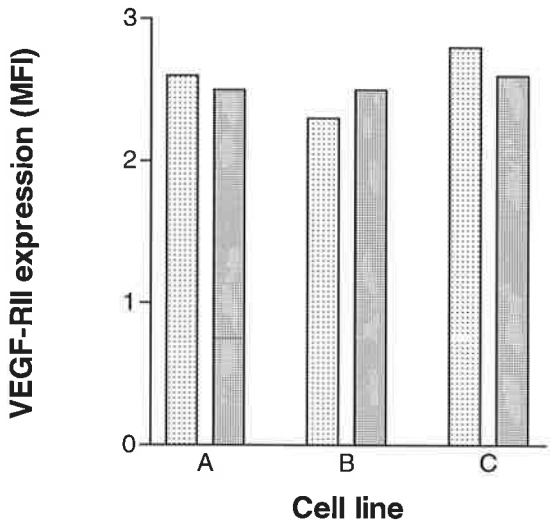


Fig 5.21 shows the effect of over-expression of SK on cell surface expression of CD34 as indicated by the median fluorescence intensity (MFI). (a) shows the time course of CD34 expression in cells over-expressing SK (dark columns) and EV control cells (light columns). (b) shows CD34 expression measured at 4-6 days post-transfection in endothelial cells from four different endothelial lines (A-D). The effect of exogenous stimulation with S1P (5μM) on CD34 expression is shown in (c) , which represents the pooled data from two separate experiments using different isolates of endothelial cells. Bars represent SEM.



(a)



(b)

Fig 5.22 shows the effect of over-expression of SK on the cell surface expression of VEGF-RI (a) and VEGF-RII (b), as indicated by the median fluorescence intensity (MFI), and measured in three separate endothelial cell lines (A-C) over-expressing SK (dark columns) or EV control (light columns).

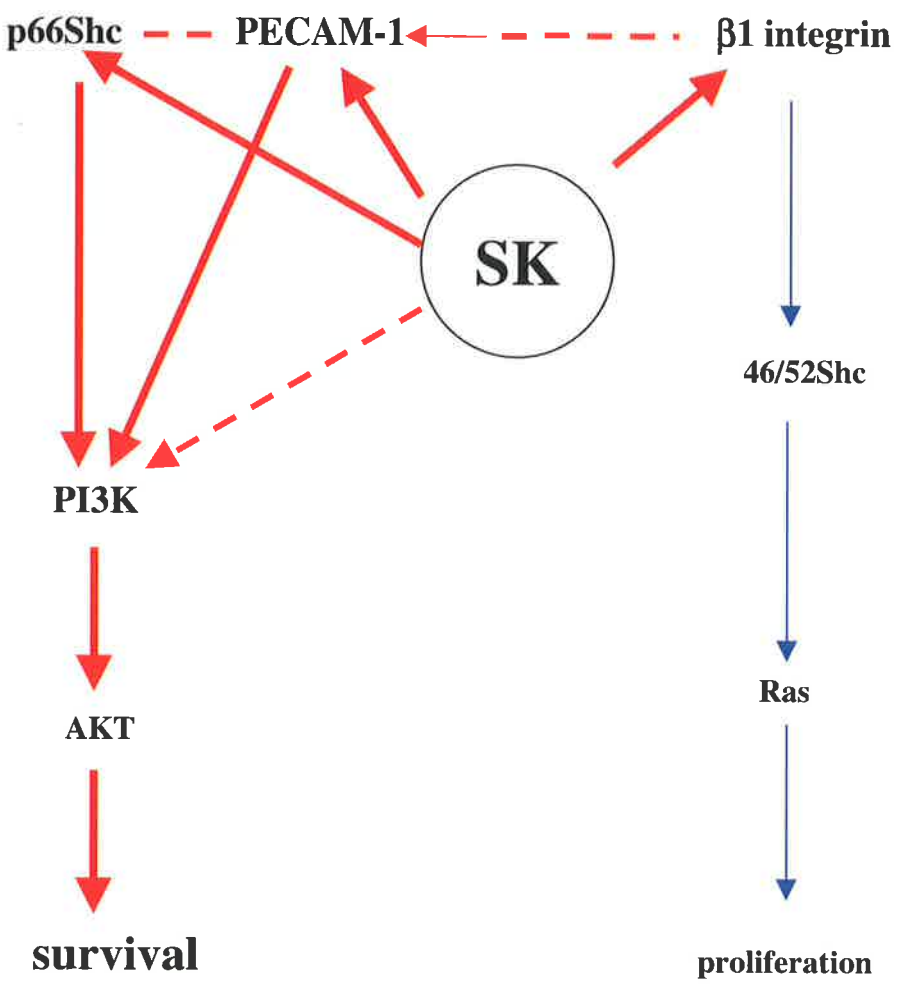


Fig 5.23 shows the signalling pathways engaged by raised intracellular levels of SK in HUVEC. Solid red arrows indicate the pathways shown in this thesis to be regulated by SK, resulting in a survival advantage under stressful conditions. Broken red arrows indicate hypothesized interactions. Blue arrows indicate the recognised pathway of integrin signalling resulting in cell proliferation which was not the dominant phenotype in HUVEC over-expressing SK.

CHAPTER 6

The Phenotypic consequences of over-expression of high levels of SK in Endothelial Cells

INTRODUCTION

The phenotypic consequences of moderately raised SK activity in endothelial cells have been described, yet the effects of higher levels of over-expression of SK have not been addressed. The observation that high levels of over-expression of SK result in reduction in relative cell accumulation over three days (Chapter 4.2) was intriguing, and suggested that endothelial cells may be more sensitive to levels of SK than other cell lines or tumor cell lines.

AIM

To investigate the phenotypic consequences of over-expression of high levels of SK in HUVEC.

METHODS

High levels of SK in HUVEC was achieved using adenoviral-mediated gene delivery (50 pfu/cell).

RESULTS

6.1 High levels of SK inhibit cell growth

It has been shown that over-expression of high levels of SK inhibits relative cell accumulation compared with control cells (Chapter 4.2). It was not however clear whether cells over-expressing high levels of SK still were able to increase in number over three days, albeit less than control cells, or whether this was a relative reduction in cell proliferation compared with control. Cell accumulation over three days in medium supplemented with 2% FCS was

measured in cells over-expressing SK and cells containing EV. In order to confirm that the observed effects were mediated specifically by SK, and not due to disruptions in the cell resulting from high levels of over-expression of a non-specific protein, cell accumulation in cells over-expressing equivalent pfu/cell of adenoviral supernatant carrying the catalytically inactive SK mutant, G82D was determined in the same experiment.

