Studies on the Endothelial Cell Specific Receptor Tyrosine Kinase, Tie-1

by

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A thesis submitted in candidature for the degree of Philosphiae Doctor

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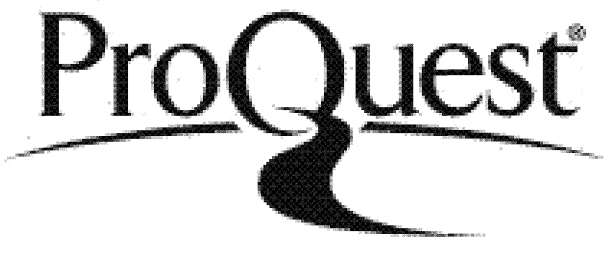

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Abstract

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Tie-1 is an endothelial cell specific tyrosine kinase receptor that is essential for the stabilisation of newly developed vessels and maintenance of endothelial cell integrity in the latter stages of angiogenesis. There is presently no known ligand for the tie-1 receptor, little is known of the factors that regulate its expression and information is lacking on downstream signalling events that occur following its activation.

This study demonstrates that tie-1 protein expression in endothelial cells is increased at the level of transcription by VEGF and low oxygen tension, factors which are known to initiate angiogenesis. The cell surface expression of the receptor is also regulated by a metalloprotease enzyme which results in tie-1 ectodomain cleavage at a region close to the transmembrane region. This event generates a tie-1 endodomain fragment that persists in the cell membrane for several hours. This results in downstream signalling events, which lead to further secretion of the metalloprotease enzyme that can cleave tie-1 ectodomain in surrounding endothelial cells. The cleavage event may well be protein kinase C dependent and can be activated by VEGF. The loss of the extracellular domain of tie-1 inhibits tie-1 ligand binding at the cell surface and probably results in destabilising the endothelial cell allowing it to undergo angiogenesis or vessel remodelling.

A potential tie-1 ligand, produced by a malignant melanoma cell line, results in activation of the tie-1 receptor by autophosphorylation of tyrosine kinase residues. Attempts at isolation of this protein suggest that it is a glycoprotein with a molecular weight of approximately 60-90kDa and this protein has been visualised on a nitrocellulose membrane and is currently awaiting N-terminal sequencing.

Work presented in this thesis demonstrates for the first time factors that regulate expression of tie-1, novel downstream signalling events and the possible isolation of the tie-1 ligand.

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Dedication

I dedicate this work to my daughter Madelyne Grace whose early appearance in this world and her ability to survive was a source of inspiration to me.

Finally, I would like to dedicate this work to my wife Lucy, whose constant support and encouragement over the years has never faltered and for 'appearing to be' genuinely interested when I demonstrated to her interesting blots.

Abbreviations

BAEC's	Bovine aortic endothelial cells
BSA	Bovine serum albumin
CHO cells	Chinese hamster ovary cells
cDNA	Complementary DNA
DEPC	Diethyl pyrocarbonate
DMEM	Dubecclos modified eagles medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra acetic acid
EGF	Epidermal growth factor
ED	Extracellular domain
FCS	Foetal calf serum
FGF	Fibroblast growth factor
HRP	Horse radish peroxidase
HMEC	Human microvessel endothelial cells
HUVE	Human umbilical vein endothelial cells
IL-1	Interleukin 1
IL-6	Interleukin 6
Ig	Immunoglobulin
ID	Intracellular domain
mRNA	Messenger RNA
PBS	Phosphate buffered saline
PAGE	Polyacrylamide gel electrophoresis
PDGF	Platelet derived growth factor
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
RT	Reverse transcription
RNA	Ribonucleic acid
SB	Sample buffer
SMC	Smooth muscle cell
SDS	Sodium dodecyl sulphate
TGF	Transforming growth factor
TAE	Tris-acetic-EDTA
TBS	Tris buffered saline
Tie	Tyrosine kinase with Ig and EGF homology domains
Tie-1 ED	Tie-1 extracellular domain
Tie-1 ID	Tie-1 intracellular domain
Trk-A	Nerve Growth Factor receptor
VEGF	Vascular endothelial growth factor
WB	Western blotting
WGA	Wheat germ agglutinin

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Chapter One

Introduction

1.1 Introduction

The development of a vascular network allows multicellular organisms to grow above a certain critical cell mass, by allowing diffusion of oxygen and nutrients to the cell with diffusion of waste products away from the cell. At the third week of development , the human embryo can no longer nourish itself adequately by the process of simple diffusion of food materials across the extraembryonic coelom and at this stage develops a primitive vascular network. The development of such a vascular network is termed vasculogenesis. This process involves the development of blood islands and a vascular plexus from the mesoderm by the differentiation of angioblasts (primitive vascular endothelial cells), this plexus then lead to the generation of primitive blood vessels and right and left endocardial tubes. The development of subsequent blood vessels by the proliferation and migration of pre-existing endothelial cells is a process known as angiogenesis. Angiogenesis occurs throughout the growth of the foetus especially during organogenesis and ultimately leads to the development of mature blood vessels by a process of remodelling and maturation. The mechanisms regulating vascular development have, until recently, been poorly understood. However, since Folkman first hypothesised that tumour blood vessel growth required a combination of angiogenic growth factors and activation of their receptors, the understanding of the mechanisms regulating and controlling angiogenesis have increased dramatically (1).

Angiogenesis is not only essential for placental and embryonal vascular development but also plays an integral role in wound healing and the female reproductive cycle (2,3). The switching on of angiogenesis also plays a role in certain disease process's such as the progression and metastases of tumours, diabetic retinopathy, psoriasis, rheumatoid arthritis and haemangiomas (4). The process of angiogenesis can be divided into 4 stages: i) an initiation phase, characterised by increased permeability ; ii) progression, constituted by the production of proteolytic enzymes that degrade the extracellular matrix and promote endothelial cell migration and the entry of cells into the active part of the cell-cycle ; iii) differentiation into new vessels and iv) the stabilisation and maturation of vessels with recruitment of mesenchymal cells to the vessel wall. Key signals regulating endothelial cell

growth and differentiation are mediated by polypeptide growth factors and their transmembrane receptors, many of which are tyrosine kinase receptors. The process of angiogenesis is a complex cascade of activating and inhibitory cellular signals that eventually lead to the development of a mature blood vessel. Knowledge of some of the growth factors that initiate angiogenesis has allowed certain anti-angiogenic agents to be tested on tumours in order to prevent tumour progression (4, 5). Furthermore, angiogenic growth factors are currently being used to promote blood vessel growth in ischaemic tissues such as the myocardium (6) and critical ischaemic lower limb (7). Further knowledge of the factors regulating angiogenesis will enhance the understanding of this complex process and may provide additional therapeutic targets for pathological states in which angiogenesis plays or could play a critical role.

1.1.1 Angiogenesis

Mature arterial blood vessels are composed of an inner intimal, medial and outer adventitial layers. The intimal layer is made up of a single layer of endothelial cells that line the luminal surface of the vessel. These cells play an important role in tissue homeostasis, fibrinolysis, coagulation and vasomotor regulation of the vessel (8). The development of the mature vessel stems from the proliferation and differentiation of the endothelial cell. In the embryo the endothelial cell is formed from progenitor cells in the mesoderm, these primitive cells are termed angioblasts. It appears that angioblasts originate from haematopoetic cells. Evidence for this is suggested by vascular endothelial growth factor receptor-2 (VEGF-R2 also known as Flk-1 and KDR in mice and humans) gene knockout experiments (9). The expression of VEGF-R2 and its ligand vascular endothelial growth factor (VEGF) are increased in situations of vascularisation (10, 11). Expression of VEGF-R2 is predominantly found on endothelial cells (11). VEGF-R2 expression can be detected in the mesodermal yolk-sac blood island as early as 7 days post-coitum, however mouse embryos that have homozygous deficient VEGF-R2 genes all die at day 8.5 and 9.5 post-coitum. Examination of these homozygous mutants revealed failure of the development of primitive blood vessels and the number of haematopoietic cells was severely reduced, consistent with the idea that the two cell lineage's develop from the same precursor, the haemangioblast (9). However, the

regulating factors which allow a progenitor cell to differentiate into either an angioblast or a primitive haematopoietic cell are currently unknown.

In the yolk-sac of the embryo, the angioblasts and haematopoietic precursor cells develop blood-islands, the development of these primitive vessels is termed vasculogenesis. A primary vascular network is developed by fusion of the blood islands and the ability of the angioblasts to form lumina (12). New capillaries are then developed by a process of sprouting angiogenesis or non-sprouting intussusception {Figure 1.1}.

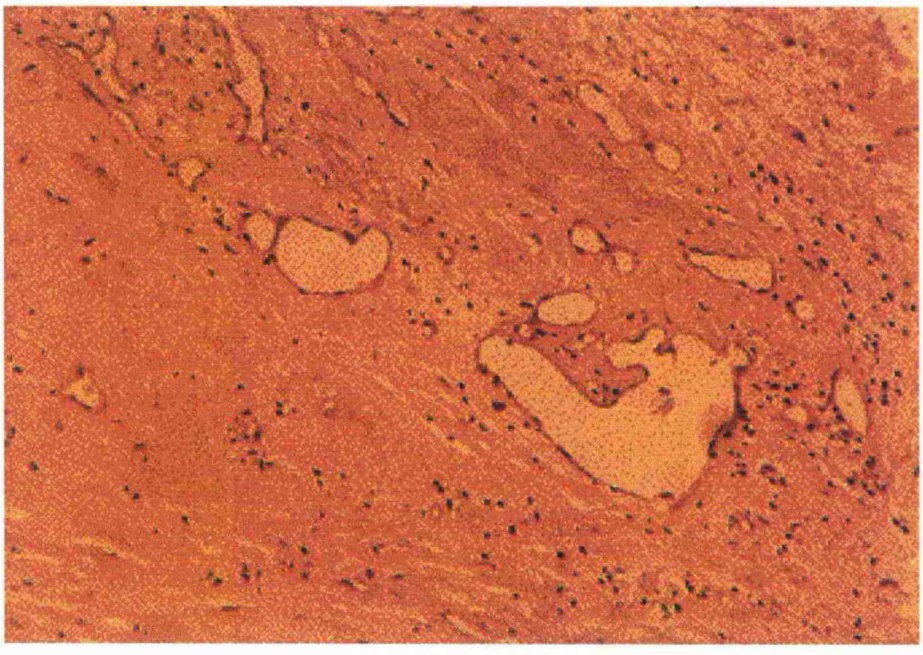


Figure 1.1 Angiogenesis occurring in an atherosclerotic plaque. Carotid endarterectomy sample that has been fixed, and endothelial cells identified with a CD31 specific monoclonal antibody. New vessels can be seen to be developing by possibly sprouting and intussusception.

Sprouting angiogenesis involves the activation of endothelial cells by angiogenic stimuli leading to breakdown of the basement membrane and extracellular matrix followed by the migration of the endothelial cell towards the source of the stimulus and their proliferation. As a result of this a cord of endothelial cells is formed which ultimately remodels into a new

capillary. In addition to elaboration of a branched vascular network by sprouting angiogenesis, vessels may branch by non-sprouting means. Non-sprouting angiogenesis occurs with the splitting of pre-existing vessels by transcapillary pillars due to ingrowing columns of connective tissue (13). Eventually this occludes the pre-existing capillary and divides the vessel into two separate vessels. It has been suggested that both sprouting and non-sprouting occur in parallel in the vascularisation of tissues and organs such as the heart, lung and yolk-sac (14). However, sprouting angiogenesis appears to predominates in the brain whereas non-sprouting predominates in the lungs where this is rich preponderance of angioblasts (14).

A period of vessel maturation then follows which involves the recruitment of pericytes and smooth muscle cells. These additional cells surround the newly formed tube and help to provide stability both physically and through signalling pathways stimulated by cell: cell interaction (15). The initiation of the remodelling process seems to involve a complex interplay of both mechanical forces and growth factor activation/inhibition. Remodelling includes the regression of non-perfused vessels and adjustment of vascular density depending on the tissue oxygen tension. Several growth factors have been shown to be required for the maturation process to occur. These include transforming growth factor (TGF)-β (15), platelet derived growth factor (PDGF) (16), tissue factor (17, 18), and angiopoietin-1 (19). Understanding of the regulatory factors that control angiogenesis have largely been generated by gene knockout experiments in mice. These will be further discussed in the following sections.

1.2 Clinical applications for the inhibition and activation of angiogenesis

The fundamental importance of regulatory polypeptide growth factors in vasculogenesis and angiogenesis was first suggested by Folkman (1). Since then several growth factors have been shown to play a regulatory role in angiogenesis. Furthermore, the importance of angiogenesis in the progression and metastases of malignant tumours has been the focus of

much research (5) and has led to the search for anti-angiogenic agents that could be used to prevent tumour progression.

Conversely, there are clinical situations in which it would be desirable to activate new blood vessel formation. During tissue ischaemia which results from atherosclerotic occlusive disease, the initiation of angiogenesis may allow the development of new blood vessels which would increase the amount of perfusion of blood to ischaemic tissues. Ischaemia commonly occur in the heart, which can result in myocardial infarction and death, and in peripheral vascular disease, where critical ischaemia can lead to gangrene and limb loss. Inducing neovascularisation in situations of local tissue hypovascularity has been termed "Therapeutic Angiogenesis" (20).

1.2.1 Inhibition of angiogenesis

The realisation that most malignant tumours need to develop a blood supply in order to grow in size and metastasise has led to a search for inhibitors of angiogenesis that can be used to halt these events, inhibit tumour growth and metastasis, and hence prolong patient survival (4). Tumours need to "switch" to an angiogenic phenotype that allows the production and secretion of growth factors that will lead to neovascularisation of the tumour (5). The ability of tumours to develop a blood supply is regulated by activating and inhibitory growth factors which are normally in a state of equilibrium. Alterations in the level of growth factors will lead to blood vessel growth or regression. Overexpression of growth factors and their corresponding receptors is a predictor of tumour progression and metastases and poor clinical outcome (5, 21, 22, 23, 24, 25). In some tumours it has been noted that neovascularisation is inhibited by certain growth inhibitors. One such factor is angiostatin which was first discovered in mouse tumours (Lewis lung carcinoma) (26). In these tumours angiostatin was produced by the primary tumour and inhibited the neovascularisation of distant metastases. However, removal of the primary led to marked neovascularisation of these metastases, but why angiostatin did not inhibit angiogenesis from occurring in the primary tumours is not known. It has been shown subsequently that

angiostatin could inhibit growth of both human and mouse tumours transplanted under the skin of mice with minimal toxicity (27).

Identification of angiogenic growth factors, their corresponding receptors and signalling pathways has provided many potential targets for the inhibition of angiogenesis (28). Potential points at which angiogenesis can be inhibited include, interference with the receptor binding and activation by the growth factor; inhibition of the release of the angiogenic factor by tumour cells; enhancement of the production of an inhibitor of angiogenesis; the use of angiogenic inhibitor agonists; interference with the signal transduction process of the angiogenic factor; and prevention of matrix degradation by protease or matrix degrading enzymes (29). Several inhibitors of angiogenesis have been identified (angiostatin (26), TNP-470 (30), platelet factor 4 (31), captopril (32)). They appear to function by preventing endothelial cell proliferation but the specific mechanisms for this process are unknown. Tumour growth and angiogenesis has been inhibited by more specific methods that involve neutralising antibodies to bFGF, using soluble vascular endothelial growth factor receptor to inhibit the effect of VEGF (33) and soluble tie-2 receptor to inhibit the effect of angiopoietin-1 (34). Folkman has postulated that angiogenic inhibitors not only lead to cessation of tumour angiogenesis but also aid the delivery of traditional chemotherapeutic agents to the tumour (4) and that angiogenesis inhibitors can be used as adjuvant therapy and also as long maintenance therapy in tumour treatment. Inhibitors of angiogenesis may also have a role in the treatment of non-malignant disease that involve proliferation of blood vessels by angiogenesis, these include ocular neovascularisation, infantile haemangiomas, adult sarcomas, psoriasis, arthritis and dysfunctional conditions of the female reproductive tract (4).

1.2.2 Therapeutic angiogenesis

The ultimate aim of therapeutic angiogenesis is to enhance collateral artery growth such that it improves perfusion in ischaemic tissues. Initial studies into augmenting collateral arterial growth used fibroblastic growth factor (FGF). Both acidic and basic FGF have been injected intramuscularly (IM) into the ischaemic hind limb of New Zealand white rabbits and serial

angiograms demonstrated statistically elevated collateral blood vessel formation in ischaemic limbs treated with the growth factors compared to controls (35, 36). Furthermore, necroscopy studies of ischaemic muscle demonstrated increased capillary densities of treated muscle compared to controls and more importantly a haemodynamic improvement in blood flow. bFGF has also been shown to enhance collateral blood vessel development and flow in the ischaemic myocardium of animal models (37, 38). However, FGF is not only mitogenic for endothelial cells but also for smooth muscle cells and this may ultimately lead to the development of intimal hyperplasia in the newly developed vessel (39). This prompted the investigation of $VEGF_{165}$ as a potential agent to augment neovascularisation. The main advantages of VEGF over FGF is that its action is endothelial cell specific. Furthermore, FGF lacks a secretory leader sequence and, therefore, is unable to be secreted from the cell, unlike VEGF (40). This allows a constant level of VEGF production following VEGF cDNA gene transfer over a longer period of time rather than giving intermittent protein injections. Augmented collateral vessel growth and angiogenesis has been achieved in the ischaemic hind limb of the rabbit with recombinant $VEGF_{165}$ when administered intra-arterially (41), intravenously (42,43) and intramuscularly (44). $VEGF_{165}$ has been shown to enhance endothelial cell proliferation which leads to collateral blood vessel growth (45). Furthermore, new collateral vessels, initiated to grow by VEGF, have increased endothelium-dependent flow and retain their vasomotor tone when tested with the vasoconstrictor, parpaverine (46). VEGF has been shown to increase the total number of collateral blood vessels visible on angiography, increase the capillary density of ischaemic muscles at necroscopy, and furthermore lead to haemodynamic improvements in blood flow. This was measured using simple improvements in the ankle-brachial pressure ratio and by using an intra-arterial Doppler wire (47), which showed increase in phasic blood flow velocity in the ischaemic limb vessels.

$VEGF_{165}$ is a secreted protein, therefore, VEGF gene transfer would allow production and secretion of its product into the local circulation and negate the need to inject foreign protein with all its incumbent risks. Transfection efficiencies *in vivo* are notoriously low (48), however, gene transfection of cDNA that encodes for a secreted protein can result in normal

physiological levels of the protein despite an almost undetectable level of transfection (49). Isner's group have described successful adenoviral gene transfer into the arterial smooth muscle cells when the plasmid DNA is applied to a hydrogel polymer coated angioplasty catheter (50). Henceforth they applied naked plasmid $VEGF_{165}$ onto a hydrogel coated catheter and dilated the angioplasty balloon at the proximal site of occlusion and were able to demonstrate enhanced collateral blood vessel development and angiogenesis in the ischaemic hind limb of rabbits maintaining localised VEGF production for up to 30 days (39). Subsequently, this technique was used on the first human case, a patient who presented with endstage critical lower limb ischaemia with constant ischaemic rest pain and a very poor exercise tolerance (51). Digital subtraction angiography performed 4 weeks following gene transfer demonstrated a marked increase in collateral vessels in the ischaemic limb with an apparent improvement in lower limb haemodynamics as measured by using intra-arterial Doppler-flow. However, although VEGF enhanced angiogenesis, the limb produced several spider angiomas and quite marked peripheral oedema. These problems highlight some of the complications that can be encountered when using VEGF therapeutically. Even though the spider angiomas regressed, it is a particular concern that administration of VEGF may lead to the progression of previously unrecognised tumours and the development of vascular malformations. Stringency in patient selection is vital and each patient has to be carefully screened prior to gene therapy for the presence of tumours or vascular malformations (52). Despite the apparent success of the balloon catheter technique the same group have injected naked plasmid $VEGF_{165}$ intra-muscularly into ischaemic muscles of rabbits and demonstrated significantly enhanced collateral vessel formation and angiogenesis (53, 54). This technique is obviously less invasive and cheaper than having to introduce an angioplasty catheter percutaneously. This has led to a phase I clinical trial injecting naked plasmid $VEGF_{165}$ into patients presenting with critical lower limb ischaemia. Early results of this non-randomised study were reported in 1997 (7).They reported 10 limbs that had undergone intramuscular gene transfer in 9 patients with critical lower limb ischaemia. Newly visible collateral blood vessels were demonstrated in 7 out of 10 limbs by angiography and ankle-brachial pressure index increased significantly in these patients. Improved tissue perfusion allowed healing of 4 out of 7 ischaemic ulcers and within this group limb salvage was achieved in 3 patients

who were scheduled for below knee amputations. In these patients circulating VEGF was assayed using ELISA and was found to be still detectable up to three weeks after gene transfer in some patients. As found with the first case report, peripheral oedema was a common complication that occurred in six patients. This complication is directly related to VEGF's ability to lead to hyperpermeable blood vessels (7, 39). No long term data is available yet on these patients who have undergone VEGF gene transfer, however gene therapy definitely appears to have a role for patients with critical lower limb ischaemia. This treatment may not be an option for diabetic patients with peripheral vascular disease as it may lead to the development of proliferative diabetic retinopathy.