Cells over-expressing high levels of SK showed reduction in cell number, relative to the number of cells plated (as indicated by reduction in optical density) over three days, indicating cell death. This is in contrast to control cells with equivalent viral load which were able to proliferate (increase in cell number), as shown in Fig 6.1. Over-expression of G82D did not alter cell accumulation compared with EV, indicating that the inhibition of cell accumulation is mediated specifically by SK, and is not an artefact of high-level protein over-expression.

As cell accumulation over a defined period of time is determined by the proliferation and death of cells, it was postulated that over-expression of high levels of SK alters this balance to favour cell death.

6.2 High levels of SK increase Caspase-3 activity

Having established that high levels of SK in endothelial cells inhibits cell accumulation with the possibility that the effect was mediated through enhanced cell death, it was sought to confirm this by measurement of caspase-3 activity.

Over-expression of high levels of SK caused a consistent increase in caspase-3 activity in four separate endothelial cell lines under basal conditions, and in two of these endothelial cell lines

subjected to serum deprivation for 24 hours as shown in Fig 6.2(a) and (b). The mean fold increase in caspase-3 activity in cells over-expressing SK compared with control under basal conditions was 1.63 (95% confidence interval 1.42-1.87), ($p<0.001$) as shown in Fig 6.2(c). There was a trend towards an exaggerated caspase-3 activation in response to serum deprivation, which due to the small sample size ($n=2$) did not reach statistical significance ($p=0.1$) (Fig 6.2(d)).

6.3 High levels of SK reduce cell survival

6.3.1 High levels of SK reduce serum-free cell survival

Having established that over-expression of high levels of SK increased basal caspase-3 activity and increased cell death over three days, it was sought to determine whether toxicity was also evident over a shorter time period. The trend towards a greater caspase-3 response to serum deprivation prompted a determination of whether this had functional consequences in inhibiting cell survival in serum free conditions. Thus cell survival was measured with the colorimetric MTS assay over one day in serum free medium.

Cells over-expressing high levels of SK showed reduced cell numbers compared with control, in serum free conditions at 24 hours (i.e. reduced cell survival) as shown in Fig 6.3(a). Although there was no difference in the numbers of cells seeded at Day 0, ($p=0.92$), at Day 1, there were significantly less viable cells over-expressing SK compared with EV ($p<0.05$). This indicates that cells over-expressing high levels of SK have greater cell death than control cells in the absence of serum, which is consistent with the greater caspase-3 activity seen in these cells. Over-expression of high levels of G82D did not alter cell survival compared with

control cells, confirming that the effects observed are mediated specifically by SK, and are not the result of cellular toxicity from a non specific increase in protein expression.

6.3.2 High levels of SK reduce cell survival in suspension

Given the reduction in cell survival in serum free conditions in cells over-expressing high levels of SK, it was sought to determine whether these cells are less resistant to the stress of loss of attachment to extracellular matrix. Cell numbers following incubation in serum free conditions within non-tissue culture, non-adhesive 96 well trays coated with BSA was measured using the MTS assay.

Cells over-expressing high levels of SK showed significantly reduced survival in suspension compared with EV-bearing control cells as shown in Fig 6.3(b). There was no difference in the numbers of cells seeded at Day 0 comparing cells over-expressing SK with EV ($p=0.92$), however at Day 1, both cells over-expressing SK and EV were less in number, with cells over-expressing SK showing significantly fewer viable cells compared with EV ($p<0.05$).

Thus I concluded that cells over-expressing high levels of SK show reduction in cell survival in serum free conditions and when unattached. This is consistent with the observed enhanced caspase-3 activity in these cells.

6.4 The effects of over-expression of high levels of SK on cell junctions

Given that cells over-expressing high levels of SK show reduced cell survival, and in light of the recognized role of endothelial cell junctions and cell-cell contact in maintaining

endothelial cell viability, permeability to FITC-dextran was measured to assess the structural integrity of monolayers of HUVEC over-expressing high levels of SK.

6.4.1 The effect of over-expression of high levels of SK on basal permeability

Cells over-expressing high levels of SK showed increased basal permeability to FITC-dextran across all time points tested (Fig 6.4(a)). The disruption of cell-cell junctions seen under these conditions contrasts with the tighter cell-cell junctions seen with over-expression of low levels of SK (Chapter 4.3.5.1), and is consistent with the observed reduction in endothelial cell viability described above.

6.4.2 High levels of over-expression of SK cause loss of thrombin response

When treated with thrombin there was no difference in permeability assessed across all time points, between cells over-expressing high levels of SK and EV ($p=0.38$), as indicated in Fig 6.4(b).

HUVEC infected with high levels of EV showed a significant response to thrombin stimulation (Fig 6.4(c)). This effect was not seen in cells over-expressing high levels of SK (Fig 6.4(d)).

6.4.3 Conclusions

I concluded that over-expression of high levels of SK disrupts the integrity of the endothelial cell monolayer resulting in enhanced basal permeability. The resistance shown by these cells to the disruptive effects of thrombin on the cell junctions may be explained by high

extracellular levels of S1P in these cells, which are known to antagonize the effects of thrombin. This is in contrast to cells over-expressing low levels of SK, which were not protected from thrombin stimulation (Chapter 4.3.5.2). It is however possible that further disruption of the cell junctions by thrombin in these cells, which are already showing enhanced permeability, is either not possible (maximal separation of cell junctions), or not within the limits of detection of the well plate reader. A further possibility is that the cells over-expressing SK are dying and are unable to respond to thrombin stimulation.

6.5 Summary

It has been demonstrated that cells over-expressing high levels of SK show a reduction in cell accumulation, reduced cell survival and enhanced apoptosis. This is in sharp contrast to the angiogenic phenotype resulting from over-expression of low levels of SK and suggests differential engagement of downstream signalling pathways by different intracellular levels of expression and activity of SK.

6.6 The cellular mechanisms involved in mediating the changes conferred by high level over-expression of SK

The cellular mechanisms underlying the loss of cell numbers over three days, reduction in cell survival, and increased apoptosis seen with over-expression of high levels of SK (50 pfu/cell) were investigated.

6.6.1 Phosphorylation of SK under basal conditions was evident with over-expression of high levels of SK

An investigation into the mechanisms underlying the activation of SK has revealed that phosphorylation of SK at Ser225 results in activation of the enzyme (120)(Chapter 1.6.3). Thus it was sought to determine whether the enzyme is in an active (phosphorylated) state when over-expressed at low level (1 pfu/cell) or high level (50 pfu/cell) in HUVEC.