Patients with critical lower limb ischaemia or ischaemic heart disease are currently managed by a combination of bypass surgery and percutaneous transluminal angioplasty (PTA) ± stenting. One of the major complications of these techniques is re-stenosis which is largely due to intimal hyperplasia (55). This leads to a significant stenosis in approximately 30 % of cases (56). It is thought that the pathophysiology of intimal hyperplasia is a direct result of de-endothelialised vein or artery due to trauma incurred on the vessel. The inhibitory effect of the endothelial cell on smooth muscle cells is removed and hence they proliferate and migrate towards the intima of the vessel resulting in luminal narrowing and, hence, vein graft stenosis. Enhancement of re-endothelialisation may prevent migration of smooth muscle cells and, hence, intimal hyperplasia. Infusion of VEGF protein and VEGF gene transfer has been shown to result in rapid re-endothelialisation after injury to a vessel with resurrection of endothelial-dependent function (57, 58, 59). Using VEGF to enhance re-endothelialisation may have a therapeutic role to play in the future with regard to the prevention of stenosis and establishing the efficacy of VEGF coated stents, prosthetic arterial grafts and angioplasty balloon catheters is currently under investigation.

Further investigation into the regulatory factors and receptors involved in blood vessel development will not only enhance the understanding of this complex process and its molecular interactions, but also allow adaptation of this knowledge into therapeutic targets/agents that could be used to activate or inhibit angiogenesis in human disease.

1.3 Endothelial cell specific receptor tyrosine kinases and their respective ligands

Many of the signals regulating angiogenesis are mediated by polypeptide growth factors and their transmembrane receptors, a variety of which are receptor tyrosine kinases. These receptors all contain a large glycosylated extracellular ligand binding domain, a single hydrophobic transmembrane region and an intracellular/cytoplasmic domain which contains a tyrosine kinase catalytic domain. Receptor tyrosine kinases have been classified according to their extracellular structural characteristics (60, 61). The complexity of the extracellular structure varies considerably between receptors and ranges from relatively simple epidermal growth factor receptor (EGFR) to the complex tie-1 and tie-2 receptors. Some growth factors which are known to stimulate angiogenesis and their respective receptor tyrosine kinases are shown in Figure 1.2 and Table 1. Both VEGFR-1, VEGFR-2, VEGFR-3 and tie-1 and tie-2 are largely endothelial cell specific in the adult, whereas the other receptors are expressed in other cell types (62, 63, 64). For all tyrosine kinase receptors the binding of a ligand to the extracellular domain leads to activation of the tyrosine kinase in the cytoplasmic domain. This leads to downstream activation of a number of signalling molecules. Proteins that are frequently activated include phospholipase C-γ, phosphatidylinositol 3-kinase (PI3-kinase), GTPase-activating protein, pp60-src, p21 ras, Raf-1 kinase and MAP kinases (ERK 1 and ERK 2) (61).

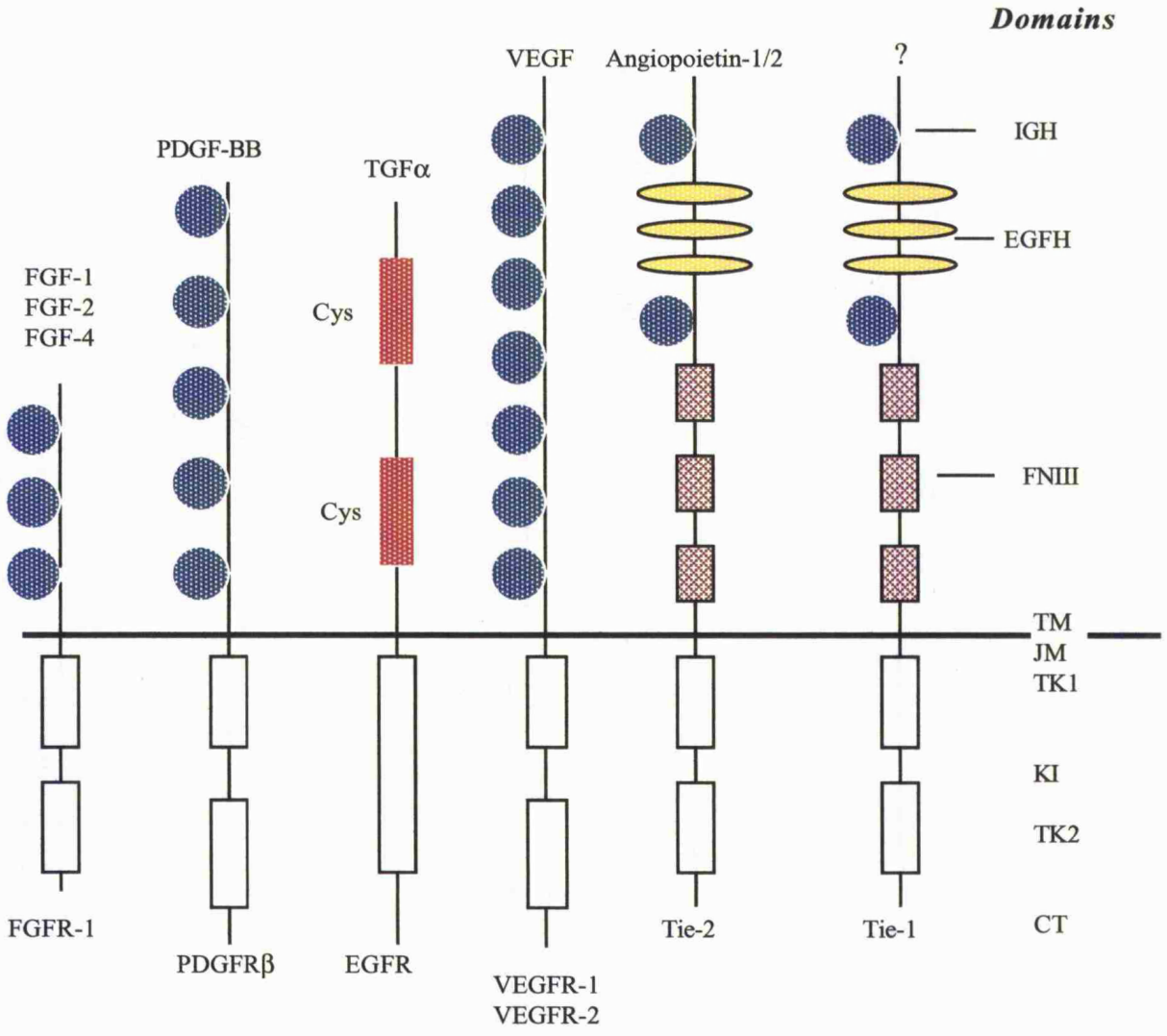


Figure 1.2 Major endothelial cell tyrosine kinase receptors and their respective ligands that are involved in vasculogenesis and angiogenesis. The receptors are fibroblast growth factor receptor-1 (FGR-1), platelet derived growth factor (PDGF)-β, epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR) -1/2 and tie-1 and tie-2 (tek). The abbreviations for the growth factors appear in the text. The structures of the receptors are shown schematically and the protein domains highlighted (IGH=immunoglobulin homology domain, EGFH= epidermal growth factor homology domain, FNIII=fibronectin type III homology domains, TM= transmembrane domain, JM= juxtamembrane domain, TK1 and TK2= tyrosine kinase catalytic domains, KI= kinase insert, CT= carboxyl terminal tail, Cys= cysteine rich domains.

Growth Factors	Growth Factor Receptors
VEGF A= VEGF $_{121, 165, 189, 206}$	VEGFR1 (Flt1) + VEGFR2 (KDR)
VEGF B	?
VEGF C	VEGFR2 + VEGFR3 (Flt4)
VEGF D	VEGFR2 + VEGFR3
Placental growth factor	VEGFR1
Angiopoietin-1 and -2	Tie-2
?	Tie-1
Fibroblast growth factor	Fibroblast growth factor receptor
Transforming growth factor	Epidermal growth factor receptor

Table 1 Known angiogenic growth factors and their tyrosine kinase receptors

1.3.1 Vascular endothelial growth factor (VEGF) and its receptors VEGFR-1, VEGFR-2 and VEGFR-3

1.3.1.1 VEGF

Vascular endothelial growth factor (VEGF-A) has been shown to be a major initiator of neovascularisation during embryonal development (65, 66), tumour angiogenesis (67) and ocular disorders (68). VEGF was first noted for its ability to produce hyperpermeability in tumour blood vessels and, therefore, was first described as vascular permeability factor (VPF) (69). Subsequently, it has been shown to be mitogenic for endothelial cells both *in vitro* (70, 71, 72) and *in vivo* (73, 59). Furthermore, VEGF enhances endothelial cell migration through the $\alpha_v\beta_5$ integrin (74) and increases endothelial cell expression of $\alpha_v\beta_3$ (75) which is essential for FGF induced angiogenesis (76). VEGF is also a chemoattractant for monocytes through its receptor VEGFR-1 (77). VEGF not only appears to lead to the generation of new blood vessels but is also essential for the normal maintenance and repair of the endothelium (78). This effect appears to be due to the stimulation of nitric-oxide (NO) release (79, 80).

There are four isoforms of VEGF: VEGF-A (81) , VEGF-B (82), VEGF-C (83) and VEGF-D (84). Placental growth factor (PlGF) is structurally related to VEGF (85) and binds to VEGFR-1(86) but at present its biological role remains unknown. VEGF-A, B and C genes are located on different chromosomes (81, 87). VEGF-A is a secreted 45 kDa glycoprotein which is coded for on human chromosome 6p21.3 (81). Due to alternative exon splicing there are four isomers for VEGF which correspond to 121, 165, 189 and 206 amino acids in size. $VEGF_{121}$ is a highly soluble acidic polypeptide that does not bind to heparin-sulphate whilst $VEGF_{185}$ and $VEGF_{206}$ are more basic compared to $VEGF_{165}$ and readily bind to heparin-sulphate and, therefore, are not freely secreted and are bound to the cell surface extracellular matrix. $VEGF_{165}$ is the predominant isoform secreted by a variety cells (endothelial cells, smooth muscle cells and pericytes) (64). VEGF-A is the natural ligand for and has a high affinity for VEGFR-1(Flt-1) and VEGFR-2(KDR) but not VEGFR-3(Flt-4) (10, 88, 89)

VEGF-B is a known endothelial cell mitogen (82). VEGF-C binds to VEGFR-2 and VEGFR-3 and has been shown to regulate endothelial migration via its activation of VEGFR-3 (Flt4) which is found on the endothelial cells of lymphatic vessels (83). VEGF-D is also an endothelial cell mitogen and is a ligand for VEGFR-2 and VEGFR-3 (90).

1.3.1.2 VEGF Receptors (VEGFR-1, VEGFR-2 and VEGFR-3)

Two receptor tyrosine kinases bind VEGF-A with high affinity, these are VEGFR-1(Flt-1) (88) and VEGFR-2 (FLK in mice and KDR in humans) (10, 11, 91). Furthermore, expression of these receptors is almost totally restricted to the vascular endothelium (64) . Both receptors contain seven immunoglobulin like regions within their extracellular domains. VEGF binding to the receptors results in activation of the tyrosine kinase catalytic domains followed by autophosphorylation of tyrosine residues, however VEGF binds to VEGFR-1 more readily than VEGFR-2 (89). VEGFR-3 (Flt-4) is closely related in structure to VEGFR-1 and 2 (92) and is found predominantly on the endothelial cells of lymphatics and venules (93). VEGF-A does not appear to bind or stimulate autophosphorylation of

VEGFR3 (94). Phosphorylation of tyrosine residues following receptor activation allow binding of downstream signalling molecules, which for VEGFR-1 and VEGFR-2 appear to be different (89). Stimulation of endothelial cells with VEGF-A leads to phosphorylation of several proteins that contain SH2 domains; phospholipase- C ; Ras GTPase activating protein (GAP) ; phosphatidylinositol 3-kinase (PI3 kinase) ; and the oncogenic adaptor Nck (95). It appears that Ras GTPase plays a significant role in the signalling events involved with vasculogenesis and angiogenesis. Homozygous mutants of mice lacking the Ras GTPase-activating protein exhibit major defects in endothelial cell organisation and fail to develop a primitive vascular network by day 9.5 post-coitum and the vessels appeared very weak exhibiting local rupture and leakage of blood (96). The failure of development of a primitive vascular network are similar to those seen in VEGFR-1 (97), tie-2 and tie-1 (98) deficient mice generated by disrupting the respective genes by homologous recombination of embryonic stem cells. Furthermore, it has been suggested that the RasGTPase activating proteins are substrates for both VEGFR-1 and VEGFR-2 and may play a crucial role in the downstream signalling events (96).

1.3.1.3 Mouse gene knockouts of VEGF-A and VEGFR-1, VEGFR-2

Evidence for the different functional roles of VEGFR-1 and VEGFR-2 are apparent when either gene coding for the receptors is disrupted (9, 97). Both receptors are essential for the development of the normal embryonal vasculature and mouse embryos with homozygous targeted mutations for either VEGFR-1 or VEGFR-2 die at day 8.5-9.5 post-coitum. However, whilst VEGFR-1 deficient embryos developed endothelial cells, VEGFR-2 deficient embryos did not. Furthermore VEGFR-1 deficient embryos failed to develop normal vascular channels, whereas VEGFR-2 deficient embryos lacked any form of vasculogenesis and failed to develop blood islands (9, 97). Targeted mutations of the VEGF-A gene have demonstrated that VEGF is essential for embryonal angiogenesis and vasculogenesis (65, 66). Interestingly both heterozygous and homozygous embryos died at day 11-12 post-coitum with a lack of blood island and normal blood vessel formation showing that even low levels of VEGF are inadequate to support normal embryonal development. These knockout experiments show the importance of VEGF-A and its

receptors (VEGFR-1 and -2) in early vascular development, and that they play an initiating role, in angiogenesis and vasculogenesis.

1.3.1.4 Regulation of VEGF expression

It appears that expression of all four isoforms of VEGF-A are regulated by different means. Expression of VEGF-A is up-regulated by platelet derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor (TGF)- β, hypoxia, and certain oncogenes. However, expression of VEGF-C mRNA is only up-regulated by the growth factors shown above while VEGF-B mRNA does not appear to be up-regulated by any of these factors (99). VEGF-D is expressed in the heart, lung, skeletal muscle, colon and small intestine, however, little is known of the factors that control its expression (90). This suggests that the VEGF family have overlapping but distinct functions and that they are differentially regulated.

VEGF-A is secreted by mesenchymal and stromal cells and its expression is comparable to that of its receptors during embryonal development. In the majority of developing organs the vasculature stains positively for VEGFR-1 and VEGFR-2 whilst the surrounding stromal cells stain positively for VEGF (10, 100). Angiogenesis has been shown to correlate with tumour progression and metastases (4, 5) and VEGF-A is thought to be one of the major regulators of this event. VEGF expression has been shown to be markedly up-regulated in many human tumours including lung, thyroid, breast, bladder, stomach, melanoma and cervix (4, 64). Furthermore, levels of VEGF expression correlate with vascular density and metastatic potential of some breast tumours (101). VEGF-positive breast tumours correlate with a poor prognosis for patients compared to patients with tumours that were VEGF-negative (102). In addition, inhibition of the action of VEGF, by a VEGF specific monoclonal antibody, inhibited angiogenesis and tumour growth in human glioblastoma multiforme and leimyosarcoma cell lines that had been injected into mice (103). Inhibition of VEGFR-2 signalling also prevented tumour invasion and allowed reversion of a malignant tumour into a benign one (21). Tumour growth has also been suppressed by dominant-

negative inhibition of VEGFR-2 (104) and this further demonstrates the important role that VEGF and its receptors have on angiogenesis and tumour growth.

Regulation of VEGF gene expression has been studied extensively both *in vitro* and *in vivo*. Several growth factors have been shown to increase the expression of VEGF, these include EGF, TGF-β, IL-1β, IL-1α, PGE_2 (71), PDGF (99), bFGF (105). Several of these growth factors (PDGF, bFGF and EGF) are themselves endothelial cell mitogens and promoters of angiogenesis (5). This illustrates the complex interplay of events that occur during angiogenesis. Importantly, expression of VEGF-A is up-regulated at the transcriptional and post-transcriptional level by low oxygen tension (hypoxia) (103, 106, 107, 108), and this appears to be regulated by hypoxia-inducible factor-(HIF) -1α (109). However, the expression of VEGF-B or VEGF-C does not appear to be up-regulated by hypoxia (99). Interestingly, the expression of both VEGFR-1 and VEGFR-2 are also up-regulated by hypoxia (110, 111). These findings are not suprising as hypoxia is a known stimulus for angiogenesis. Furthermore, increased VEGFR expression has been demonstrated in areas of tissue hypoxia, such as around the necrotic core of tumours (103) and in the ischaemic myocardium (107) and it is in these tissues that angiogenesis and neovascularisation occurs.

1.3.2 Fibroblast Growth Factor family

The fibroblast growth factors (FGF's) are a family of pleiotropic heparin binding molecules containing ten members (29), the most common of which are acidic FGF and basic FGF. The single bFGF gene encodes for multiple isoforms with molecular weights ranging from 24-18 kDa (112) and is expressed in endothelial cells (29) but exert its effects on many different cell types (smooth muscle cells, endothelial cells, fibroblasts). However, all these isoforms exert angiogenic activity in certain *in vivo* models and induce cell proliferation and chemotaxis of endothelial cells *in vitro* (113). The FGF's are specific ligands for the FGF receptors, of which there are four encoded (FGFR1-4) for by four different genes. These are receptor tyrosine kinase receptors whose extracellular domains are comprised of three immunoglobulin like regions (61). FGF binding leads to tyrosine phosphorylation followed

by downstream signalling. Some of the intracellular molecules that become phosphorylated on FGFR activation are Raf-1, ERK-1 and ERK-2, PLC-γ (61, 114)

The precise role that bFGF has on vascular development and angiogenesis is still not well defined. Whilst recombinant FGF's have been shown to have angiogenic effects both *in vitro* and *in vivo* (72, 115, 116, 117), endogenous FGF's have no secretory leader sequence and, therefore, the precise mechanism by which FGF's exert a paracrine response is not known. Mice deficient in bFGF exhibit no overt developmental abnormalities (15) and, therefore, the importance of FGF in vascular development has been questioned. Furthermore, the use of a dominant-negative mutant kinase deficient FGFR developed agenesis/dysgenesis of the kidney, lung, specific cutaneous structures, exocrine and endocrine glands, craniofacial and limb abnormalities, but no vascular abnormalities were described (118). However, it has been shown that by using antisense targeting of bFGF and FGFR-1, intratumoral angiogenesis and tumour growth can be inhibited (119) and bFGF is thought to play a role in the growth and neovascularisation of solid tumours (120). The reason for this paracrine effect is thought to be due to the fact that FGF gets released from tumour cells as they undergo necrosis, allowing it to interact with neighbouring endothelial cells (29). FGF has been shown to be both mitogenic and a chemoattractant for smooth muscle cells *in vivo* (121) and, therefore, it may play a role in maturation of blood vessels during vascular assembly.

1.3.3. Platelet-Derived Growth Factor

Platelet derived growth factor (PDGF) is a potent mitogen for smooth muscle cells, fibroblasts and some endothelial cells (122). PDGF consists of three isoforms each consisting of disulfide linked A chains or B chains in either homodimeric (AA or BB) or heterodimeric (AB) combinations (15). There are two PDGF receptors PDGFαR and PDGFβR (122). These receptors are tyrosine kinase receptors that contain five immunoglobulin like regions in the extracellular domains. There is very similar sequence homology between the two receptors, however there are differences in their binding capacity

for the different PDGF isoforms. The pattern of dimer formation has suggested that the A chain ligand binds to only PDGFαR whereas the B-chain ligand binds to both PDGFαR and PDGFβR (61). Knockout experiments of PDGF and PDGF receptors have given some insight into the role of PDGF in vascular development. Both PDGF-B and PDGFβR null mice reveal similar phenotypes with the development of immature leaky vessels and an absence of mesangial cells in the kidney with aneurysmal endothelial lined sacs in the renal glomerulus (123, 124). Furthermore, due to lack of pericytes capillary aneurysms developed in the brains of PDGFB null mice (16). PDGF-A has also been shown to be important for normal vascular development and it appears to play a role in smooth muscle cell migration towards the immature blood vessel (125). Therefore, it appears that PDGF plays a role in the maturation of blood vessels during vasculogenesis and angiogenesis.

1.3.4 Transforming Growth Factor - β

Transforming growth factor-β's (TGF-β) are comprised of a large family of homodimeric peptides that play an integral role in cell growth and differentiation (126). The receptors for this family of growth factors consist of two subfamilies type I and type II serine/threonine kinase receptors, which are structurally similar, with small cysteine-rich extracellular regions (127). *In vitro* TGF-β inhibits endothelial cell and smooth muscle cell proliferation and migration (128, 129). Approximately 50% of TGF-β homozygous deficient mice that die *in utero* have defective haematopoiesis and vasculogenesis (130). There was defective blood vessel formation in the yolk sac of these mice with failure of contact between endothelial and smooth muscle cells and this led to distended capillaries. Similar findings were seen in TGF-β receptor deficient mice (131). Furthermore the function of TGF-β in vasculogenesis has been suggested to be related to *in vitro* findings which demonstrate that TGF-β is activated by interactions between endothelial cells and smooth muscle cells and in turn this activation inhibits endothelial proliferation and migration and induces the differentiation of

mesenchymal cells to a smooth muscle cell/pericyte lineage (15, 132). This then leads to stabilisation and the maturation of the newly developed vessel.

1.3.5 Tie-1, tie-2 receptors and the angiopoietins

1.3.5.1 Tie-1 and Tie-2 receptor tyrosine kinases

The tie receptor family consist of tie-1 and tie-2 (tek) (133, 134, 135). Tie-1 was first isolated and cloned from a chronic myeloid leukaemia cell line, and its name was derived from its complex extracellular structure (tie= tyrosine kinase with Ig and EGF homology domains) (63), (Figure 1.2). The extracellular domain is unique in that it contains three fibronectin homology domains close to the transmembrane region followed by two immunoglobulin like homology domains interspersed with three epidermal growth factor homology domains. Tie mRNA is 4.4 kb in length and codes for a 135 kDa glycosylated protein expressed predominantly on endothelial cells and in some haematopoietic cell lines (62, 63). Tie-1 expression is increased during neovascularisation and appears to be down regulated in quiescent adult tissues (62, 136). Tie-2 (tek) was first cloned from mice (tek, standing for tunica externa kinase after its site of expression in blood vessels) (137, 138). In humans, tie-2 mRNA, is 4.5kb in length which codes for a 145 kDa glycosylated protein and its expression is up-regulated in embryonal endothelial cells and angioblasts (139).

Tie-1 and tie-2 possess very similar structural characteristics, however, most divergence occurs within the extracellular domains. For the amino acid sequence there is 78 and 84% identity between tie-1 and tie-2 intracellular tyrosine kinase domains with a 40 % identity at the transmembrane region (134). The identity between tie-1 and tie-2 in the extracellular domain is shown in Figure 1.3 and it can be seen that most divergence occurs within the fibronectin homology domains nearest the transmembrane region.

The high divergence within the extracellular domain is probably due to the fact that tie-1 and tie-2 have different ligands and thus different ligand-binding sites. At the present time only

ligands for tie-2 have been identified (Angiopoietin-1 and -2) (140, 141) and these ligands do not bind to the tie-1 receptor.

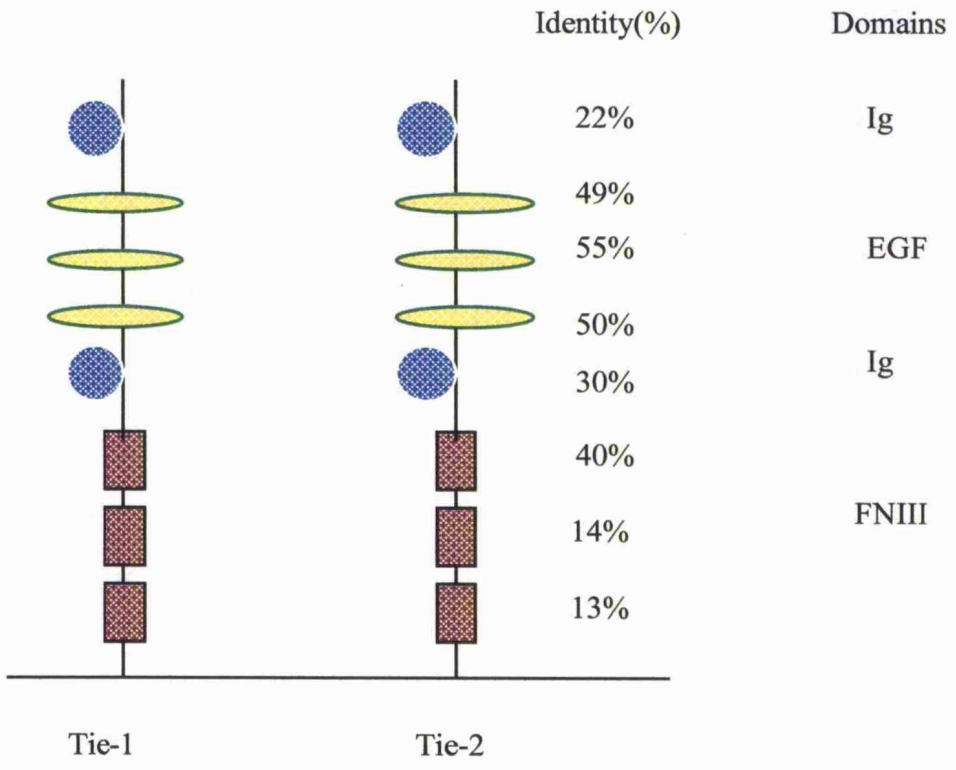


Figure 1.3 Extracellular domain structure of tie-1 and tie-2. The amino acid identities between mouse tie-1 and tie-2 are indicated as percentages.

1.3.5.2 Mouse gene knockouts for tie-1 and tie-2 receptors

Insight into the precise functional roles of tie-1 and tie-2 have been gained by examining the development of mouse embryos that have undergone mutations of the tie-1 and tie-2 genes by homologous recombination of the targeted genes. Embryos deficient in tie-1 develop normally until embryonal day 13 after which they fail to establish structural integrity of endothelial cells, all develop severe oedema and localised haemorrhage of subcutaneous tissues and internal organs leading to death between day 13.5 and birth (98, 142, 143) (normal mouse gestation is approximately 22 days). These defects appear to be due to large

transcellular holes within the endothelial cell (98). Patan showed that tie-1 deficient mice developed excessive vascular branching and density in organs undergoing angiogenesis (lung, brain, skin, kidney, small intestine, atrium of the heart), however, there was no increase in endothelial cell number (144). Patan also demonstrated that the endothelial cells had many holes within them in addition to multiple extensions and filopodia (144). Considered together all the tie-1 deficient mice studies suggest that the function of tie-1 appears to be involved with maintaining endothelial cell integrity, inhibiting endothelial cell motility and inhibiting blood vessel splitting, overall leading to stabilisation of the newly developed vasculature (145, 146, 147).

Embryos deficient in tie-2 all die *in utero* at embryonal day 10.5. These embryos had severe growth retardation and malformation of their vascular network with a lack of capillary sprouting being noted in the neuroectoderm. Thus, tie-2 is important for normal angiogenesis to occur (98). However, these findings are somewhat contradictory to work published by Dumont et al , who showed by dominant-negative and targeted null mutations that tie-2 also played a critical role in vasculogenesis or endothelial cell survival (148). The reason for these latter findings may have been because an incomplete tie-2 promoter was used as a marker for endothelial cells. Failure of this promoter to reflect endothelial cell number may have led to underestimates of vessel formation and endothelial cell survival (98). Patan demonstrated that tie-2 deficient mice had poorly developed hearts with occlusions of the sinus venosus, the endothelial cells were rounded and there was a lack of surrounding periendothelial cells with mesenchymal cells being found at a distance from the endothelial cells and in addition there was no vascular loop formation (144). Therefore, tie-2 seems to mediate endothelial cell stretching/motility and is essential for endothelial cell and extracellular matrix interactions and results in blood vessel splitting.

The function of tie-2 thus appears to be counteracted by tie-1. Tie-2 promotes vascular network growth and remodelling through intussusceptive vessel growth, whilst tie-1 inhibits these processes and results in vessel stabilisation.

1.3.5.3 Expression of tie-1 and tie-2

Embryonal expression of tie-2 mRNA appears between 7.5 and 8.0 days post-coitum (137), whereas tie-1 is expressed on day 8.5 in angioblasts of the head of the mesenchyme, in the splanchnopleure, dorsal aorta and vitelline veins and in haematopoietic precursor cells (63). Both have been shown to play an integral role in the later stages of embryonal angiogenesis and vascular development. Wong et al demonstrated that tie-2 protein and mRNA were up-regulated in skin wounds and in tissues undergoing angiogenesis (149). In addition the immunoprecipitated tie-2 protein was tyrosine phosphorylated indicating active downstream signalling (149). Tie-2 was also found to be expressed throughout the vascular system in arteries, veins and capillaries and, furthermore, it was tyrosine phosphorylated in all vessels. This suggests that tie-2 plays a role in the normal maintenance of vessel integrity and angiogenesis. These findings were confirmed by Witzenbichler et al who showed by RT-PCR that tie-2 mRNA was present in human specimens of the internal mammary and radial arteries and the saphenous vein (150). Not suprisingly, tie-2 mRNA expression is also increased during breast tumour angiogenesis (151) and tumour angiogenesis has been inhibited using a soluble extracellular domain of tie-2 (tek) (34). This technique blocks the tie-2 pathway by binding free ligand and this, in particular, demonstrates the importance of tie-2 in tumour angiogenesis. The maintenance of the adult vasculature appears to be one of the major roles of tie-2 (149), therefore it is interesting that an activating mutation of tie-2 has been discovered in two families with venous malformations. These malformations are characterised by dilatation of the affected vein and lack of supporting smooth muscle cells. The activating mutation in tie-2 resulted from a transition from C to T nucleotides at position 849 of the kinase domain of the receptor which resulted in an arginine to tryptophan substitution. This point mutation led to increased autophosphorylation of the tie-2 receptor which perhaps leads to altered downstream signalling (152).

Further evidence that the tie family of receptors have a role to play in angiogenesis is demonstrated by the fact that there is increased expression of tie-1 mRNA in the endothelia of malignant brain tumours (153), in highly vascular capillary haemangioblastomas and

haemangiopericytomas of the central nervous system (154) , in cerebral arteriovenous malformations (155), metastatic melanomas (156) and breast carcinomas (157). In the work by Salven et al, an attempt was made to correlate tie-1 expression in breast carcinomas with prognostic factors (157). However, using immunohistochemical staining (with a monoclonal antibody to the extracellular domain of tie-1) on various breast tumours and comparing tie-1 staining to that of CD31(an endothelial cell marker), it was found that not all malignant angiogenic microvessels expressed the tie-1 protein. However, an immunohistological stain using antibodies to the extracellular domain of tie-1 may not allow a true picture of the level of tie-1 expression because regulation of tie-1 expression may be partly regulated by protein kinase C mediated release of the extracellular domain (158) into the surrounding tissues. Although this process was stimulated by activation of protein kinase C with a phorbol-ester (PMA) *in vitro* ectodomain cleavage of tie-1 does occur *in vivo* in human placenta as discussed in chapter 5.

Despite the demonstration that the expression of tie-1 and tie-2 mRNA and protein are up-regulated in certain physiological and pathological situations, very little is known at the present time concerning the factors that regulate this expression. However, upstream promoter regions of tie-1 and tie-2 have been identified (159, 160) and this will enable investigation into what regulates the expression of these receptors.

1.3.5.4 Signalling intermediates associated with tie-1 and tie-2

Ligand binding to receptor tyrosine kinases leads to receptor dimerisation and intracellular domain tyrosine-phosphorylation (161). Autophosphorylation creates high affinity binding sites for signalling intermediates which contain motifs that recognise phosphotyrosine. Precisely how the tie-1 receptor exerts its effects intracellularly is currently unknown, however, knowledge of the downstream tie-2 signalling intermediates is unfolding. Huang et al reported that GRB-2 and SH-PTP2 associated with tie-2 and were potentially important downstream signalling molecules (162). The same group recently reported that tie-2 associated with the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-kinase) and that this leads to activation of both PI3-kinase and Akt/protein kinase B. Interestingly,

activation of Akt/protein kinase B has been associated with cell survival and anti-apoptosis (163, 164). This may partly be responsible for the role that tie-2 has in blood vessel maturation and maintenance of cell integrity, although Witzenbichler et al have shown that the natural ligands for tie-2 (Angiopoietin-1 and 2) do not exert any pro or anti-apoptotic effects on HUVE cells in culture (150). Therefore, the role of Akt/protein kinase B in tie-2 signalling needs to be further investigated. Using a yeast two-hybrid system, Jones et al have shown that a novel docking protein, Dok-R interacts with the activated intracellular domain of tie-2 and when Dok-R becomes phosphorylated it interacts with rasGAP, Nck and Crk (165). Whilst the precise endothelial role of Nck and Crk is not known, rasGAP is thought to be required for endothelial cell motility and rasGAP-null embryos develop vascular defects similar to those seen with angiopoietin-1 and tie-2 knockout mice (96).

At the time of writing, there is no known activating ligand for tie-1 and furthermore, nothing is known of the downstream signalling intermediates involved with the tie-1 receptor signalling.

1.3.5.5 Angiopoietin -1 and -2

Tie-2 has two ligands, one is an activating ligand (angiopoietin-1) and the other is angiopoietin-2, which is a natural antagonist for angiopoietin-1(141). Both the genes for these ligands are localised to chromosome 8 (166).

Angiopoietin-1 is a secreted activating ligand for the tie-2 receptor (140). Angiopoietin-1 is a 70 kDa glycosylated protein which is unique in structure and contains a coiled-coil motif at the N-terminus linked to a fibrinogen-like region at the carboxyl terminus. The exact site that binds to the receptor is currently unknown. Its expression appears to be first evident in the embryo at around day 9 to 11 post-coitum and is found most prominently in the myocardium, later in embryonal development it is widely expressed in the mesenchyme surrounding developing blood vessels in close association with endothelial cells.

Angiopoietin-2 is a naturally occurring antagonist of angiopoietin-1 and it is very similar in size and structure containing a coiled-coil and fibrinogen like region. There is 60% homology in the amino acid sequence between angiopoietin-1 and -2 (141). Transgenic overexpression of angiopoietin-2 in mice leads to disrupted blood vessel formation and heart underdevelopment which almost mirrors the defects seen in tie-2 and angiopoietin-1 deficient embryos. In the embryo, expression of angiopoietin-2 is similar to that seen by Davies et al with angiopoietin-1. However in the adult, where angiopoietin-1 is almost universally expressed in all arterial vessels, angiopoietin-2 expression seems to be limited to the ovary, placenta and uterus suggesting that angiopoietin-2 expression is increased at sites of vascular remodelling. Both angiopoietin-1 and -2 are expressed in endothelial and smooth muscle cells of both artery and vein throughout the whole of the adult vasculature (150).

Angiopoietin-1 does not result in endothelial cell proliferation but induces the formation of capillary sprouts *in vitro* (167) and is chemotactic for endothelial cells (150). These effects can be blocked by angiopoietin-2 (150). Homologous recombinant targeted mutations of the angiopoietin-1 gene result in defects similar to those seen in tie-2 deficient embryos (19, 98). Homozygous angiopoietin-1 gene disrupted embryos all died in utero at day 12.5 post-coitum, all embryos had underdeveloped hearts with trabeculated defects and there was a lack of vascular branching and remodelling of the primary vascular network (19). These findings suggest that angiopoietin-1 plays a crucial role in mediating interactions between the endothelial and mesenchymal cells and the surrounding matrix and, thus, has a role to play in the later stages of angiogenesis involved with the stabilisation of immature blood vessels. Furthermore, the fact that both angiopoietin-1 and tie-2 are expressed in normal human arteries suggest that the angiopoietin/tie-2 system plays a role in maintaining the integrity of the artery and vein throughout the whole of the adult vasculature (150). Angiopoietin-1 expression, unlike VEGF expression, appears to be downregulated by hypoxia (99), this observation may reflect the stabilising influence of angiopoietin-1 on blood vessel maintenance. Hypoxia is known to activate angiogenesis and so by decreasing the expression of angiopoietin-1 this could possibly lead to the destabilisation of endothelium

and promote angiogenesis. Using a mouse corneal micropocket assay both angiopoietin-1 and angiopoietin-2 failed to stimulate an angiogenic response when administered alone. However, when given in combination with VEGF both angiopoietin-1 and -2 augment the formation of neovessels. In addition, angiopoietin-1 increased microvascular density and resulted in an increase in the number of patent vessels which were surrounded by smooth muscle cells (168).

Despite the tie-1 receptor being isolated and cloned in early 1992 (63) there is still no known ligand for this receptor.

1.4 Possible model of neovascularisation

The understanding of the molecular mechanisms involved in blood vessel development are far from complete. However, the investigation and gene knockout experiments for VEGF, VEGFR-1, VEGFR-2, Angiopoietin-1, tie-1 and tie-2 have led to suggested models of blood vessel development (14, 132, 169). VEGF appears to be one of the major regulators (initiators) of vasculogenesis and angiogenesis. This leads to endothelial cell proliferation and migration and the development of immature, hyperpermeable capillary like vessels. In order for vessels to become truly functional they must undergo a period of maturation and remodelling. It appears that angiopoietin-1 regulates maturation and remodelling by activation of its receptor, tie-2. This activation is suggested to lead to an as yet unknown signalling event, which signals to the endothelial cells to produce smooth muscle cell mitogens such as PDGF-BB and bFGF, however there is no data to support this. This allows recruitment of smooth muscle cells and pericytes to the immature vessel thus allowing the maturation process to occur by activation of TGF-β. The survival and integrity of the endothelial cell is then maintained via tie-1 activation which results in stabilisation of the newly developed vessel. In the long term, tie-2 signalling is also thought to play a role in the maintenance and stability of adult vessels.