To this end, a polyclonal antibody recognizing a phospho-peptide centred around phospho-ser225 of human SK1 was generated in rabbits (by Dr. S.M. Pitson at the Hanson Institute). This antibody was used to probe lysates of cell preparations over-expressing both low and high levels of SK by Western blot analysis.

In cells over-expressing high levels of SK, phosphorylation of the enzyme was evident under basal conditions (Fig 6.5). A band which migrated at 45kDa was evident on probing the membrane with anti-phospho-SK peptide antibody. The size of this band was ascertained by comparing its relative migration with that of both pre-stained molecular weight markers, and recombinant SK. The identity of this band was confirmed by stripping the membrane and re-probing with anti-flag antibody. This resulted in a much more intense band, which overlay the initial band, providing further evidence that this band is SK. Thus it was concluded that over-expression of high levels of SK is associated with a basal level of activation of the enzyme.

In contrast, in cells over-expressing low levels of SK, phosphorylation was not detected in cell lysates, even after stimulation with TNFα for ten minutes (Fig 6.4). Definite expression of SK was confirmed in these cells by re-blotting the membrane with anti-flag antibody.

The possibility however, that in cells over-expressing low levels of SK, the level of expression of the enzyme is below the lower detection limit of the anti-phospho-SK peptide antibody, was recognised. It was thus decided to immunoprecipitate SK (under basal conditions and in response to stimulation with TNFα for ten minutes) using anti-flag antibody, and to assess in the same manner, whether there is phosphorylation of the enzyme. Even with immunoprecipitation of SK (and in response to activation by TNFα), under the conditions employed, phosphorylation of the enzyme was not detected (data not shown). Again it is noted that this does not exclude entirely the possibility of phosphorylation of SK, however makes it unlikely that a significant fraction of the enzyme is phosphorylated when over-expressed at low levels in endothelial cells.

6.6.2 Over-expression of high levels of SK does not alter β1 integrin expression

Having demonstrated that over-expression of high levels of SK results in reduced cell survival and cell number, and in view of the recognized role of the β1 integrins in the regulation of the cell cycle and cell proliferation, alterations in the cell surface expression of β1 integrin was sought. Due to very high GFP expression in cells infected with 50 pfu/cell, and the possibility of GFP emission being detected in the channel set for detection of phycoerythrin emission on the flow cytometer, β1 integrin expression was measured by Western blot analysis rather than flow cytometry.

Cells over-expressing high levels of SK showed no change in total β1 integrin expression as shown in Fig 6.6(a). The mean fold increase in β1 integrin expression above control was 0.84 (95% CI 0.67-1.07), (p=0.39), as shown in Fig 6.6(b). This was in contrast to the up-regulation of β1 integrin observed in cells over-expressing low levels of SK (Chapter 5.1.1).

6.6.3 Over-expression of high levels of SK down-regulates PECAM-1 expression

Cells over-expressing high levels of SK were shown to have increased basal permeability and reduction in cell survival. The known role of PECAM-1 in regulation of cell junctions and protection from apoptosis prompted a determination of total PECAM-1 levels in cells over-expressing SK.

By Western blot analysis, cells over-expressing high levels of SK showed a substantial reduction in total PECAM-1 expression, as indicated in Fig 6.6(c). Quantification of PECAM-1 expression in two separate endothelial cell lines using ImageQuant software confirmed that cells over-expressing high levels of SK reduce PECAM-1 expression by 42% (95% CI 0.41-0.42), ($p<0.05$) (Fig 6.6(d)). Over-expression of high levels of SK reduced PECAM-1 expression selectively, as there was no change in VE cadherin expression in four separate endothelial cell lines (mean fold increase in VE cadherin above EV 0.92, 95% CI 0.76-1.11, $p=0.45$).

The reduction in total PECAM-1 levels in cells over-expressing high levels of SK is in contrast with the increase in PECAM-1 noted in cells over-expressing low levels of SK (Chapter 5.6.1).

6.6.4 Over-expression of high levels of SK reduces cell cycling

Although it has been established that over-expression of high levels of SK shifts the balance of cell survival/ proliferation and cell death to favour cell death, it has not yet been determined whether there is an accompanying reduction in cell cycling, or true increase in cell proliferation which is of insufficient magnitude to overcome the enhanced apoptosis in these

cells. Thus expression of cyclin D1 and cyclin E was measured by Western blot analysis in cell lysates made under basal culture conditions.

Over-expression of high levels of SK resulted in a significant reduction in expression of both cyclin D1 (mean fold change compared with EV 0.75 ± 0.03, n=3, $p<0.05$) and cyclin E (mean fold change compared with EV 0.78 ± 0.01, n=3, $p<0.05$) (Fig 6.7), indicating that these cells show a reduction in cell cycling and cell proliferation.

Thus over-expression of high levels of SK not only enhances cell death, but it also inhibits cell cycling.

6.6.5 Cells over-expressing high levels of SK failed to engage the PI-3Kinase pathway

Having established that high levels of SK in HUVEC enhance caspase-3 activity and reduce cell survival, an investigation into the activation of the PI-3kinase/Akt pathway, the main cell survival pathway, by these cells was conducted. Phosphorylation of Akt was measured in these cells and control cells, both under basal culture conditions and in response to the stress of serum deprivation for six hours.

Cells over-expressing high levels of SK showed a reduction in the proportion of Akt which is phosphorylated both under basal conditions and when stressed with serum deprivation (Fig 6.8). This was confirmed in two separate endothelial cell lines. Moreover, cells over-expressing high levels of SK showed a further down-regulation in phosphorylation of Akt with serum deprivation, as did the control cells (Fig 6.8). This is in contrast to cells over-expressing low levels of SK which responded to serum deprivation with a further increase in the fraction of phosphorylated Akt (Chapter 5.3.5).

The reduction in phosphorylation of Akt under basal conditions and the failure to engage this pathway in response to serum deprivation are consistent with the enhanced cell death and reduction in cell survival seen in these cells.