1.5 Aims of study

The endothelial specific, tie-1 receptor tyrosine kinase receptor, plays a critical role in both vascular development and angiogenesis, however at the time of writing very little is known about (a) factors controlling its expression (b) intracellular signalling pathways and (c) ligands for this receptor . The aim of this work was to investigate factors that regulate the expression of this receptor, search for a ligand for tie-1 and, hence, investigate possible intracellular signalling pathways. Identification of a ligand for the tie-1 receptor will allow a greater understanding of the function of this receptor during blood vessel development and may be a possible therapeutic agent that could be utilised in therapeutic angiogenesis.

Chapter Two

Materials and Methods

Section I

2.1 Materials

2.1.1 General Reagents

All chemical reagents were obtained from Fisons (Loughborough, Leicestershire, UK) or Sigma Chemical Company Ltd., (Poole, Dorset, UK) unless otherwise stated.

For cell culture, all media, antibiotics and other reagents were from GibcoBRL (Paisley, UK) with the exception of foetal calf serum (FCS), gelatin and Minimum Essential Medium Eagle, alpha modification (α-MEM) which were from Sigma. All tissue culture plastics were obtained from Nunc Products (supplied by GIBCO BRL).

2.1.2 Solutions

All solutions were prepared with double distilled deionised water. The pH of solutions was measured using a Whatman PHA 230 pH meter. Unless otherwise stated all solutions were pH adjusted using Na OH or HCl. For DNA and RNA work all solutions were autoclaved for 15 minutes at 121 ^{0} C. Furthermore all solutions used in RNA work were DEPC treated prior to autoclaving.

Phosphate Buffered Saline (PBS)

140mM NaCl, 2.7mM KCl, 10mM $NaHPO_4$, 1.8mM KH_2PO_4, pH 7.3.

Tris Buffered Saline (TBS)

25 mM Tris, 144mM Na Cl, pH (7.4).

Tris-acetate (TAE)

40mM Tris-base, 40mM glacial acetic acid, 1mM EDTA (pH 8.0)

Tris-EDTA

10mM Tris-HCl, 1mM EDTA (pH 7.5)

Agarose gel loading buffer Type IV

0.25% bromophenol blue and 40% sucrose in water

Protein Transfer Buffer

25mM Tris-HCl pH 8.3, 0.15 M glycine, 20% methanol

Protein Electrophoresis Buffer

250mM glycine, 25mM Tris-base, 0.1% SDS

Protein Loading Buffer

50mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 100mM DTT (DTT was added just prior to use)

Luri-Bertoni (LB) medium

1% tryptone, 0.5% yeast extract, 1% Na Cl

SOC Media

2% tryptone, 0.5% yeast extract, 10mM Na Cl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose

2.1.3 Growth Factors

Human recombinant Vascular Endothelial Growth Factor ,$VEGF_{165}$ was obtained from Genzyme Diagnostics (Kent, UK) and diluted with PBS into 100ng/ml aliquots.

2.1.4 Antibodies

Unless otherwise stated all antibodies were stored at 4-8 ^{0} C.

Polyclonal affinity-purified antibodies raised against peptides corresponding to amino acids 1121-1138 of human tie-1 and corresponding to amino acids 576-593 of SH-PTP2 at the carboxy termini, were obtained from Santa Cruz Biotechnology. The anti-tie-1 and SH-PTP2 antibodies were stored in 0.05M sodium phosphate buffer with 0.1% sodium azide and 0.2% gelatin at a concentration of 0.1μg/μl. One microgram of anti-tie-1 antibody was used for immunoprecipitation of the tie-1 protein, and it was used in Western blotting at a dilution of 1:1000 in blocking buffer (5% Bovine Serum Albumin in TBS+ 0.1% triton x-100). The anti-tie-2 antibody was used in Western blot analysis at a dilution of 1:1000 in blocking. The anti-SH-PTP2 antibody was used for immunoprecipitation of the SH-PTP2 protein from cells and for detection of the protein in Western blot analysis at a dilution of 1:1000 in blocking buffer. Where used for blocking of tie-1 antibody binding, the tie-1 peptide was used at a concentration of 1μg/ml (Santa Cruz Biotechnology). The specificity

of the tie-1 antibody was also tested with a peptide derived from tie-2 (see Figure 5.2). The amino acid sequence of these peptides is given below:

Tie-1 blocking peptide (TIE-1 1121-1138) SLFENFTYAGIDATAEEA

Tie-2 blocking peptide VNTTLYEKFTYAGIDCSAEEA

No other protein was identified as having sequence homology at the amino acid level to the tie-1 peptide in a BLAST search.

A recombinant anti-phosphotyrosine-RC20: Horse Radish Peroxidase conjugated antibody (Transduction Laboratories) was used to detect phosphorylated proteins by Western Blot analysis, this was stored at -20 ^{0} C.

For immunoprecipitating and detecting the tie-1 extracellular domain-myc/His fusion protein in Western Blots and Dot Blots an anti-*myc* mouse monoclonal IgG_1 antibody was used (Invitrogen, San Diego, U.S.A.) at a dilution of 1: 5000 in blocking buffer for Western analysis and 1µl of antibody per 75µl of transfected culture media was used to immunoprecipitate the fusion protein.

A rabbit polyclonal antibody, VEGFAb-1(Neomarkers, Fremont, U.S.A) was used to neutralise the bioactivity of VEGF at a concentration of 10µg/ml.

Horseradish peroxidase (HRP) conjugated antibodies were all obtained from Amersham these included:

Anti-mouse Ig, HRP linked whole antibody (from sheep)

Anti- rabbit Ig, HRP linked whole antibody (from donkey)

Section II

Methods

2.2. Cell Culture

2.2.1 Human Umbilical Vein Endothelial Cell's (HUVECS)

HUVEC'S were isolated from umbilical cords by firstly washing the cord with PBS containing penicillin (100U/ml) and streptomycin (0.1mg/ml). The umbilical veins were flushed through with PBS to remove any debris, clamped at one end and then distended with collagenase solution (Dulbecco's Modified Eagle Medium{DMEM} containing 10mM Hepes buffer pH 7.4 and 0.5mg/ml collagenase-Clotridiopeptidase A from Clostridium histolyticum type II). Cords were then incubated at 37°C for 15 minutes, kneaded gently, then the collagenase solution was drained into a Universal container followed by flushing the cord with PBS to ensure complete removal of the endothelium. Cells were then pelleted by centrifuging for 7 minutes at 300g, resuspended in complete medium, (medium-199,with Earle's salts, supplemented with 2mM glutamine, 50U/ml penicillin, 50μg/ml streptomycin and 20% batch-tested FCS) and placed into a 25cm^2 flask coated with 1% gelatin. Cells were routinely cultured in complete medium supplemented with 0.5μg/ml endothelial cell growth supplement (ECGS), passaging 1:3 when reaching confluency. For immunofluorescence studies, cells were seeded on glass coverslips coated with 1% gelatin in 35mm diameter 6 well tissue culture dishes. Cells were used for no longer than 6 passages after isolation. Cells were quiesced by allowing them to grow to 50% confluence, washing them with PBS and then culturing the cells in Optimem 1 reduced serum media(GIBCO BRL, Life Technologies).

2.2.2 Human Microvessel Endothelial Cells (HMEC's)

HMEC-1 cells were a kind gift from Dr. R. Bicknell (ICRF, University of Oxford) and were cultured in MCDB 131 medium with 15% FCS, 1% penicillin and streptomycin, 10ng/ml of Epidermal Growth Factor, 1μg/ml of hydrocortisone and 1% L-glutamine.

2.2.3 Bovine Aortic Endothelial Cells (BAEC' S)

BAEC'S were a kind gift of Dr. M Boarder (University of Leicester) and were routinely cultured in DMEM supplemented with 1% glutamine, 1% streptomycin, 1% penicillin and 10% FCS.

2.2.4 Chinese Hamster Ovary cells (CHO's)

CHO cell lines were purchased from the European Collection of Animal Cell Cultures. Cells were cultured in α-MEM supplemented with 10% New Born Calf Serum and 1% streptomycin, 1% penicillin and 1% L- Glutamine.

2.2.5 Malignant Melanoma Cell lines (SK-Mel 24, G361 and RPMI-7951)

Melanoma cell lines were a kind donation from Mr. S. Thirdborough (Dept. of Surgery, University of Leicester). All melanoma cell lines were cultured in DMEM supplemented with 10% FCS, 1% streptomycin, 1% penicillin, 1% L-Glutamine and 2-mercaptoethanol 10.4mol/l. Cells were quiesced using serum-free DMEM containing 1% streptomycin, 1% penicillin, 1% L-Glutamine and 2-mercaptoethanol 10.4mmol/l, which was added to the cells after allowing growth to 60-80% confluence and washing with PBS. Cells were quiesced for 24 hours and the conditioned medium collected, centrifuged in a Sorvall tube at 300g for six minutes. The conditioned medium was either used on the day or stored at - 80 °C. One protease inhibitor cocktail tablet (Boehringer Mannheim) / 10mls of media was added to all conditioned media after centrifugation.

All cell lines were passaged by using trypsin (0.1%) and EDTA (0.02%), followed by centrifugation at 300g for six minutes. Cell pellets were resuspended in the appropriate volume of culture media and plated onto culture dishes at an appropriate dilution.

2.3 HUVEC DNA Synthesis

The measurement of cell proliferation or DNA synthesis in HUVEC's was determined by using 5-Bromo-2'-deoxy-uridine (BrdU) which is incorporated into DNA in place of thymidine. Denaturation of the labelled DNA allows access to anti-BrdU antibodies. The BrdU labelling and detection kit was purchased from Boehringer Mannheim Biochemica.Malignant melanoma cell lines SK-Mel 24, G361 and RPMI-7951 were cultured as described and conditioned media were collected and centrifuged as described in section 2.2.1. Concurrently HUVE cells on coverslips in 6 well plates were cultured until reaching 50% confluence and were then quiesced for 12 hours using Optimem 1. After this period of quiescence the cells were either a) left in a quiescent state b) had 2.5nM of VEGF added to the cells or c) had Optimem 1 removed, were washed 3 times with sterile 1x PBS, followed by culture of the cells in conditioned melanoma medium. After 24 hours the HUVE cells were then investigated for differing levels of cell proliferation.

HUVEC's were grown to 50% confluence on glass slide coverslips and were placed in serum free medium, Optimem 1, for 12 hours to remove the effects of serum. The desired concentrations of agonists or conditioned medium were added to the cells for 24 hours and incubated at 37 °C. During the last hour of this incubation period, BrdU was added (final concentration 10μM) to the cells. Cells were washed with PBS and fixed in 70% ethanol (in glycine buffer, 50mM pH 2.0) for 30 minutes at -20 °C. The coverslips were then washed in PBS followed by incubation with anti-BrdU mouse monoclonal antibody (diluted 1:10 with 66mM Tris buffer, 0.66mM Mg Cl_2 , 1mM 2-mercaptoethanol) in a humidified chamber for 30 minutes at 37 °C. After further washing the coverslips were incubated with anti-mouse Ig-alkaline phosphatase (the antibody was diluted 1:10 with PBS) for 30 minutes at 37 °C. Following this incubation the coverslips were washed with PBS and covered with colour substrate solution (13μl of nitroblue tetrazolium salt, 75mg/ml, in dimethylformamide

70%(v/v) plus 10µl of 5-bromo-4-chloro-3-indolyl phosphate, toluidium salt 50mg/ml, in dimethylformamide plus 3ml of Tris-HCl-buffer 100mM, $MgCl_2$ 50mM, pH 9.5.) and incubated at room temperature for 30 minutes. The coverslips were then washed in dH_2O and incubated with 10% Nuclear Fast Red for 3 minutes. Nuclear Fast Red enhances the nuclear staining of non-proliferating cells which makes these cells easier to identify during microscopy.

The coverslips were then mounted onto glass slides by placing them cell side down onto approximately 4-5 µl of glycerol. To prevent dehydration the coverslips were sealed with clear nail varnish. The cells were visualised using a Nikon diaphot microscope and photographed on Kodak ASA400 colour film. Cells undergoing active proliferation were stained black, Figure 2.1. HUVE cells were visualised at 200x magnification and counts of stained to non-stained cells were made in 10 different visual fields. Proliferating cells were then expressed as a percentage of the total number of cells seen in a visual field and the collective mean and standard deviation was calculated.

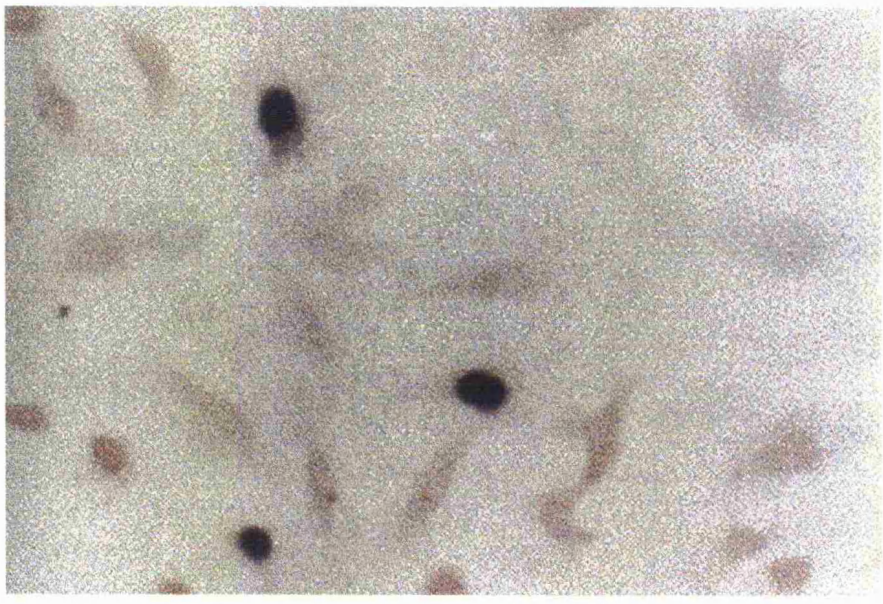


Figure 2.1 Proliferating HUVE cells. HUVE cells that are undergoing proliferation and DNA synthesis readily incorporate BrdU into the nucleus and stain black. Magnification x200

2.4 Cloning

2.4.1 Cloning of tie-1 cDNA

Umbilical vein endothelial cells have been shown to express the tie receptor tyrosine kinase (136), (170). Therefore, HUVEC's were used to extract RNA which would have adequate levels of tie mRNA to allow reverse transcription and amplification of tie cDNA by the polymerase chain reaction using tie-1 specific primers.

2.4.2 RNA and mRNA isolation

To inhibit RNase activity all glassware and solutions were treated with 0.1% of the RNase inhibitor Diethyl Pyrocarbonate (DEPC) for at least 2 hours and then autoclaved to breakdown the DEPC.

RNA was initially extracted from cells using the Guanidium thiocyanate-phenol-chloroform extraction technique (171) and the amount of RNA was quantified using spectrophotometric analysis (see section 2.4.3). However, greater levels of extraction of total RNA were achieved using the RNeasy® total RNA isolation system (Qiagen Ltd., Crawley, U.K.). The RNA was extracted using the manufacturers protocol. This system utilises the guanidium-thiocyanate extraction method combined with silica-gel membrane centrifugation. The extracted total RNA binds to the silica-gel membrane whereas other contaminants are washed away. The total RNA was readily eluted using DEPC water. Messenger RNA was further isolated from total RNA by using oligotex mRNA isolation kits (Qiagen Ltd., Crawley, U.K.). The method used for this technique was as described in the manufacturers protocol. The principle of this technique utilises the unique polyadenylated tail of mRNA. This poly A^+ hybridises to oligo dT primers which are coupled to a solid phase matrix (oligotex latex particle suspension) following washing to remove non-mRNA. The mRNA is then eluted by lowering the ionic strength (with Tris-HCl pH 7.5) and destabilising the dT:A hybrids.

2.4.3 Spectrophotometric quantification of RNA and DNA (172)

RNA and DNA absorb in ultraviolet wavelength between 250 and 270nm. At 260nm an absorbance of 1 measured in a cuvette with a 1cm path length is indicative of RNA at approximately 40μg/ml and double stranded DNA at 50 μg/ml. Pure RNA and DNA has an A260/A280 absorbance ratio >2.

2.4.4 Reverse Transcription (RT)

First strand cDNA was transcribed from mRNA using SUPERSCRIPT ™ II Reverse Transcriptase (Gibco BRL). Fifty nanogrammes of mRNA was mixed with 0.5μg of oligo dT, adding sterile distilled water to a final volume of 12μl. This was incubated at 70 ^{0} C for 10 minutes and then quickly chilled on ice, followed by centrifugation at 12,000g for one minute. To this mixture was added 4μl 5x first strand buffer (250mM Tris-HCl, 375mM KCl, 15mM $MgCl_2$), 2μl of 0.1M dithiothreitol (DTT), 1μl of 10mM of deoxynucleotide mix (10mM each of dATP, dGTP, dCTP and dTTP) and this was incubated for 2 minutes and 42 ^{0} C after which time, 200 units of the reverse transcriptase was added and the reaction mixture was incubated at 42 ^{0} C for 50 minutes followed by 70 ^{0} C for 15 minutes to inactivate the reaction. To remove RNA complementary to the cDNA 2 units of *E.Coli* RNase H was added and incubated at 37 ^{0} C for 20 minutes.

2.4.5 Polymerase Chain Reaction (PCR)

The amplification of DNA was performed as described by Mullis *et al* (173) with modification. This technique involves hybridisation of specific primers to opposite strands of the target sequence. DNA is synthesised in the 5' to 3' direction by repeated cycles of denaturation of the template, annealing of the primers and extension of primers using a proof reading (3'to 5' exonuclease activity) thermostable DNA polymerase, ELONGASE™ enzyme mix (Life Technologies Inc., GibcoBRL, U.K). This contains both Taq and Pyrococcus species GB-D thermostable DNA polymerases.

The amplification reaction was performed in a final volume of 50µl on ice. The reaction mix (1) contained target template, tie-1 specific forward and reverse primers (400 nM) and dNTP's (200µM of dATP, dCTP, dGTP and dTTP) made up to a volume of 20µl with deionised and distilled water (ddH_2O). In a separate reaction tube, reaction mix (2) tube contained Buffer B (60mM Tris-SO_4 pH 9.1, 18mM $\{NH_4\}_2$ SO_4 and 2mM Mg SO_4), 5 units of ELONGASE enzyme mix and the final volume was made up to 30µl with ddH_2O. Reaction mix(1) was pipetted into reaction mix (2), reaction mixtures were overlaid with 50µl of mineral oil and mixed by vortexing followed by centrifugation at 12,000g for 3 seconds. PCR was performed in a DNA Thermal Cycler 480 (Perkin-Elmer). The samples were preheated to 94^0C for 30 seconds to allow pre-amplification denaturation. DNA was then amplified over 40 cycles by denaturing for 30 seconds at 94^0C, combined annealing and extending at 68^0C for 3 minutes followed by a final single extension of 10 minutes at 68^0C. PCR products were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 0.5 x volume 7.5 M ammonium acetate and 2 x volume absolute ethanol for 1 hour at -20^0C.

Primers for amplification of tie-1 cDNA and trk A cDNA by polymerase chain reaction (PCR) were produced by Genosys, Cambridge, UK (page 55-56 and page 44 respectively). β-Actin primers (below) were used to optimise PCR reactions and as a controls (Stratagene, U.K.). The β-Actin PCR product produced represents a 661 base pair nucleotide from nucleotides 1038 to 1905 in the β-Actin sequence.

Forward primer: 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'
Reverse primer: 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'

2.4.6 Agarose Gel Electrophoresis

DNA was size fractionated on a 2% agarose gel. When DNA electrophoretic bands were to be excised from the gel a low melting point 1.5% agarose gel was used and electrophoresed

at 4°C. Agarose was heated in 1x TAE until dissolved. After cooling to approximately 50°C the agarose solution was poured into an electrophoresis block, well comb added and allowed to cool to room temperature. 1-4 volumes of sample were mixed with 1 volume of gel loading buffer (see section 2.1.2), loaded into the gel and electrophoresed at 100 volts in 1x TAE buffer until the tracking dye had reached the end of the gel. The size of DNA fragments in base pairs resolved by agarose gel electrophoresis were determined by comparison with a 1Kb DNA ladder (GibcoBRL). This standard has a range of fragments between 500bp to 12 Kb base pairs of DNA and was detected by immersion of gels in a solution of 5µg/ml ethidium bromide in water for 30 minutes, destained in water and visualised by transillumination by ultraviolet light. Gels were photographed using a Polaroid MP-4 land camera with black and white Polaroid 667 film. The amount of PCR product was quantified by comparing band intensities with comparable bands of known molecular weight from the 1Kb standard. PCR products and digested vector fragments were purified from LMP agarose gel using the QIAquick gel extraction kit as recommended by the supplier (Qiagen Ltd., Crawley, U.K.)

2.4.7 TA-cloning of PCR products

PCR products were cloned directly into the eukaryotic vector pCR 2.1 by TA- cloning (Invitrogen). This approach utilises the terminal transferase activity of non-proofreading polymerases such as *Taq* DNA polymerase, which add a single 3' deoxyadenosine (A)-overhang to each end of the PCR product. pCR 2.1 is supplied linearised with single 5' deoxythymidine (T)- overhangs to enable direct ligation of PCR products at high efficiency. The vector map of pCR 2.1 with the multiple cloning site is shown in Figure 2.2. In order to carry out TA-cloning, blunt ended products generated by the ELONGASE™ enzyme mix were treated with non-proofreading *Taq* DNA polymerase in 1x PCR buffer, containing 200µM dATP, for 20 minutes at 72°C. PCR products were ligated to 60ng of linearised pCR 2.1 (molar ratio of 2:1) by incubation overnight at 15°C with 4 units of T4 DNA ligase in 10µl of 1x ligation buffer (6mM Tris-HCl [pH 7.5], 6mM $MgCl_2$, 5mM NaCl, 7mM β_2 mercaptoethanol, 0.1mg/ml BSA, 0.1mM ATP, 2mM DTT, 1mM spermidine).

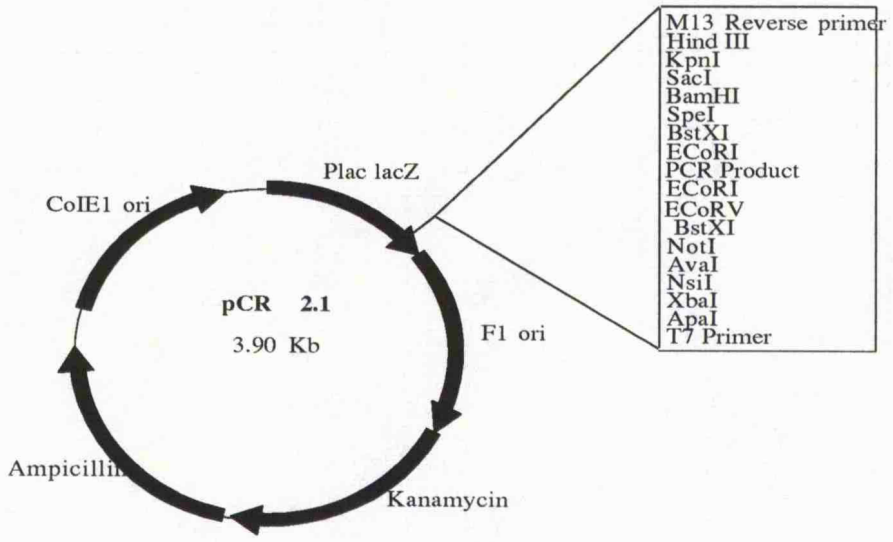


Figure 2.2 Vector map of pCR 2.1 and its multiple cloning sites and sites of ampicillin and kanamycin resistance genes.

Ligation reactions were centrifuged briefly and 2µl used to transform One Shot competent cells (TOP10F') according to the protocol provided with the TA-cloning kit. After transformation, 50µl and 200µl aliquots of the cells were spread onto Luria-Bertoni (LB) agar plates containing 50µg/ml of ampicillin and 40µl each of 40mg/ml of X-Gal and

100mM IPTG (to help express LacZα by inhibiting the *lac* repressor). The plates were inverted and incubated overnight at 37^{0}C. Individual white colonies were picked and placed in LB broth containing 50μg/ml of ampicillin and shaken overnight at 37^{0}C. The cultures were screened by PCR, with the appropriate primers, to check for correct the cDNA insert. PCR products were examined by agarose gel electrophoresis, ethidium bromide staining and ultraviolet transillumination. From positive cultures, 10μl of broth was placed into LB broth containing 50μg/ml of ampicillin and shaken overnight at 37^{0}C.

2.4.8 Plasmid purification

High quality plasmid DNA for restriction analysis and gene transfection was prepared by alkaline lysis/Qiagen® anion-exchange chromatography (25ml midi-prep yielding up to 100μg of DNA) as recommended by the supplier (Qiagen, Ltd., Crawley, U.K.). The amount of DNA yield was calculated using spectrophotometric analysis as described in section 2.4.3.

2.4.9 Restriction analysis and the orientation of the insert

The orientation of inserts was performed using restriction map analysis. Restriction analysis was performed using 0.2-0.3μg of DNA . The conditions used were specific for each particular restriction digest enzyme used (172).

For further manipulations of insert DNA, the plasmid DNA was linearised using restriction digest and recircularization and religation (only with complementary ends) was prevented by using calf intestinal alkaline phosphatase to dephosphorylate both 5' termini. This enzyme catalyses the hydrolysis of 5'-phosphate groups from DNA. This reaction was performed as directed by the suppliers protocol (Promega, Madison, U.S.A).

2.4.10 Storage of bacterial stock cultures

Aliquots of bacterial culture (850 µl) were mixed with sterile glycerol (150 µl) in a sterile 1.5ml freezing vial (Sarstedt) and snap-frozen in liquid nitrogen. For long-term storage the vials were stored at -80^{0}C.

2.4.11 Sequence analysis

The sense and anti-sense strands of cloned tie-1 ED cDNA were sequenced using the ABI PRISM ™ dRhodamine terminator cycle sequencing ready reaction kit, marketed by Perkin-Elmer Ltd. High quality double-stranded DNA was purified using the Qiagen plasmid purification kit as described. 100ng of plasmid DNA template was sequenced using the protocol supplied with the ABI PRISM ™ kit, employing T7 and M13 reverse primers which bind to complementary sites within the multiple cloning sequence(MCS) of pCR2.1. Sample electrophoresis was performed by the Protein and Nucleic Acid Central Lab (PNACL, University of Leicester), excess dye terminators having been removed by ethanol precipitation, The resulting sequence data was analysed using Gene Jockey II (Biosoft, Cambridge) and Sequence Navigator software (Perkin-Elmer Ltd). Internal primers were designed to enable full-length sequencing.

2.4.12 Subcloning of tie-1 extracellular domain into an Expression Vector

In order to identify which tissues express the tie-1 ligand and aid its cloning, a method described by Hwai-Jong Cheng *et al* was utilised (174). Essentially this involved the use of the extracellular domain of a receptor (ligand binding region) as a probe, to identify tie binding i.e. tie ligand. In order to express and secrete the tie-1 extracellular domain protein, the tie-1 DNA sequence had to be subcloned into a suitable expression vector. The expression vector pSecTag (Invitrogen) was chosen because the proteins expressed from pSecTag are fused at the N-terminus to the murine Ig κ- chain leader sequence for protein secretion from the cell and at the C-terminus to a peptide containing the *myc* epitope and six tandem histidine residues for detection and purification onto a nickel chelating resin. PsecTag

B was chosen as it allowed correct in-frame fusion with the Ig κ- chain leader sequence, Figure 2.3.

Tie-1 ED DNA was excised out of pCR2.1/tie-1ED DNA using restriction enzyme digestion. In order to allow the correct in-frame reading sequence within pSecTag the pCR2.1/tie-1ED was linearised using the Nsi I enzyme. The cDNA was then extracted and purified using the phenol:chloroform:isoamyl alcohol method and redissolved in 10μl of ddH_2O. The 3' terminus was then polished using Pfu DNA polymerase (Stratagene Ltd, Cambridge) to generate a blunt end. The 10μl of DNA was added to 1.3 μl of 10x polishing buffer, 1 μl of dNTP mix (2.5mM each) and 0.5 units of Pfu DNA polymerase, mixed and 20μl of mineral oil was overlaid. This was then incubated at 72^0C for 30 minutes. cDNA was then further precipitated using the phenol:chloroform:isoamyl alcohol method.

Further incubation with Kpn I restriction enzyme generated a 2199 bp fragment which corresponded to tie-1ED cDNA sequence with 49 bp of pCR2.1 MCS both upstream and downstream of the cloned sequence. The DNA fragment was cut out of a 2% LMP agarose gel and purified using the Qiagen DNA purification kit. Synchronously 5μg of pSecTag B vector was incubated with Kpn I and ECORV(which produces blunt ends) restriction enzymes in multicore buffer for 90 minutes at 37^0C. The linearised band was then excised from the 2% agarose gel and purified with the Qiagen method as above. Estimates of pSecTag B DNA and tie-1 ED DNA were made using spectrophotometric analysis. Tie-1ED cDNA was then ligated into pSecTag B using T4 DNA ligase as previously described in section 2.4.7 (1:1 vector:insert ratio). Transformation and screening of colonies with PCR were performed as described in sections 2.4.7.

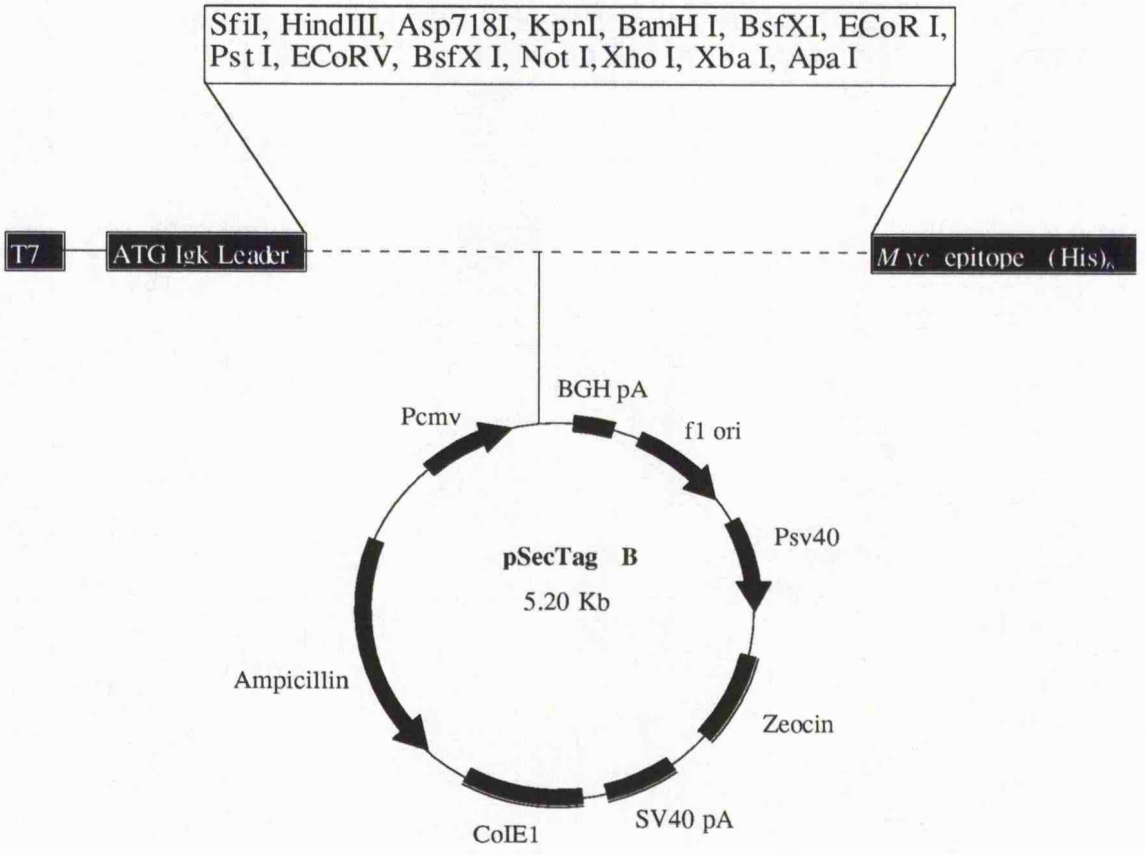


Figure 2.3 Vector map of the expression vector pSecTag B. Fusion-protein expression is driven by the CMV promoter. Expressed protein has an Ig k leader sequence and has a *myc-His*$_6$ epitope Tag.

From positive colonies further sub-cultures were set up in LB broth containing ampicillin. The plasmids were purified using the Qiagen plasmid purification system.

2.4.13 Cloning of the Trk A receptor extracellular domain into PCDNA 3.1

Trk A is one of the receptors for Nerve Growth Factor (NGF), and is specific to neuronal tissue. A trkA extracellular domain (trk A ED) tagged fusion protein was used as a control for tie-1 ED myc-His when assessing specific binding to potential tie-1 ligands. As far as is known malignant melanomas do not produce or secrete NGF. Previously cloned whole receptor Trk A DNA sequence within $pCDNA^{neo}$ was a kind gift of Dr. E. Gunn-Moore (University of Bristol). The extracellular domain was amplified by PCR using in sequence forward and reverse primers as shown below. The primers were designed from the published Trk A nucleotide sequence, Genbank: accession number M23102.

Forward primer: 5'- GCCGCCGCGATGCTGCGA-3'

Reverse primer: 5'- GACGAAACACCTTTTGGGGGTCTGG-3'

This was further sub-cloned into the pCR Script vector (Stratagene) following restriction digest of the vector with Srf I and blunt end ligation of the amplified DNA into the vector.

Subsequently the TrkA ED DNA was further subcloned into the expression vector PCDNA 3.1 by using restriction digest of the pCR Script/ TrkA ED with Not I and Bam H I. The resultant band of Trk A ED (1.2kb) was excised from a LMP agarose gel, purified and ligated with pCDNA 3.1(Invitrogen) that had also undergone restriction digest with the above enzymes. Transformed cells were plated onto LB plates as previously described, section 2.4.7. Colonies were screened with PCR and if positive were further subcultured in LB broth and the plasmid then purified using the Qiagen plasmid purification method. The correct insert orientation was confirmed with restriction analysis. The pCDNA3.1/TrkA ED plasmids were then used for transfection into CHO cells using conditions identical to those described for pSecTag/tie-1 ED. The secreted TrkA ED-myc/His fusion-protein appeared as a 70kDa protein.

2.5 Transient Transfection

2.5.1 Transient transfection of pSecTag/tie-1ED into Chinese Hamster Ovary(CHO) cells

CHO cells were chosen for transfection because they are a very stable immortal cell line, have relatively high transfection efficiencies and are not known to constitutively express the tie receptors. Superfect transfection reagent (Qiagen) was used for transfection of tie-1ED DNA into CHO cells. Superfect transfection reagent is a polycationic sphere of defined shape and size. It possesses a spherical architecture, with branches radiating from a central core and terminating at charged amino groups. The Superfect reagent assembles the DNA into compact structures, thus optimising the entry of DNA into the cell. Superfect-DNA sequence complexes possess a net positive charge, which allows them to bind to negatively charged receptors on the surface of eukaryotic cells. Once inside the cell Superfect reagent buffers the lysosome after it has fused with the endosome, leading to pH inhibition of lysosomal nucleases, which ensures the stability of the transfected DNA complex.

CHO cells were transfected when they had reached 40-50% confluence. Optimisation of transfection conditions were performed on cells in 6-well plates. Optimisation of transfection conditions were achieved for pSecTag/tie-1ED by adjusting the DNA concentration and also the DNA:Superfect reagent ratio (see results Chapter 3 section 3.2.4). Transfection was achieved using methods described in the suppliers protocol (Qiagen Ltd., Crawley, U.K.).

2.5.2 Expression of the tie-1 ED-myc/His fusion protein

The estimated molecular weight of the unglycosylated tie-1 extracellular domain is 79.3 kDa, therefore it was calculated that the molecular weight of the tie-1 ED-myc/His fusion protein would be 89 kDa.

To elucidate if the tie-1 ED myc-His fusion protein was being expressed by CHO cells and secreted into the surrounding cell growth medium, CHO cells were transfected for 24 hours in serum containing growth medium, after which time the medium was removed, the cells washed in sterile 1x PBS and they were then grown in serum free medium for 48 hours.

The culture medium was collected and concentrated using the centriprep-10 ultrafiltration device (Amicon Ltd., Stonehouse, Gloucestershire) which has a 10kDa filter. The medium was added to the centrifuge tube which was then centrifuged at 3000g for 30 minutes, after which time the retenate was collected and either utilised or frozen at -80^{0}C after the addition of protease inhibitor cocktail tablets, one tablet/10mls of media (Boehringer Mannheim).

Both the CHO cell lysates and the concentrated serum free medium were examined for the presence of tie-1 ED-myc/His fusion protein by Western Blot analysis. Transfected cells were lysed by adding 50µl of 1x Sample Buffer (1M Tris pH6.8, 20% SDS, 100% glycerol, 100mM EDTA pH 6.8 and bromophenol blue) and 100mM DTT (1x SB-DTT) to each well, scraping the cells off the flask, pipetting the lysate into an eppendorf and then sonicating the lysate for 30 seconds to cause cell disruption. The samples were then boiled and centrifuged and loaded onto a 12% SDS-PAGE gel.

Tie-1ED-myc/His fusion protein was recovered from the media in a variety of ways. Firstly, an equal volume of x2 SB-DTT was added to a sample of medium, boiled and then centrifuged. Secondly, the tie-1 ED-myc/His protein was immunoprecipitated by an anti-myc antibody (Invitrogen) using a method similar to that used in section 2.6.1 except using Protein-G-Sepharose. The third method utilised the six tandem histidine residues in the N-terminus of the tie-1ED-myc/His protein which have a high affinity for nickel chelating resins i.e. ProBond™ resin (Xpress™ system protein purification, Invitrogen, Leek, Netherlands). The medium was incubated with the Probond columns for 10 minutes under native conditions as specified in the suppliers protocol. The column was then washed using native wash buffer (20mM Na PO_4, 500mM Na Cl at a pH 6.0). The tie-1ED-myc/His protein was eluted off the column either by simply adding an equal volume of 2xSB-DTT and boiling or by using a pH elution technique as described in the suppliers protocol, using a Native-pH elution buffer (20mM Na PO_4, 500mM Na Cl at pH 4.0).