DISCUSSION

It has been demonstrated that markedly raised intracellular levels and activity of SK in HUVEC result in alterations in endothelial cell phenotype, which are clearly distinct from those seen when enzyme activity is increased more modestly. High levels of over-expression of SK in endothelial cells result in enhanced apoptosis with corresponding loss of cell numbers. Whereas cells over-expressing low levels of SK induced PECAM-1 expression and signalled through PECAM-1 to activate the PI-3K/Akt pathway, cells over-expressing high levels of SK down-regulated PECAM-1 and failed to engage the PI-3K/Akt pathway in response to the stress of serum deprivation. This, together with reduction in G-phase cyclins resulted in a phenotype of inhibited cell growth and cell survival. This cellular 'toxicity' resulting from high levels of SK in HUVEC is distinct to HEK293 cells, NIH3T3 cells and tumour cell lines which do not show such toxicity with high levels of SK (199). The reasons for this difference have not been addressed specifically, but indicate a need for cellular levels of SK to be regulated in endothelial cells. It is possible that moderately enhanced intracellular SK activity in endothelial cells results in enhanced inflammatory potential and aberrant angiogenesis, whilst more dramatic elevations in SK activity may result in vascular regression. While the physiological relevance of a 50-fold increase in SK expression is questionable, the divergence in effects of high and low expression of SK is consistent with the biphasic dose response curve of many active metabolites when given through a range from zero to conditions of over-abundance.

The reduction in cell numbers over three days seen with over-expression of high levels of SK is a net result of both enhanced cell death (as indicated by greater caspase-3 activity, Fig 6.2) and reduced cell cycling (as determined by measurement of cyclin D1 and cyclin E, Fig 6.7). This effect was mediated specifically by SK, as over-expression of a similar level of the inactive SK mutant (G82D) did not alter cell accumulation (Fig 6.1). Similarly the inhibition of serum-free cell survival seen with markedly raised SK activity (Fig 6.3) appeared to be mediated specifically by SK activity, rather than the non-specific effects of increased cellular protein synthesis, as over-expression of high levels of G82D did not alter survival compared with control cells. The SK mutant, G82D does not alter basal catalytic activity, but rather prevents agonist-induced activation of SK (127).

Whereas cells over-expressing low levels of SK showed up-regulation of PECAM-1 expression (Chapter 5.6.1), and an ability to engage the PI-3K/Akt pathway (Chapter 5.3.5), which was shown to be mediated by PECAM-1 signalling (Chapter 5.6.6), cells over-expressing high levels of SK showed reduced PECAM-1 expression, and did not activate the PI-3K/Akt pathway. It is tempting to speculate that the failure to engage the PI-3K/Akt pathway is due to a reduction in PECAM-1 signalling consequent upon high levels of over-expression of SK, however this has not been specifically addressed. The differential effects on PECAM-1 expression are also consistent with opposing effects on the structural integrity of endothelial cell monolayers, with disruption of cell-cell junctions seen with high levels of over-expression of SK (Fig 6.4), and tightening of cell-cell contacts with low levels of SK over-expression (Fig 4.8). Indeed the junctional molecule PECAM-1 is involved in anti-apoptosis(189;217), and it is intriguing that this protein is differentially regulated by variable levels of SK.

A critical difference between high and low level over-expression of SK is likely to be the degree to which the enzyme is phosphorylated under basal conditions. Sphingosine kinase 1 was phosphorylated under basal conditions when over-expressed at high levels in endothelial cells, however no phosphorylation was evident with over-expression at low levels (Fig 6.5). Although a small degree of phosphorylation of SK with over-expression of SK at low levels is not excluded, the failure to detect phosphorylation even in immunoprecipitated SK (Fig 6.5) makes it unlikely that this occurs to any significant degree. The fivefold elevation in SK activity achieved with enforced expression of low levels of SK, although not substantially dissimilar to the level of basal intrinsic activity, still produced significant functional changes in endothelial cells. By contrast transfection with the non-phosphorylatable hSK1 mutant (containing the substitution Ser225→Ala), while enhancing SK catalytic activity, did not result in functional alterations when over-expressed in HEK293 cells(120). Indeed under basal conditions in HEK293 cells, SK is not phosphorylated (120), and it is likely that endothelial cells are comparable in this regard, with absence of phosphorylation of SK in the unstimulated state. As for other protein kinases which are activated by phosphorylation, it is conceivable that phosphorylation of SK alters its ability to interact with downstream targets, and indeed, activation of SK above its basal catalytic level, is obligatory for its role in signal transduction cascades (127).

Another possible explanation for the observed differences in phenotype resulting from over-expression of high or low levels of SK is the subcellular localisation of the enzyme. Phosphorylation of SK has recently been shown to result in its translocation from the cytosol to the plasma membrane (120), and it is possible that over-expressed SK at high levels may become localized to a distinctive site, possibly the plasma membrane, thereby changing the profile of its intracellular interactions. This issue has not been specifically addressed in this thesis.

The levels of S1P resulting from obligatory expression of SK at high or low levels have not been measured. Normally, S1P generated by virtue of intrinsic catalytic activity of SK, undergoes degradation by S1P lyase (278;279) and S1P phosphatases (280). These enzymes are involved in regulating the cellular levels of S1P, and in the basal state, act to maintain low cellular levels of S1P (135). This regulatory mechanism may be sufficient to contain the small increase in S1P formation resulting from over-expression of low levels of SK, but may be insufficient to counteract grossly raised intracellular levels of S1P resulting from obligatory expression of high levels of SK. It may be postulated that chronic exposure to high intracellular levels of S1P may result in loss of responsiveness to the stimulant, with loss of the normal response of cell survival and protection from apoptosis.

As SK2 has recently been shown to inhibit cell proliferation and enhance apoptosis (110), the question was raised whether markedly raised activity of SK1 induces SK2. Determination of SK2 expression by RT-PCR however did not confirm this, and no increase in SK2 expression was detected when SK1 activity was greatly increased (data not shown).

In summary, markedly raised intracellular levels of SK render the cell susceptible to apoptosis and to the cellular stresses of serum deprivation. Phosphorylation of the enzyme under basal conditions when subject to obligatory expression at high levels is postulated to be critical in accounting for the phenotypic differences compared with those resulting from low levels of over-expression of SK. This work highlights the fact that variable levels of enzymatic activity can result in distinct phenotypic consequences, and indicates that cellular levels of SK are subject to control in endothelial cells. The possibility exists that such control is lost in diseases of enhanced cell death, and that profoundly elevated SK activity plays a pathogenic role in such conditions.