2.6 Protein Analysis

2.6.1 Immunoprecipitation

To analyse expression and phosphorylation, proteins were precipitated using specific antibodies, size fractionated using electrophoresis and then visualised using immunodetection and enhanced chemiluminescence. Confluent cells in T-80 flasks were washed twice with 1x PBS, followed by addition of 0.75ml of cell lysis buffer/dish:(50mM Tris pH 7.4, 50mM Na Cl, 1mM Na orthovanadate, 1mM Na F, 1mM EGTA, 1 protease inhibitor cocktail tablet (Boehringer Mannheim)/ 10mls of lysis buffer). Prior to use, 50µl/ml of 20% Triton X-100 was added to the lysis buffer to give a final concentration of 1%(v/v). The flasks were scraped, the lysates removed and pipetted into eppendorf tubes and these were vortexed vigorously at 4 ^{0}C for 1 minute. After the lysates had been centrifuged at 12,000g for 10 minutes at 4 ^{0}C, the supernatants were removed and pipetted into fresh eppendorf tubes. Primary antibody was then incubated with the lysates for a minimum of 2 hours at 4 ^{0}C with gentle agitation. For tie-1 and SH-PTP2 immunoprecipitations 10µl of antibody was added to 750 mls lysate, phosphorylated proteins were immunoprecipitated using 25ml packed volume of monoclonal anti-phosphotyrosine antibody on agarose beads(Sigma). The antibody-protein complex was recovered with addition of 50µl of Protein-A-Sepharose (50mg/ml) in lysis buffer and incubated for a further 2 hours at 4 ^{0}C. The immunoprecipitate was collected by centrifuging at 12,000g for 30 seconds and the supernatant discarded. The immunoprecipitate was washed three times with wash buffer (lysis buffer, 1 protease inhibitor cocktail tablet/ 10mls lysis buffer and 5 µl of Triton X-100/ml to give a final concentration of 0.1% {v/v}). Proteins were solubilised in 50µl of 2x sample buffer containing 100mM DTT and boiled for 5 minutes. Samples were centrifuged for 5 minutes at 12,000g and the supernatant removed and loaded onto an SDS-PAGE to resolve the proteins. Unless otherwise stated each track resolved on a SDS-PAGE gel represented the protein immunoprecipitated from a single 80cm^2 tissue culture flask of confluent cells.

2.6.2 Determination of protein concentration

Protein concentrations in cell lysates were determined using the Micro BCA protein assay reagent kit (Pierce, USA). The assay utilises the ability of bicinchoninic acid (BCA) to bond with Cu^{1+}. This assay incorporates the biuret reaction (protein reacting with Cu^{2+} in an alkaline medium to produce Cu^{1+}) and the interaction of two BCA molecules to one cuprous ion (Cu^{1+}) is water soluble and leads to a colour change which exhibits a strong absorbance at 562nm. Individual samples were assayed as per the manufacturers protocol. BSA was used as a standard in this assay.

2.6.3 Separation of proteins by SDS-polyacrylamide gel electrophoresis(SDS-PAGE)

Separation of proteins was performed under denaturing conditions. The proteins were resolved on 10% or 12% polyacrylamide gels depending on the size of the proteins to be examined. The gels contained the appropriate amount of acrylamide{30% stock 37.5:1 acrylamide to bisacrylamide, Protogel National Diagnostics}, 0.1% SDS, 0.37 M Tris-HCl pH 8.8. Polymerisation was catalysed by the addition of TEMED (1µl/ml gel solution) and 10% ammonium persulphate (10µl/ml gel solution). The gel solution was mixed by gently swirling and cast between two glass plates using syringe and needle. The gel solution was overlaid with water to exclude oxygen. Following polymerisation the water was removed and the stacking gel cast on top. The stacking gel contained 5% acrylamide (30% stock of 37.5:1 acrylamide:bis, 0.125 M Tris-HCl pH 6.8, 0.1% SDS, 10µl/ml TEMED and 10 µl/ml of 10% ammonium persulphate). A comb was inserted and the stacking gel allowed to polymerise. Boiled and reduced samples were then loaded onto the gel with a Hamilton syringe and proteins separated by electrophoresis at 200V in running buffer (250mM glycine, 125mM Tris-base, 0.1% SDS). Prestained molecular weight markers (Novex) were electrophoresed on each gel and electrophoresis stopped when the optimum separation achieved as judged by the markers position.

2.6.4 Western Blotting

Following electrophoresis, the proteins were transferred to a nitrocellulose membrane by electroblotting. The gel was positioned cathode side of a membrane (Hybond ECL, Amersham) and sandwiched between Whatman 3MM filter paper (prior to loading, the membrane and filter paper had been pre-soaked in transfer buffer). These were then inserted into a mini-Trans Blot (Biorad) and proteins transferred by applying a maximum current of 0.1 A over 12 hours using 1x transfer buffer (39mM glycine, 48mM Tris-base, 0.037% SDS and 20% methanol). Blots were used directly or blocked and stored wet at 4 ^{0}C wrapped in Saranwrap.

2.6.5 Immunoblotting and Enhanced Chemiluminescence (ECL)

Detection of Horse Radish Peroxidase (HRP) conjugated antibodies was carried out using the technique of Enhanced Chemiluminescence (ECL, Amersham) and detection of light emission on x-ray film.

Following electroblotting, the free protein binding sites on the membrane were blocked by incubating the membrane in a blocking agent for one hour at room temperature. For membranes probed with anti-human tie, anti-phosphotyrosine, anti-SH-PTP2 antibodies the blocking agent was 5% Bovine Serum Albumin (BSA) and 0.1% Triton X-100 in TBS. Primary antibodies were diluted at the appropriate dilution in blocking buffer and incubated at room temperature for one hour with the membrane. The membranes were then washed in 0.1% TBS-T for 5 minutes at least three times. A HRP conjugated secondary antibody (diluted in blocking buffer) specifically reacting against the primary antibody was incubated with the membrane for 30 minutes at room temperature. The membrane was then washed 3 times for 10 minutes to remove any unbound antibody. Immunoreactive bands were detected by the ECL chemiluminescent detection system (Amersham International Plc, Bucks, UK) as per the protocol provided. Equal amounts of reagent 1 and 2 were mixed and added to the membrane immediately and incubated with agitation for one minute. The membrane was removed and excess liquid was drained off before wrapping the membrane in Saranwrap,

being careful to eliminate all air bubbles. The membrane was then placed in a light tight cassette and light emission was visualised on Kodak XAR5 film for varying time periods. Bands on films were then quantified by scanning with an LKB Ultrascan densitometer. Peak heights of each band scanned were recorded.

Membranes were reprobed with differing antibodies following stripping of the membrane of previous primary and secondary antibodies. The antibodies were removed by incubating the membrane with stripping buffer (100mM 2- Mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) for 45 minutes at 50 ^{0}C during gentle agitation. Membranes were then washed twice for ten minutes in TBST at room temperature and then blocked and reprobed as already described.

2.6.6 Protein staining with Protogold and silver enhancement

Proteins immobilised on a Western blot were stained using Protogold stain (British Biocell, Cardiff, UK). Protogold contains a gold colloid. These gold particles are negatively charged and bond both hydrophobically and ionically to proteins immobilised on a Western blot. The protein then stains dark red. The use of a silver stain enhances the staining of these bands. These methods were used as instructed by the manufacturers protocol.

2.7 In vitro cell hypoxia

All experiments presented in chapter 4 were performed on BAEC's but similar results were obtained with HMEC's. For experiments under low oxygen conditions, cell culture medium was pre-gassed and flasks were flushed with 2% oxygen, 5% CO_2 , the balance being made up with N_2 (BOC Ltd., London, U.K.). Partial oxygen tension (pO_2)was measured using an electronic oxygen meter (Model 9071, Jenway Ltd., Essex, UK) as per the manufacturers instructions. Flasks were sealed and the pO_2 was monitored at hourly intervals. Where the effects of conditioned medium was tested, cells were made hypoxic or maintained under normoxic conditions for 18 hours. Media from flasks was collected, centrifuged at 1000g for 10 minutes and passed through a 0.2μm filter. After gassing to restore normoxia the

conditioned media was added to fresh cells that had been cultured under normoxic conditions.

2.7.1 Assessment of tie-1 protein expression

After exposure to experimental or control conditions, cells were washed twice with PBS and then lysed with 0.75% (m/v) sodium dodecyl sulphate (SDS). Cell lysates were then scraped from the flasks and sonicated. An aliquot was removed to allow calculation of the protein content from each sample by using a Micro BCA protein assay (Pierce and Warriner , Cheshire, UK) as described in section 2.6.2 and the suppliers protocol. The protein content was determined by reading the absorbance at 562 nm for each protein sample and comparing this to a standard curve of known protein standard concentrations. To the remainder of the samples an equal volume of 2x SB-DTT was added and boiled for 5 minutes. Equal amounts of protein (approximately 100μg/track) were then loaded onto a 10% SDS-PAGE gel, electrophoresed and the proteins were transferred to a nitrocellulose membrane and probed with an anti-tie-1 antibody. Immunoreactive bands were detected with a peroxidase-conjugated secondary antibody and the ECL chemiluminescent detection system. Bands were then scanned and quantified using an LKB Ultrascan densitometer.

2.8 Tie-1 immunoprecipitation from human tissues

In order to determine whether tie-1 is truncated *in vivo*, cell lysates from various human breast and lung tumours, and placenta were immunoprecipitated with an anti-tie-1 antibody. Fresh tissue samples were collected and immediately snap frozen in liquid nitrogen and frozen at -80 ^{0} C. Frozen samples were crushed to powder in a mortar and pestle and then mixed with an equal volume of lysis (extraction) buffer. This method was adapted from Marchini et al (216). The lysis buffer was made up of: 100mM Na Cl, 10mM Tris-HCl pH 7.6, 1mM EDTA and one tablet/10 mls of lysis buffer, of protease inhibitor cocktail tablets (Boerhinger, UK). Once the tumour lysates had fully defrosted and resuspended in lysis buffer, the samples were centrifuged at 3,000 g for 10 minutes. The resultant supernatants were removed and immunoprecipitation of tie-1 was performed with an anti-tie-1 antibody (see section 2.6.1). Immunoprecipitates were washed three times with PBS. An equal volume of 2x SB-DTT was then added to the sample, vortexed for 15 seconds, sonicated for

30 seconds, boiled for 5 minutes and centrifuged for 2 minutes at 13,000g. The samples were analysed by SDS-PAGE followed by Western blotting. Tie-1 immunoreactive bands were identified by incubation with an anti-tie-1 antibody following blocking of the membrane with 5% BSA in x1 TBS-T.

Chapter Three

Identifying a ligand for the tie-1 tyrosine kinase receptor

3.1 Introduction

The tie-1 receptor tyrosine kinase has an essential role in vascular development (143, 98) however at the time of writing, a ligand has yet to be identified for this receptor. Cloning and characterisation of such a ligand would not only lead to a greater understanding of tie-1 receptor function but also allow the investigation of potential intracellular signalling mechanisms. In contrast to tie-1, at least two ligands, the angiopoietins, have been described for tie-2 (19, 140, 141).

Angiopoietin-1and 2 are secreted ligands for the tie-2 receptor and given the similarity between the tie-1 and tie-2 receptor, it is likely that the ligand for tie-1 is also secreted out of the cell rather than being membrane bound. Therefore, strategies to clone a secreted ligand were adopted. Angiopoietin-1 was cloned and characterised by a novel method called secretion-trap expression cloning (140). Identification and cloning of secreted ligands is notoriously difficult and time consuming (175, 176) whilst membrane bound ligands are slightly easier to identify (177). A secreted ligand is at least temporarily trapped inside the vesicular compartments of cells until it is exocytosed. Davis *et al* (140) utilised this concept when cloning and characterising angiopoietin-1. Having fixed and permeabilised selected cell lines, they then used a tie receptor-FC fusion protein as a probe to identify the tie-2 ligand producing cells. From this they were able to identify cell lines that secreted the ligand and this led to the eventual cloning and characterisation of angiopoietin-1. The use of a receptor-epitope tagged fusion-protein probe has also been used by Cheng and Flanagan to identify ELF-1, a developmental ligand for the Mek4 and Sek receptor tyrosine kinases (174).

The aim of the work described in this chapter was to identify a ligand for tie-1. In order to identify cells which secrete the tie-1 ligand two strategies were adopted. Firstly, by using a molecular approach to construct and use a tie-1 extracellular domain-epitope tagged fusion protein as a probe to allow screening of potential ligand producing cell lines and to establish binding to a potential ligand (similar to the methods described above). Secondly, the ability of an activating ligand to induce autophosphorylation of a receptor tyrosine kinase was used

to probe for the presence of ligand. Once a cell line that produced the tie-1 ligand was identified, attempts were made to purify the ligand for N-terminal sequencing and characterisation. The experiments in this chapter are divided into two sections, the first section concentrating on the production of a tie-1 extracellular domain (ED) fusion protein and the second section describes experiments to try and isolate the tie-1 ligand.

Section I

3.2 Production of a secreted tie-1 extracellular domain fusion protein

3.2.1 Generation of Human tie-1 Receptor Extracellular Domain cDNA

Overall tie-1 is comprised of a 4400 base pair nucleotide sequence. The extracellular domain sequence is coded for by nucleotides 1-2300. The first 102bp nucleotides code for the secretory sequence of the receptor. To clone the human extracellular domain of the tie-1 receptor (tie-1ED), mRNA from HUVE cells was reverse transcribed into cDNA and amplified by PCR using specific oligonucleotides encompassing the tie-1 ED open reading frame from nucleotide 103 to 2301. The following primers were used, the design of which was based on the published sequence for human tie-1 mRNA from Genebank, accession number X60957;S89716.

Forward primer (T5): 5'- GTGGACCTGACGCTGCTGG-3' Tm = 69.6
Reverse primer (T3): 5'- CTCTTCAGCTGCCCGGCTC-3' Tm = 69.8

PCR amplification was attempted using the conditions as described in section 2.4.5. PCR products were electrophoresed on a 2% agarose gel.

PCR amplification of the 2.2 kb tie-1ED was achieved as shown in Figure 3.1. However as lanes 2-4 demonstrate there was great variability in the amplification of the 2.2kb tie-1 ED fragment even under standardised PCR conditions and this amplification was very sporadic and difficult to reproduce, so much so that it was not possible to ligate the amplified cDNA into a cloning vector.

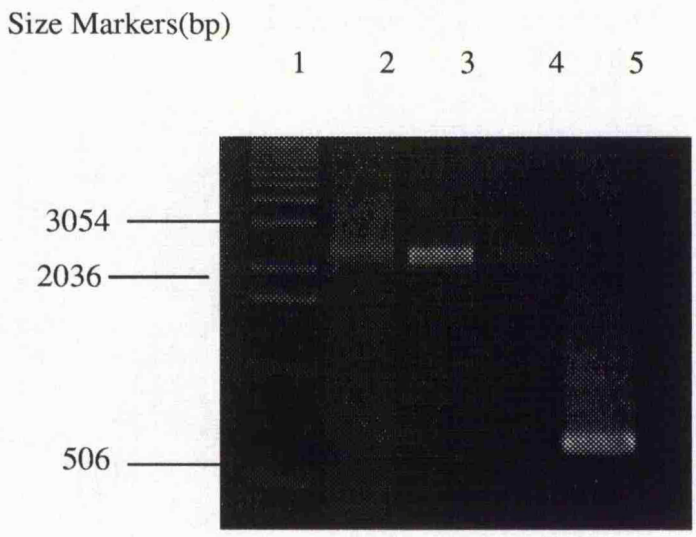


Figure 3.1 RT-PCR of human tie-1ED (2.2 kb) and β-actin (661bp). 2% agarose gel stained with ethidium bromide showing PCR amplification products using tie-1ED specific oligonucleotide primers for 3 separate reverse transcribed HUVEC mRNA's. Lane 1 is the 1kb ladder. Lane 2-4 amplification of tie-1 ED. Lane 5 amplification of β-Actin.

Shorter sequences amplify more efficiently by PCR (172) so it was decided to amplify the 2.2 kb tie-1 extracellular domain (tie-1 ED) as two smaller fragments which could be ligated. Therefore, in order to generate sufficient cDNA for ligation two further internal oligonucleotide primers were designed which would allow amplification and TA- cloning of two smaller cDNA fragments which had a sequence overlap that contained a HIND III restriction site. This would allow a point of ligation for the two clones thus generating an entire cloned tie-1ED cDNA sequence, as illustrated in Figure 3.2.

The primers were designed using the same tie-1ED sequence as for primers T5 and T3:

Forward primer (Inner): 5' - GCTGGAGAGGAAGCCAGTGCC-3' Tm = 71.6
Reverse primer (Outer): 5'- GGGGCACAAGCTTCTTGGCA-3' Tm = 71.7

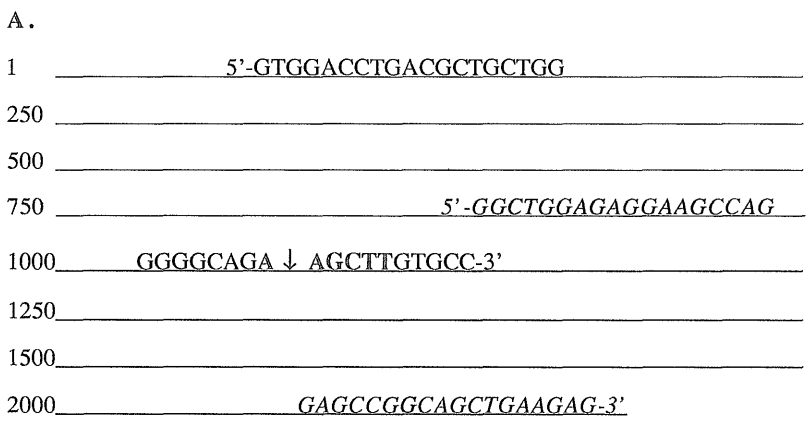


Figure 3.2 Cloning of tie-1 cDNA

A. Tie-1 Extracellular Domain cDNA sequence (2.2kb) with 18bp internal primer designed to incorporate a Hind III restriction site into both individual clones. Hind III site shown in bold in the reverse primer "outer".

B. Hind III Restriction digest site for the two independently cloned portions of tie-1 (named tie-1 ED *outer* portion and tie-1 ED *inner* portion) revealing the point of ligation of the two sequences into pCR2.1 following Hind III restriction digest. Therefore this generates a tie-1 ED outer portion cDNA sequence from nucleotide 103 to 2301.

PCR amplification using reverse transcribed HUVEC mRNA was achieved for both the tie-1 ED outer sequence and the tie-1 ED inner sequence. The products were electrophoresed on a 2% agarose gel and stained with ethidium bromide, Figure 3.3.

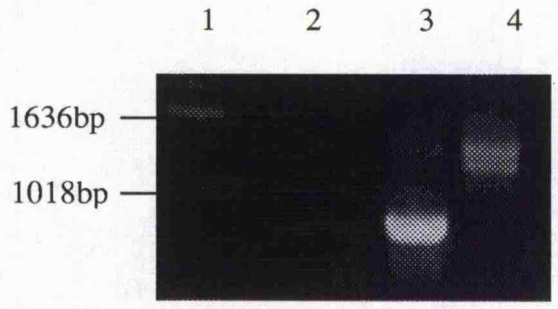


Figure 3.3 PCR amplification of tie-1 ED outer and inner sequences. Lane 1-1 kb ladder, Lane 2- tie-1 ED amplification using T5 and T3 primers, Lane 3-tie-1 ED outer sequence(854bp), Lane 4- tie-1 ED inner

The tie-1ED outer sequence and tie-1 inner sequence were independently ligated into the pCR 2.1 vectors using the TA-cloning method. Following ligation and transformation, white colonies were screened for the presence of insert. Figure 3.4 shows the positive clones identified by PCR for the tie-1ED outer sequence (A) and tie-1ED inner sequence (B). Plasmid purification was performed on these positive clones, followed by restriction analysis to check for the correct orientation of the inserts. The tie-1 ED outer sequence was digested with Hind III and if the cDNA insert was in the correct orientation then this would generate fragments of 907bp and 3880bp in size. All clones digested had the insert in the correct orientation as shown in Figure 3.5 A. Restriction analysis was performed on the tie-1 ED inner sequence with SpH I. If the cDNA insert was in the correct orientation this would generate fragments of 3645bp, 1486bp and 190bp in size. As shown in Figure 3.5 clone H generated fragments of the appropriate size and therefore this clone had the insert in the correct orientation.

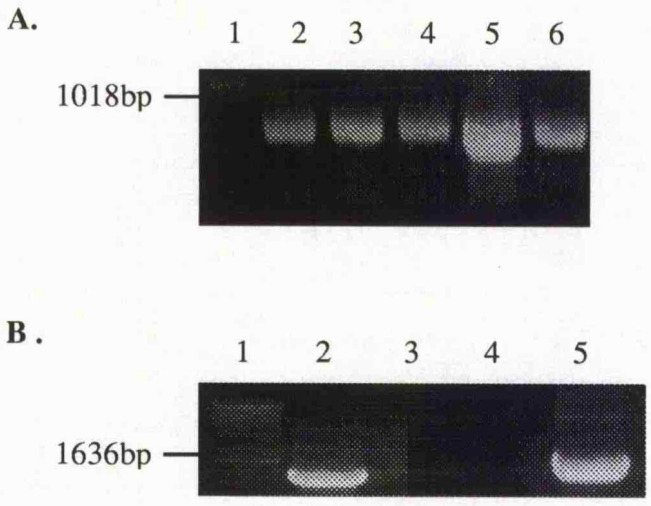


Figure 3.4 PCR screening of plasmid cultures for tie-1 ED outer (A) and inner (B) sequences. A) tie-1 ED outer sequence: Lane 1- 1 kb ladder, Lane 2-6 tie-1 ED positive clones. B) tie-1 inner sequence: Lane 1- 1 kb ladder, Lane 2- positive clone E, Lane 3-4 empty pCR 2.1 plasmid, Lane 5- positive clone H

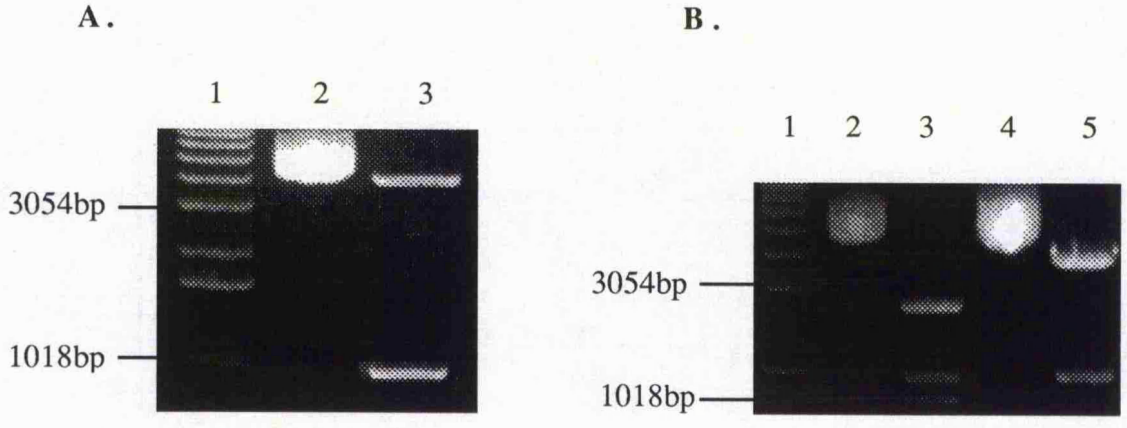


Figure 3.5 Orientation of cDNA insert of clones from (A) tie-1 ED outer sequence and (B) tie-1 ED inner sequence using restriction analysis. A) Lane 1- 1 kb ladder, Lane 2- undigested pCR 2.1/tie-1ED outer plasmid, Lane 3- pCR2.1/tie-1 ED outer plasmid digested with Hind III to give bands of 907 bp and 3880 bp. B) Lane 1-1kb ladder, Lane 2- undigested clone E, Lane 3-

SpH I digested clone E, Lane 4- undigested clone H, Lane 5- SpH I digested clone H.

The vector pCR2.1 has only one Hind III restriction site within the multiple cloning site (bases 205-339) at base position 235. This restriction site is positioned 59 base pairs(bp) upstream of the PCR product ligation point, whilst tie-1 ED has a Hind III restriction site at position 951. pCR2.1/tie-1 ED outer was digested with Hind III to generate two fragments of 907bp and 3880bp. The 907bp fragment contained the tie-1ED outer sequence (848 bp) plus the digested downstream fragment of pCR2.1(59bp). Hind III digestion of pCR2.1/tie-1ED inner generated two fragments of 88bp and 5223bp. The 5223 bp fragment contained the tie-1ED inner sequence (951bp- 2301bp) plus the pCR2.1 vector but without the 59bp sequence from site 235-294bps. The 907bp tie-1ED outer fragment was ligated with the 5223bp tie-1ED inner fragment/pCR2.1 vector to generate a clone of the entire tie-1 ED sequence from 103bp to 2301bp within the pCR2.1 vector. Figure 3.6 shows Hind III digest of tie-1 ED outer/pCR2.1(1.5μg) and tie-1 ED inner/pCR2.1(1.5μg). The products were electrophoresed at 4^0C on a 1.5% LMP agarose gel. The appropriate bands were then excised from the gel and the cDNA fragments purified using the Qiaquick gel extraction kit (Qiagen Ltd., Crawley, U.K.). To prevent recircularisation and religation of the linearised tie-1ED inner/pCR2.1 the 5' protruding end of the purified fragment was dephosphorylated using calf intestinal alkaline phosphatase. Following this, ligation of the two fragments was performed with a 1:1 insert:vector ratio. Transformation was performed as described in section 2.4.7 and white colonies were picked and screened for the entire tie-1ED sequence (103-2301bp) with PCR using the T5 and T3 oligonucleotide primers. This demonstrated several clones that had the entire tie-1 ED sequence within the pCR2.1 vector, Figure 3.7 A. The clones were analysed by restriction digestion with SmaI. SmaI cuts tie-1 ED at position 238 and 1752 but there is no restriction site within pCR2.1. The correct insertion and orientation of tie-1ED outer would generate fragments of 1494 and 4636bp in size. As shown in Figure 3.7 B all plasmids had the insert in the correct orientation.

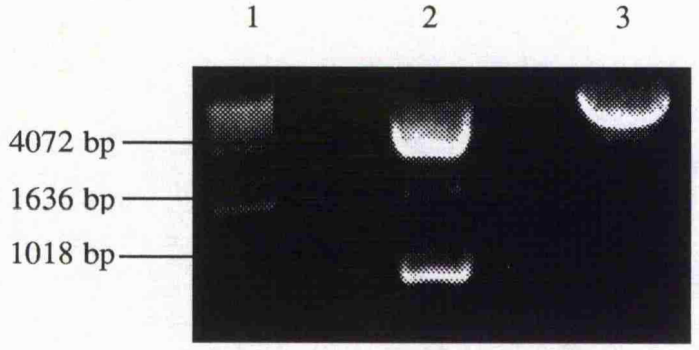


Figure 3.6 Hind III restriction digest of tie-1ED outer/ pCR2.1 and tie-1ED inner/ pCR2.1.

Lane 1- 1kb DNA ladder, Lane 2- Hind III digested tie-1ED outer/pCR2.1, Lane 3- Hind III digest of tie-1ED inner/pCR2.1.

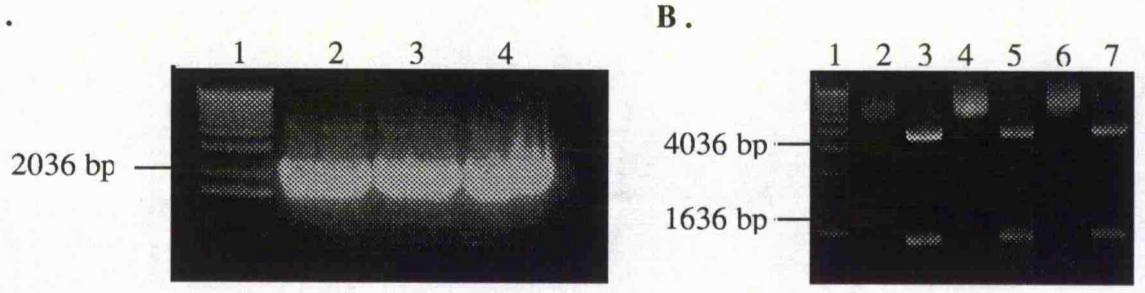


Figure 3.7 Screening of cultured colonies with PCR and restriction analysis using Sma I. A) PCR amplification of tie-1 extracellular domain (2.2 kb) from tie-1ED/pCR2.1 clones. Lane 1- 1kb ladder, Lane 2-4 screening of 3 cultured colonies following ligation and transformation of tie-1 ED outer with tie-1ED inner/pCR2.1.B) Sma I restriction analysis of the 3 purified plasmids from the 3 screened colonies. Lane 1-1kb ladder, Lane 2, 4, 6- undigested plasmids, Lane 3, 5, 7- digested tie-1 ED/pCR2.1 showing bands of 1494bp and 4636bp in size.

3.2.2 Sequencing of cloned tie-1 extracellular domain

The cloned tie-1 extracellular domain cDNA was fully sequenced using cyclical sequencing as described in section 2.4.11. Tie-1 ED cDNA was identical to the reported tie-1 sequence in Genbank accession number X60957;S89716 except for a mutation at position 1918 where in the cloned tie-1 ED sequence there was a cytosine instead of the published thymidine nucleotide (see appendix 1). However this mutated nucleotide was in a codon (CCT) which codes for leucine, the published codon sequence is CTT which also codes for the amino acid leucine, therefore the mutation was inconsequential.

3.2.3 Subcloning of tie-1 extracellular domain into the expression vector pSecTag B

Tie-1 ED was subcloned from pCR2.1 in frame into pSecTag B as described in section 2. Cultured colonies were screened by PCR with T5 and T3 primers, Figure 3.8(A). The purified plasmids were then restriction digested with Hind III to confirm correct insert and orientation, Figure 3.8 (B). The purified plasmids were then used in gene transfection.

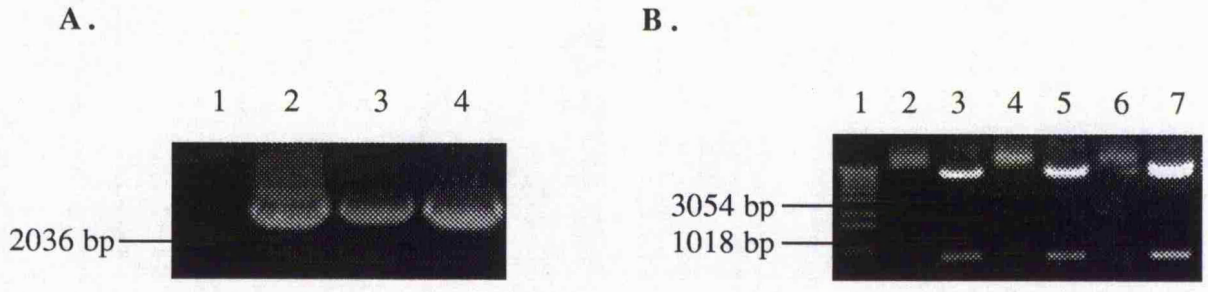


Figure 3.8 PCR amplification of tie-1 ED from pSecTag/tie-1ED (A) and restriction analysis with Hind III of pSecTag/tie-1 ED plasmids (B).
(A) Lane 1- 1kb ladder, Lane 2-4- amplified tie-1ED(2.2 kb) from pSecTag/tie-1ED positive clones.
(B) Lane1-1kb ladder, Lanes 2,4,6- undigested pSecTag/tie-1ED, Lanes 3, 5, 7- Hind III digested pSecTag/tie-1ED giving bands of 962bp and 6026bp in size which suggest the insert was in the correct orientation.

3.2.4 pSecTag/tie-1 extracellular domain transfection into Chinese Hamster Ovary (CHO's) cells- Transfection optimisation

Transient transfection of pSecTag/tie-1 ED cDNA and empty pSecTag vectors were performed into CHO cells in 6-well plates using Superfect reagent (Qiagen) as directed by the suppliers protocol and expression determined by Western blot analysis of cell lysates and samples of medium conditioned by transfected cells. A band of the correct size for the tie-1 ED-myc/His fusion protein (90 kDa) was seen, using Western blot analysis with an anti-myc antibody, in both cell lysate and conditioned medium (Figure 3.9). No bands were demonstrated of such size in the cells or medium following transfection with the empty pSecTag vector. As shown, the size of the protein band in the medium was larger than that in the cell. This is contrary to what would be expected due to the loss of the secretory sequence on secretion of the protein from the cell. It is presumed that this change in molecular weight was due to glycosylation of the protein just prior to secretion from the cell .

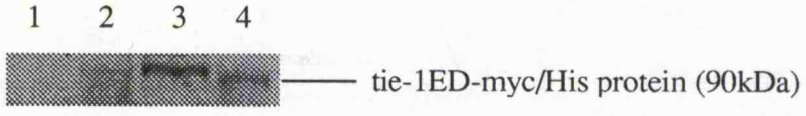


Figure 3.9 Expression of tie-1 ED-myc/His fusion protein (representative of 10 similar experiments).

Lane 1: Cell medium supernatant from cells transfected with empty pSecTag B vector. Lane 2: Cell lysate from cells transfected with empty pSecTag B vector. Lane 3: Cell medium supernatant from cells transfected with pSecTag/tie-1 ED. Lane 4: Cell lysate from cells transfected with pSecTag/tie-1 ED. Each track of cell lysate corresponded to approximately 1/10 of a single well of a 6 well plate. Each track of medium corresponded to 1/25 of the medium conditioned by a single well of a 6 well plate.

Using a DNA: Superfect ratio of 1:13, optimisation of the amount of DNA to transfect CHO cells was sought. Figure 3.10 shows the resultant increase in fusion protein expression and secretion associated with increasing the amount of DNA that was used to transfect cells.

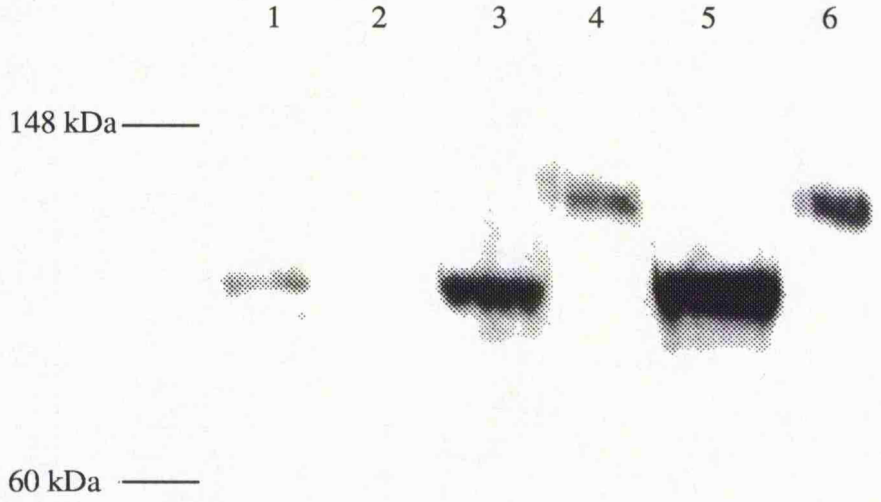


Figure 3.10 The effect of increasing transfected DNA concentration on the level of expression and secretion of the tie-1 ED-myc/His fusion protein in CHO cells.

CHO cells in a 6 well plate were transfected with 1.5μg, 2.0μg or 3.0μg of pSecTag/tie-1 ED. The ratio of DNA:Superfect was 1:13 for all transfections. Cell lysates and cell medium supernatants from equal numbers of cells were mixed with sample buffer containing 100mM DTT. Each track of cell lysate corresponded to approximately 1/10 of a single well of a 6 well plate. Each track of medium corresponded to 1/25 of the medium conditioned by a single well of a 6 well plate. Immunoblotting was performed with an anti-myc antibody. This experiment was performed once.

Lane 1: Cells lysates from cells transfected with 1.5μg of DNA.

Lane 2: Cell medium supernatants from cells transfected with 1.5μg of DNA

Lane 3: Cells lysates from cells transfected with 2.0μg of DNA.

Lane 4: Cell medium supernatants from cells transfected with 2.0μg of DNA

Lane 5: Cells lysates from cells transfected with 3.0μg of DNA.

Lane 6: Cell medium supernatants from cells transfected with 3.0μg of DNA

Optimisation of the DNA:Superfect ratio was also performed as shown in Figure 3.11. Ratios of DNA:Superfect that were tested were 1:5, 1:10, 1:13, 1:15 and 1:18. As shown in Figure 3.11, this clearly demonstrated that the optimum DNA: Superfect ratio for transfection lies between 1: 10 to 1:18. Thus all further transfections in 6 well plates were performed using 3.0μg of pSecTag/tie-1ED DNA and at a DNA:Superfect ratio of 1:13.

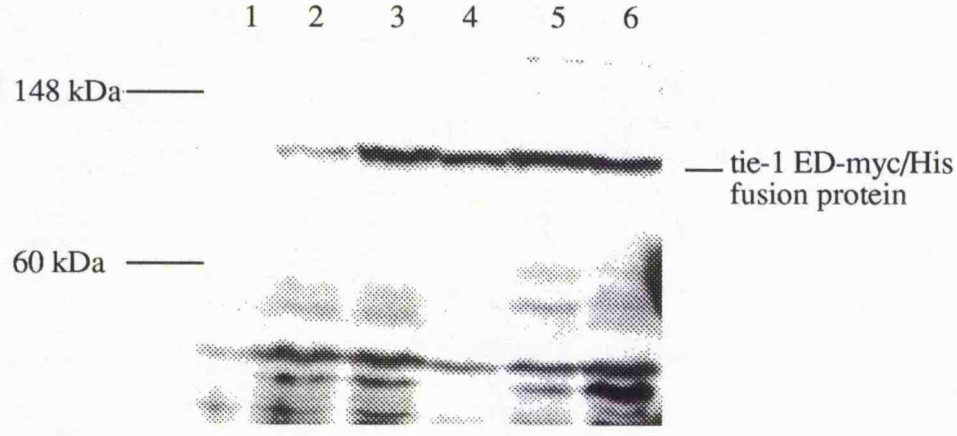


Figure 3.11 The effect of differing pSecTag/tie-1ED:Superfect ratio on the expression of tie-1ED-myc/His fusion protein in CHO cell lysates. CHO cells in a 6 well plate were transfected with pSecTag empty vector or pSecTag/tie-1 ED. Cell lysates from equal numbers of cells were mixed with sample buffer containing 100mM DTT. Each track of cell lysate corresponded to approximately 1/10 of a single well of a 6 well plate. Immunoblotting was performed with an anti-myc antibody. This experiment was performed once.

Lane 1: CHO cells transfected with pSecTag empty vector (control) Lane 2-6: CHO cells transfected with pSecTag/tie-1ED. Lane 2: DNA:Superfect ratio of 1:5 Lane 3: DNA:Superfect ratio of 1:10, Lane 4: DNA:Superfect ratio of 1:13. Lane 5: DNA:Superfect ratio of 1:15 and Lane 6: DNA:Superfect ratio of 1: 18.

3.2.5 Recovery of tie-1 ED-myc/His fusion protein from transfected CHO cell media

Figure 3.9 shows that cells transfected with pSecTag/tie-1 ED expressed a tie-1 ED-myc/His fusion protein and that this fusion protein was secreted into the surrounding cell medium. In this experiment an aliquot of cell media was simply mixed with an equal volume of 2x SB-DTT followed by boiling for 5 minutes and centrifugation.

In order to use the fusion protein as probe for a tie-1 ligand, it was necessary to recover the ectodomain fusion protein under non denaturing conditions. This could be achieved either by anti-myc immunoprecipitation or utilising the C-terminal His ability to bind to a nickel chelating resin (Probond column).