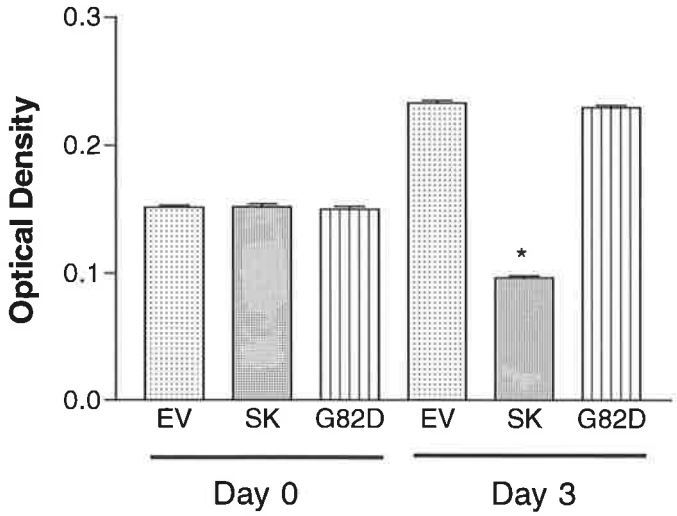


Fig 6.1 shows cell numbers over three days as reflected by the optical density, in cells over-expressing SK, G82D, or EV control. The figure shows the results of five observations from a single experiment which is representative of at least two separate experiments. Bars represent SEM. *$p<0.001$ SK compared with EV at Day 3.

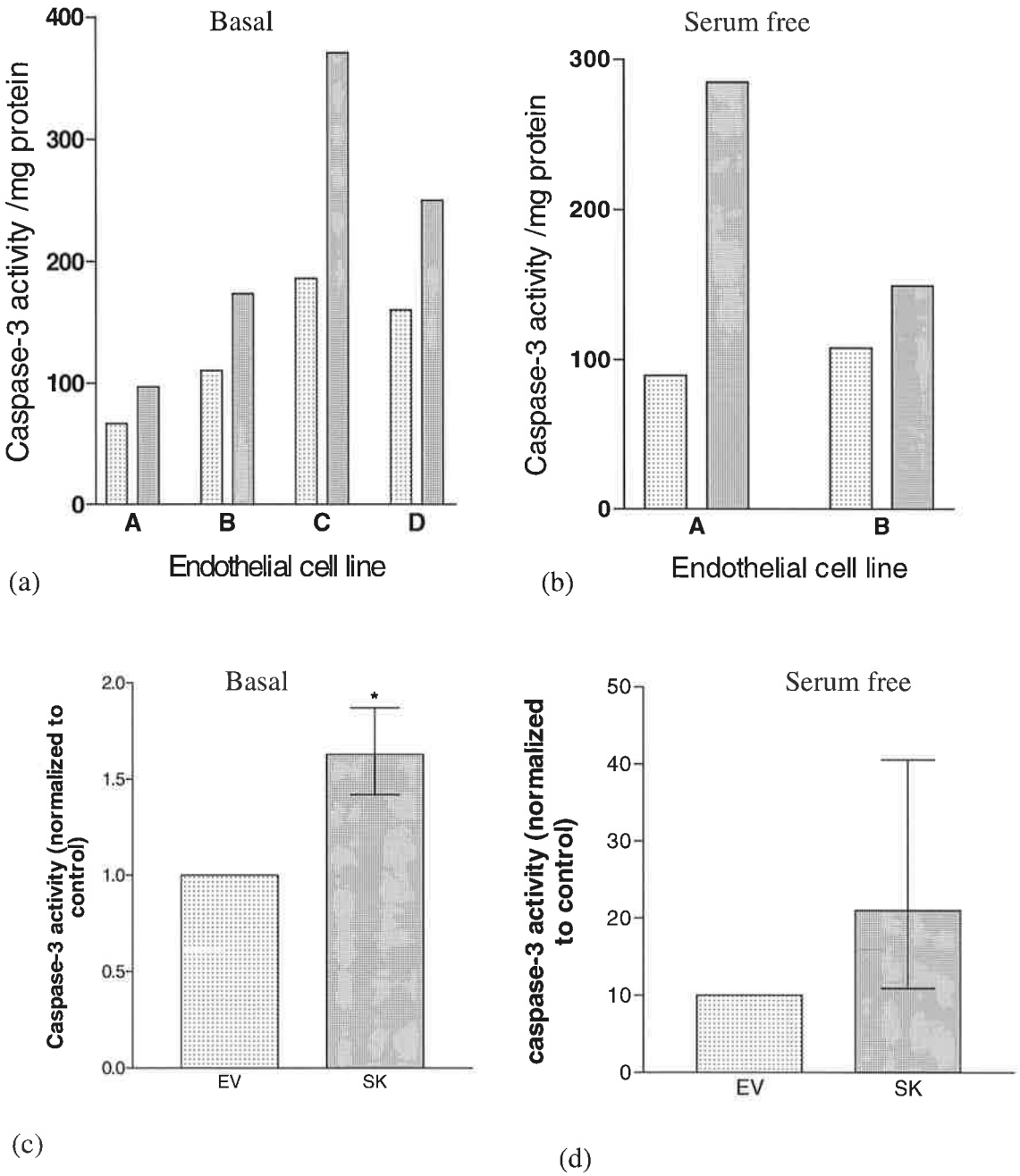
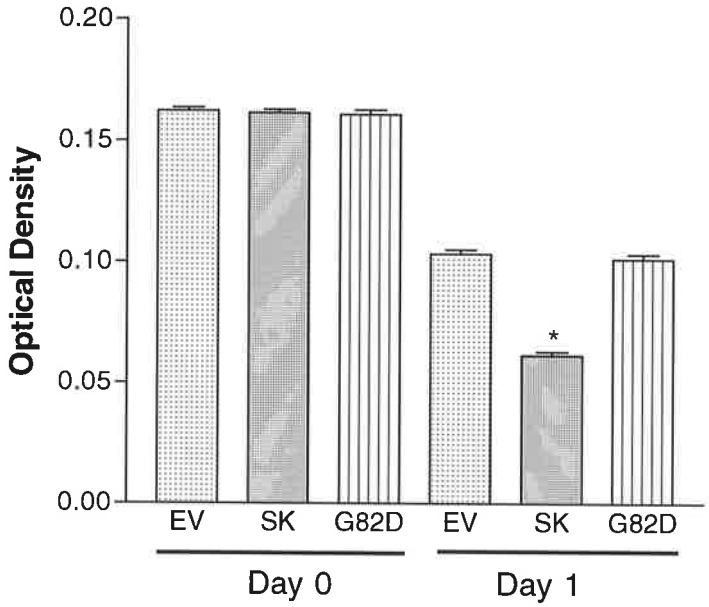


Fig 6.2 shows the effect of over-expressing high levels of SK (dark columns) compared with EV (light columns) on caspase-3 activity under basal conditions or in response to serum deprivation for 24 hours. (a) shows caspase-3 activity in four separate endothelial cell lines (A-D) under basal conditions, (b) shows the response of two of these lines (A,B) to serum deprivation. (c) and (d) show the composite analysis of (a) and (b) respectively, normalized to the control using Statistica Version 6.1 (Statsoft, Inc.) thus indicating the mean fold increase in caspase-3 activity in cells over-expressing SK compared with EV. *$p<0.001$ compared with EV. Bars represent 95% confidence intervals.

(a)



(b)

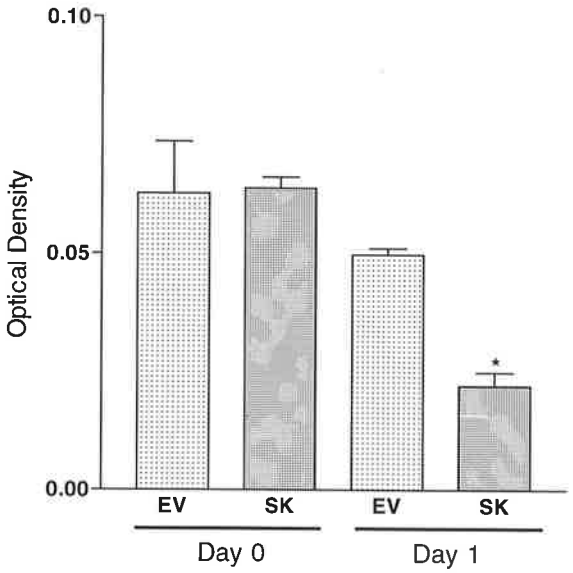
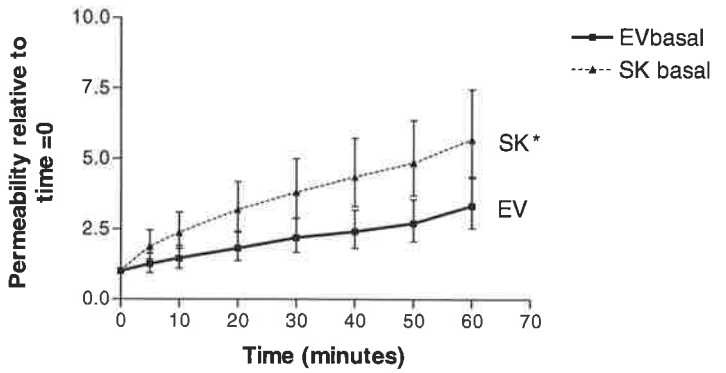


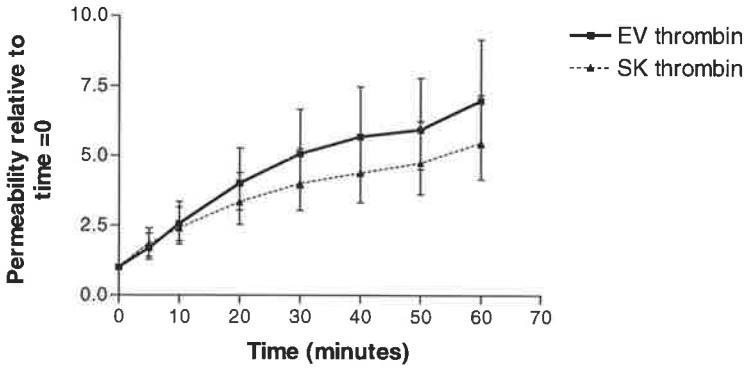
Fig 6.3 shows cell number maintained in the presence of over-expression of high levels of SK, compared with EV, in (a) serum free conditions and (b) in the absence of both an adherent extracellular matrix and serum, as reflected by the optical density. In (a) the effect of high levels of over-expression of G82D is also indicated. *$p<0.05$ SK compared with EV at Day 1. The figure shows the results from a single experiment done in quadruplicate, which is representative of four separate experiments. Bars represent SEM.

Fig 6.4 shows permeability (normalized to time =0) of cells over-expressing high levels of SK and EV, to FITC-dextran, across different time points (a) under basal conditions or (b,c,d) in response to thrombin stimulation (0.2 units/ml). (b) shows a comparison of permeability of EV and SK in response to treatment with thrombin, (c) and (d) show the response of cells transfected with EV and SK respectively to thrombin stimulation. *$p<0.001$ SK compared with EV under basal conditions across all time points (a), or untreated EV (c) compared with thrombin treated vector across all time points. The figure shows the composite analysis of 7 observations from 3 separate experiments using Statistica Version 6.1 (Statsoft, Inc.). Bars represent 95% confidence intervals.

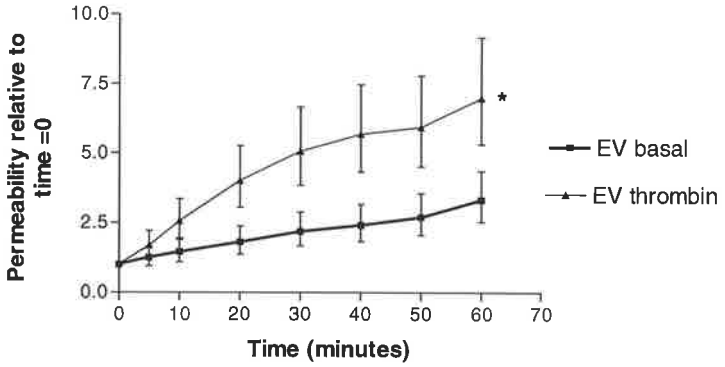
(a)


Permeability relative to time =0
10.0
7.5
5.0
2.5
0.0
0
10
20
30
40
50
60
70
Time (minutes)
EVbasal
SK basal
SK*
EV

(b)


Permeability relative to time =0
10.0
7.5
5.0
2.5
0.0
0
10
20
30
40
50
60
70
Time (minutes)
EV thrombin
SK thrombin

(c)


Permeability relative to time =0
10.0
7.5
5.0
2.5
0.0
0
10
20
30
40
50
60
70
Time (minutes)
*
EV basal
EV thrombin

(d)

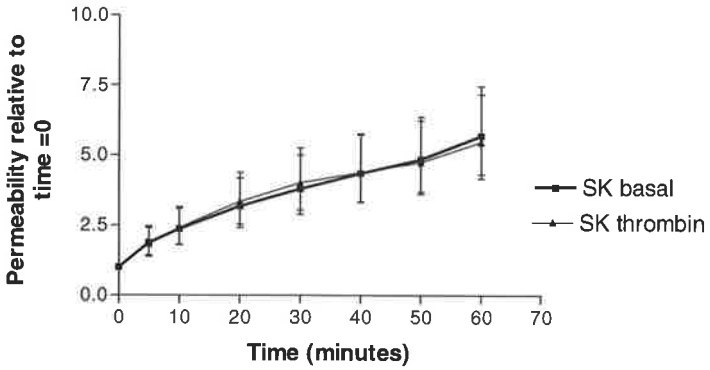

Permeability relative to time =0
10.0
7.5
5.0
2.5
0.0
0
10
20
30
40
50
60
70
Time (minutes)
SK basal
SK thrombin

Fig 6.4

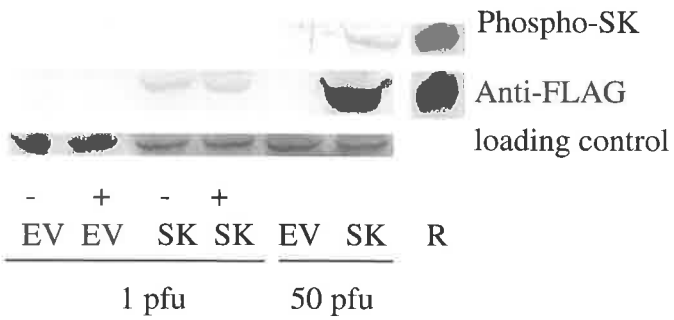
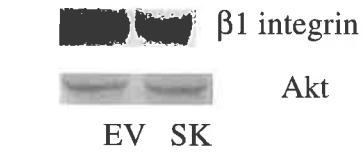
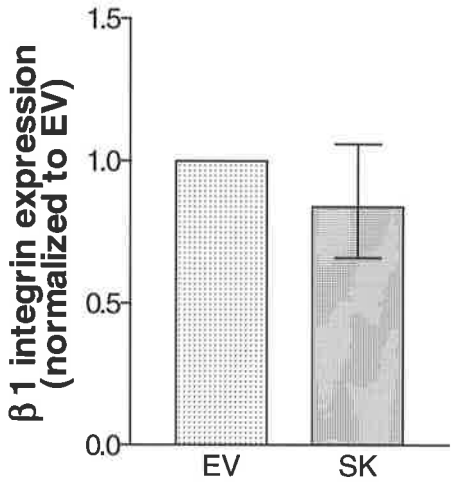


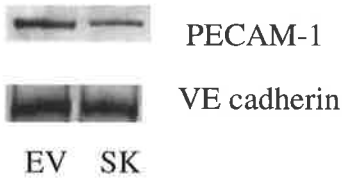
Fig 6.5 shows by Western blot, phosphorylation of SK in HUVEC infected with 1 pfu/cell or 50 pfu/cell of adenoviral supernatant carrying SK or EV. Cells infected with 1 pfu/cell were analysed in the presence (+) or absence (-) of stimulation with TNFα (0.5ng/ml) for ten minutes. 'R' indicates recombinant SK which is constitutively active and used as a positive control. The membrane was immunoblotted with rabbit anti-phospho SK peptide antibody, and then re-blotted with anti-flag antibody. The loading control of a non-specific background band detected by the anti-FLAG antibody is included.



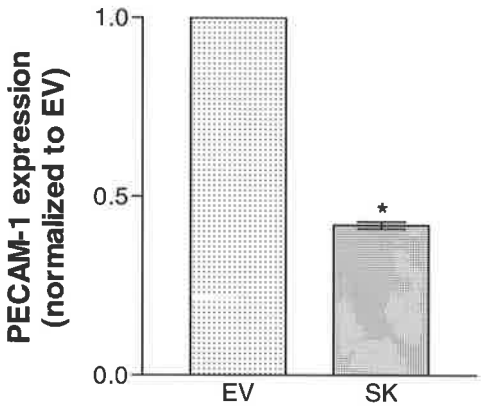
(a)



(b)



(c)



(d)

Fig 6.6 shows β1 integrin expression and PECAM-1 expression by Western blot in cells over-expressing high levels of SK and EV control. (a) shows a Western blot for β1 integrin and the loading control Akt. PECAM-1 and VE cadherin expression are shown in (c) . (b) and (d) shows the composite analysis of two separate experiments for β1 integrin (b) and PECAM-1 expression (d), normalized to EV using Statistica Version 6.1 (Statsoft, Inc.). Bars represent 95% confidence intervals. *$p<0.05$ SK compared with EV.

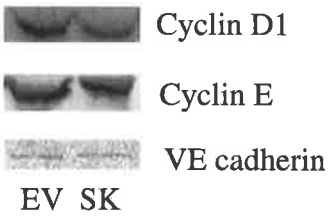


Fig 6.7 shows cyclin D1 and cyclin E expression in cell lysates made from HUVEC over-expressing high levels of SK and EV, under basal culture conditions. A loading control of VE cadherin is indicated.

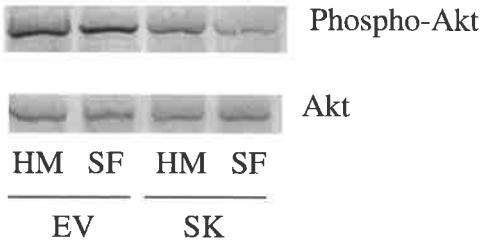


Fig 6.8 shows a Western blot of phosphorylated Akt (phospho-Akt) and total Akt in cells over-expressing SK and control (EV) cells, under basal conditions (culture in HUVE medium, HM) and after incubation for six hours in serum free (SF) medium.

CHAPTER 7

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The consequences of over-expression of SK in endothelial cells achieved with retroviral and adenoviral-mediated gene delivery have been ascertained. Mild increases in SK activity were shown to enhance the ability of the endothelial cells to undergo angiogenesis, as indicated by resistance to apoptosis, enhanced cell survival, migration, and stimulation of the ability to arrange into capillary like networks. Induction and dephosphorylation of PECAM-1 was shown to play a critical role, and a candidate signalling pathway in endothelial cells whereby, SK may act through PECAM-1-PECAM-1 to engage the PI-3K/Akt pathway has been identified. Whereas low levels of over-expression of SK enhanced angiogenesis, more profound increases in SK activity were shown to inhibit cell survival and enhance apoptosis. A factor believed to be critical in explaining the phenotypic differences between high and low levels of over-expression of SK was phosphorylation of SK when over-expressed at high levels. The phenotype of enhanced cell death and apoptosis resulting from high levels of over-expression of SK also highlights the need for cellular levels of SK and S1P to be appropriately regulated, and raises the question whether marked increases in SK activity may be present in conditions involving tissue atrophy.

In addition to enhancing the ability of endothelial cells to undergo angiogenesis, over-expression of low levels of SK also conferred upon the endothelium a pro-inflammatory phenotype, and sensitized the cells to very low concentrations of TNFα. These differences translated into dramatic increases in neutrophil adhesion, suggesting that SK is an important regulator of the inflammatory potential of endothelial cells.

This work provides substantial evidence that intracellular levels of SK regulate inflammation and angiogenesis. Indeed the significance of the *in vitro* results will best be assessed by

studying tissues, in both health and disease. These studies may be especially productive in sites of proliferative inflammation such as synovium in rheumatoid arthritis. To this end, it is intended to develop polyclonal antibodies against SK1. The immunogen to be used is 100μg of recombinant SK1, histidine tagged, generated by Factor X cleavage of a Nus-A fusion protein, generated in E Coli. The ability of the antiserum generated against SK to bind to SK protein will be analysed by enzyme-linked immunosorbent assay, and then Western blot analysis will be used to determine whether the antisera can detect the native / denatured protein.

Polyclonal antibodies provide a useful tool in clinical research. They generally have higher affinity than monoclonal antibodies, and bind multiple antigenic epitopes. Antiserum being a crude preparation, contains antibodies of multiple specificities, and thus assays such as immunofluorescence may lead to significant non-specific antibody binding. However this may be partially overcome by purification of the antibody. For example, ammonium sulphate precipitation, which is suitable for IgG antibodies only, allows the removal of contaminating proteins. Affinity chromatography purification by Protein A-Sepharose (elution of antibodies that bind Staphylococcus aureus Protein A) or specific-ligand affinity purification (elution of antibodies which bind the specific ligand only) are two further means of purification of polyclonal antibody.

It is intended to generate a polyclonal antibody against SK in chickens as well as the more traditional host of the rabbit, as with the dual system (using both chicken and rabbit antibodies) immunoprecipitation may be undertaken with one antibody, and Western blotting with the other, thus obliterating the concern of obliterating Ig bands. In addition, IgY (the avian homologue of IgG) has several advantages over mammalian antibodies (281). Firstly, as chickens are phylogenetically distant from mammals, good antibody responses can be

obtained against highly conserved mammalian proteins. Secondly, chicken antibodies show reduced cross reactivity with IgG antibodies, and fewer false positive results in mammalian sera, as IgY is not bound by rheumatoid factors. Thirdly, smaller quantities of antigen (usually 20-30 μg) are required to elicit an immune response in chickens. Fourthly, large quantities of IgY can be purified from a single egg yolk (50-150 mg), making the chicken a very efficient host. Furthermore, the purification of antibodies is inexpensive, and egg collection is non-invasive, in contrast to bleeding animals(281;282).

As SK1 and SK2 have a high degree of structural similarity, yet clearly distinct distribution, temporal pattern of expression, and function (Chapter 1.6.2) the ability of antibodies directed to SK1 to specifically bind SK1 and not SK2 will be important.

These antibodies are intended for future use in the biochemical analysis of endogenous SK and, to define the subcellular localization of SK. The main interest for using these antibodies will be in immunohistochemical surveys of normal and diseased tissue and the specific questions to be addressed relate to the levels of SK in inflamed versus normal synovium. Collection of synovium from patients with active RA, inactive disease, osteoarthritis, as well as control normal synovium is intended for this purpose. These antibodies will be used in conjunction with the antibody directed against active (phosphorylated) SK (anti-phospho-SK peptide antibody), generated in our Division (Dr. Stuart Pitson), to determine whether the level, or activation of SK is enhanced in inflamed synovium from patients with rheumatoid arthritis compared with normal control.

In addition to studies of tissue levels of SK in health and disease, the functional significance of the results presented may be further confirmed by the study of transgenic mice over-

expressing SK, specifically to determine whether they develop arthritis de novo, or are more sensitive to collagen induced-arthritis.

If the results of animal models are in keeping with the results presented in this thesis, of enhanced angiogenesis and inflammatory potential due to increased SK activity, then this research should prompt the development of inhibitors of SK. Potentially, the therapeutic application of inhibitors of SK could be extensive and encompass the numerous diseases of aberrant angiogenesis and inflammatory aetiology of which rheumatoid arthritis is but one example.

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Amendments

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Page 38-40 (2.3.2, 2.3.3, 2.3.4, 2.3.5)
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Typing amendments

Abstract Line 7 "subliminal" should be changed to "suboptimal".
Abstract Line 20 "Thus" should be changed to "This".
Page 7 Line 12 "produced" is correctly spelled and not typed as "produce" as stated by the examiner.
Page 9, Line 3: (23),(1,24)(1) should read (1:23:24).
Page 9 Line 22: (37)) should read (37).
Page 86, paragraph 3 line 10 (183),(184),(185) should read (183-185).
Page 98 Line 20 should read "It however does **not** correlate"
Page 99 Line 2 should read "It was the reduced number of apoptotic nuclei stained by DAPI rather than reduced staining of DNA that provided evidence for resistance to apoptosis."
Page 137, paragraph 3, Linc 10 (0.79-0.84)) should read (0.79-0.84).
Page 150 Line 22 "fro" should read "from".
Page 163 Line 21 "fro" should read "for".
Fig 5.2 legend Line 3,4 "ECGs" should be changed to growth factor, (GF).

Additional comments

Page 6 Line 23: The examiner has stated that bFGF and VEGF do not signal by binding integrins. It has not however been stated that these growth factors bind integrins, but rather that they signal through specific integrins.

As is standard practice for publication, for Western blots, only the "relevant" band has been depicted, rather than the entire gel. It is to be noted that the molecular weight of all proteins (e.g. Bad 23kDa, Bax 22kDa, Bcl-2 26kDa, Bim 23kDa, Akt 60kDa, PECAM-1 130 kDa) was ascertained by comparison with the relative migration of pre-stained molecular weight standards.

Statistical analysis for Fig 6.6 although only representative of two separate data sets, was performed by analysis of the normalized values, with estimated 95% confidence intervals, rather than standard error of the mean.