Therefore to investigate the recovery of the fusion-protein from the cell culture media, the binding of the tie-1ED-myc/His fusion protein to the Probond resin was examined alongside the ability of this fusion-protein to be immunoprecipitated by an anti-myc antibody.
CHO cells were transfected with pSecTag/tie-1 ED, cultured and the medium collected as described in section 2.5.2. The binding of the tie-1ED-myc/His fusion protein to the Probond resin and the ability of this fusion-protein to be immunoprecipitated by an anti-myc antibody are shown in Figure 3.12. In the case of binding to the Probond resin, the ability of the bound tie-1 ED-myc/His fusion protein to be eluted from the resin was also examined. To examine this, resin was incubated with conditioned medium from tie-1 ED-myc/His expressing cells, then recovered, washed and equal amounts subjected to pH elution as described in the manufacturers protocol (pH= 4.0) or mixed with 2x SB-DTT and boiled to elute all bound fusion-protein from the beads.

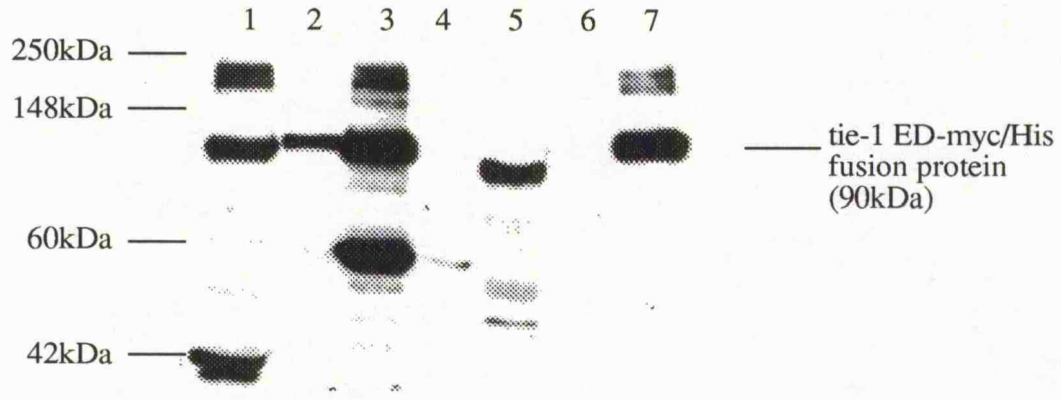


Figure 3.12 Recovery of tie-1ED-myc/His fusion protein from transfected CHO media. CHO cells in 6 well plates were transfected with tie-1 ED. Immunoblotting was performed with an anti-myc antibody. Shown is a representative immunoblot of 3 similar experiments.

Lane 1: Whole cell lysates from transfected CHO cells mixed with 1x SB-DTT. Each track of cell lysate corresponded to approximately 1/10 of a single well of a 6 well plate.

Lane 2: 5x concentrated transfected cell culture media mixed with an equal volume of 2x SB-DTT. For all protein recovered from conditioned medium the medium contained secreted protein corresponding to 1/5 of a well of a 6 well plate.

Lane 3: Immunoprecipitation of fusion-protein from transfected cell culture media with anti-myc antibody, Lane 4: Blank.

Lane 5: Supernatant of transfected cell culture media after incubation with Probond resin, mixed with an equal volume of 2x SB-DTT.

Lane 6: Eluate after pH elution (pH=4.0) method to remove fusion-protein off the resin mixed with an equal volume of 2x SB-DTT.

Lane 7: Fusion-protein remaining on resin following pH elution determined by denaturing elution in 2x SB-DTT.

Lanes 1 and 2 of Figure 3.12 demonstrate both the expression and secretion of the tie-1ED-myc/His fusion protein respectively, with the secreted protein being slightly larger than the cellular protein. Lane 3 shows that the fusion protein could be immunoprecipitated from the media, the large immunoreactive band seen at 60kDa corresponds to immunoglobulin used in the immunoprecipitation. Lanes 5-7 demonstrate the ability of the Probond resin to bind the fusion protein and its capacity to be eluted from the resin. Lane 7 shows that tie-1ED-myc/His fusion proteins binds quite readily to the resin however none was eluted off the resin by lowering the pH to 4.0. This proved to be the case for all subsequent experiments. In the blot shown the fusion-protein in lane 5 has a molecular weight of 75kDa. This may represent fusion protein that has undergone proteolysis, but it is intriguing that this is the only band in this lane, proteolysis would presumably produce multiple bands in this track. In addition a band at 180kDa is seen in lanes 1,3 and 7. The identity of this was not known, though it could represent a dimeric form of the tie fusion protein.

Therefore overall, tie-1 ED-myc/His fusion protein was expressed and secreted from transfected CHO cells and could be recovered by both immunoprecipitation from the culture media and incubation and binding to a nickel chelating resin. However, it was not possible to elute appreciable amounts of fusion protein from the resin under native conditions using the pH elution method. Therefore, when using the tie-1ED-myc/His as a probe the fusion protein was not recovered by the Probond resin but used directly in conditioned medium from expressing cells after a ten-fold concentration. This is a similar strategy to that used by Davis et al (140).

Section II

3.3 Identification of cells secreting a ligand for the tie-1 receptor

3.3.1 Malignant melanoma cell lines as a potential source of the tie-1 ligand

It has been recognised that for tumours to grow above a critical size and to metastasise they need to undergo angiogenesis (1, 4). Furthermore tumours are known to produce and secrete a wide range of angiogenic growth factors which are thought to initiate and potentiate the angiogenic process (178, 179). It has now been demonstrated that tie-1 mRNA and protein expression are increased in the endothelia of malignant tumours, particularly highly malignant brain tumours (153, 180) and in invasive breast carcinomas (157). Furthermore, Kaipainen *et al* have found increased expression of tie-1 mRNA and protein in primary cutaneous malignant melanomas and in melanoma metastases compared to skin and normal melanocytes (156). Cutaneous malignant melanomas are highly virulent tumours that have marked angiogenic activity. Increased expression and secretion of bFGF (181, 182, 183), PDGF (181) and VEGF (67) and numerous other growth factors (181, 182) have all been shown to occur in malignant melanoma cell culture lines *in vitro*. In addition, many malignant melanoma cell lines have been shown to have abnormal receptor protein tyrosine kinase gene expression (KDR, FGR-4, IGF-1R), including the tie-1 receptor tyrosine kinase, in the DX3-LT5.1 melanoma cell line (184, 185). Therefore, malignant melanoma cell lines were the first cells investigated for the presence of a tie-1 receptor activating ligand.

3.3.2 Results

3.3.2.1 The effect of medium conditioned by SK-Mel 24 cells on endothelial cell proliferation

To confirm that the malignant melanoma cell lines secrete growth factors that result in the proliferation of endothelial cells, conditioned media from these cells was added to quiesced HUVE cells for 24 hours. The extent of HUVE cell DNA synthesis was determined using the incorporation of BrdU into cellular DNA in place of thymidine. Figure 3.13 shows the proliferative effect of conditioned media from SK-Mel 24 cells on HUVE cells. For this experiment conditioned melanoma media from Sk-Mel 24 caused a 3 fold increase in proliferation rate of proliferation of HUVE cells when compared to control cells, this increase was comparable to that caused by VEGF. Similar results were achieved with two other melanoma cell lines, G361 and RPMI-7951 cells (data not shown).

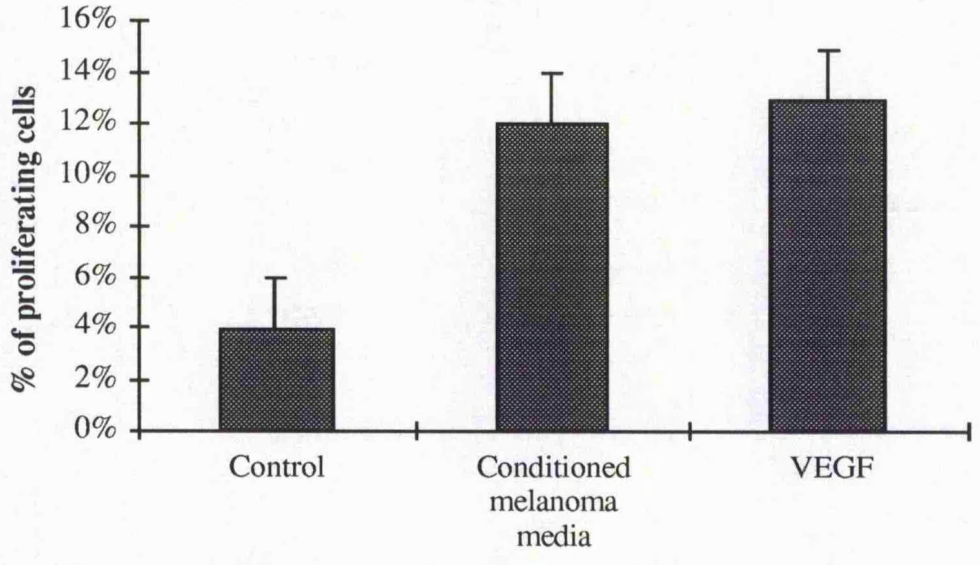


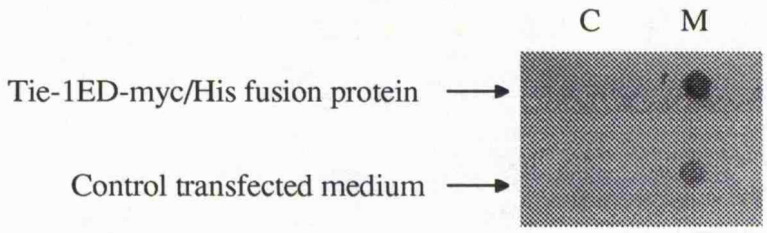
Figure 3.13 The effect of SK-Mel 24 melanoma cell conditioned medium and VEGF on the proliferation of HUVE cells using BrdU incorporation as a marker of DNAsynthesis. Values shown are means and SEM for 4 independent experiments. Serum free medium was conditioned by melanoma cells for 12 hours at 37^{0}C.

The medium was collected and centrifuged at 3000g for 10 minutes. Confluent monolayers of HUVE cells were quiesced for 24 hours in serum free medium. Melanoma conditioned medium was placed on HUVE cells for 24 hours and proliferation detected by BrdU incorporation as described in section 2.3.

3.3.2.2 Screening SK-Mel 24 conditioned media with tie-1ED-myc/His fusion protein

To investigate the possibility that SK-Mel 24 cells secrete a tie-1 ligand, conditioned SK-Mel 24 media was immobilised onto a nitrocellulose strip and probed with the tie-1 ED-myc/His fusion protein. For the purpose of this experiment, fusion protein was generated from CHO cells in T-80 flasks. Following transfection, CHO cells were cultured in complete medium for 24 hours and then used to condition serum free medium for 48 hours. The collected medium was then concentrated 5 fold and diluted 1:1 in PBS with 2% FCS and 0.1% Tween 20. To determine whether SK-Mel 24 cells secreted a ligand for tie-1, 10 fold concentrated conditioned SK-Mel 24 media (70% confluent cells cultured for 24 hours in serum free medium) was immobilised onto nitrocellulose strips and probed with either tie-1 ED-myc/His fusion protein or with media from cells that had been transfected with empty pSecTag B vector. The Dot-Blot in Figure 3.14 demonstrates that tie-1ED-myc/His binds proteins immobilised on nitrocellulose from Sk-Mel conditioned medium. There was no binding to proteins immobilised from the spot that had immobilised media that had not been in contact with SK-Mel 24 cells. Non-specific binding was determined by incubation of membranes with conditioned medium from cells transfected with pSecTag empty vector.

A.



B.

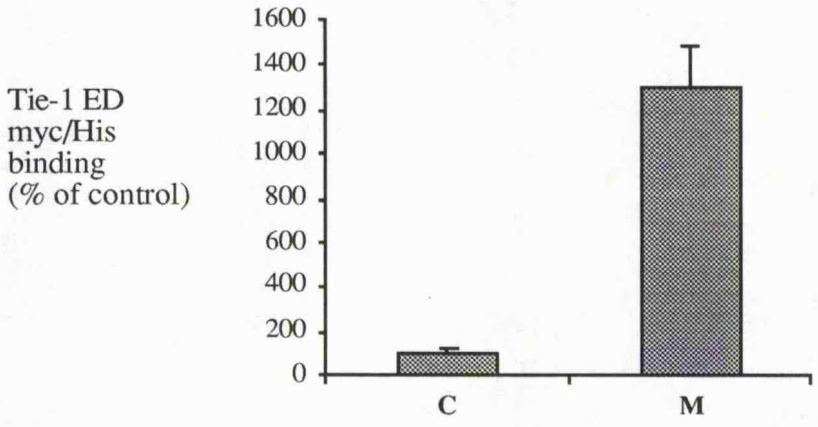


Figure 3.14 Tie-1 ED-myc/His binding to immobilised SK-Mel 24 medium on nitrocellulose strips. Serum free medium conditioned for 24 hours by a 80cm^2 flask of confluent SK-Mel 24 cells, was concentrated ten fold and immobilised onto nitrocellulose strips. Following blocking the membranes were probed with the tie-1 ED-myc/His fusion protein or media from CHO's transfected with pSecTag, and binding of the fusion-protein determined by anti-myc immunodetection. Tie-1 ED-myc/His fusion protein was used as a probe to investigate binding to a potential tie-1 ligand in SK-Mel 24 conditioned medium. Binding of the fusion protein and control medium from cells transfected with empty pSecTag vector were tested on conditioned medium from quiesced SK-Mel 24 cells (M) and on control medium (C) that had never been in contact with cells. Immunoreactive spots were scanned using a LKB ultrascan densitometer. The results shown in (B) are for three independent experiments and values represent means and SEM for the percentage increase in tie-1 ED-myc/His binding compared to control medium after correcting for non-specific binding seen with control transfected medium.

Therefore, it appeared that the tie-1 ED-myc/His protein bound to a component in the SK-Mel 24 conditioned medium that was not present in medium that had not been in contact with SK-Mel 24 cells. An additional control that could have been used was an ectodomain other than tie-1 e.g. Trk A ED-myc/His fusion protein, to establish the specificity of the tie-1 ED-myc/His binding. However, as it is possible that the melanoma cells produce a wide range of growth factors and ligands, such a fusion protein may well have reacted with the medium. An alternative negative control would have been to use a fusion protein not expected to have binding partners in melanoma conditioned medium, e.g. BSA-myc-His.

3.3.2.3 Receptor phosphorylation as an indicator of binding of an activating ligand to a receptor tyrosine kinase

Tie-1 is a tyrosine kinase receptor and, therefore, it will autophosphorylate on activation by a specific ligand (63). It would be anticipated that rapid activation of phosphorylation of tie-1 in endothelial cells would be consistent with the presence of an activating ligand for tie-1. To determine whether conditioned medium from SK-Mel 24 cells was capable of initiating phosphorylation of tie-1, quiesced HUVE cells were challenged with melanoma cell conditioned medium and tie-1 phosphorylation state examined. As shown in Figure 3.15 A. Probing anti-phosphotyrosine immunoprecipitates from HUVE cells with an antibody to tie-1 demonstrated an increase in the 135 kDa tie-1 immunoprecipitated by anti-phosphotyrosine antibody. The specificity of the anti-tie-1 antibody is described in Figure 5.2 (see later). This suggested that melanoma medium contained a component which increases the number of tie-1 molecules that are tyrosine phosphorylated. The anti-phosphotyrosine probe of Figure 3.15 A. demonstrates there was increased phosphorylation of an immunoreactive band corresponding to the size of the tie-1 receptor, following contact of HUVE cells with melanoma conditioned media. This experiment was repeated on 5 independent occasions. Immunoreactive bands were scanned using an LKB Ultrascan densitometer. There was marked variability in the level of activation of tie-1 by the conditioned melanoma media, with a mean level of phosphorylation of 2.5 times above that of control cells. This did not reach statistical significance. However, overall this suggests that there was an activating ligand for

the tie-1 receptor within the melanoma conditioned media of Sk-Mel cells. Interestingly, the presence of a 40 kDa tie-1 reactive band was consistently observed in anti-phosphotyrosine immunoprecipitations of endothelial cells following treatment with conditioned medium (this finding will be discussed in Chapter 5).

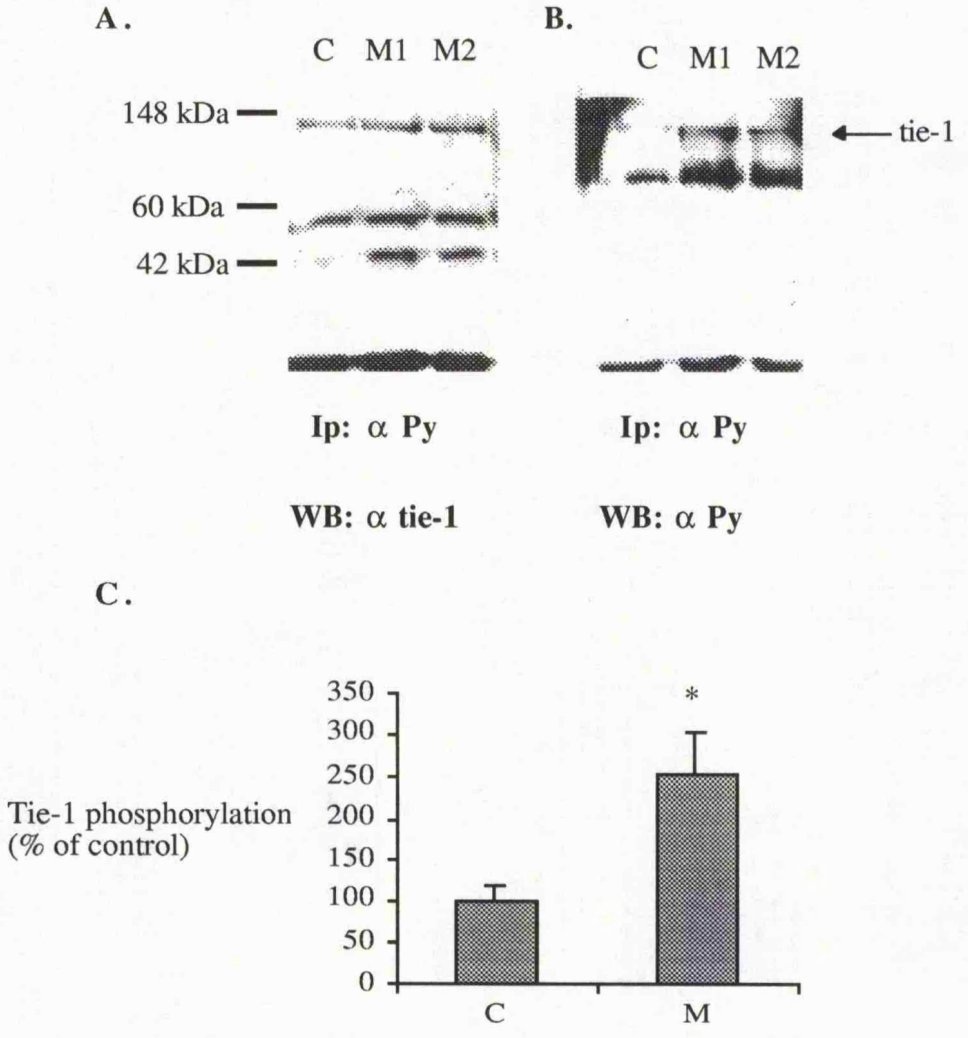


Figure 3.15 Phosphorylation of tie-1 following addition of Sk-Mel 24 conditioned medium onto HUVE cells. Quiesced HUVE cells were placed in contact with melanoma conditioned medium for 5 minutes (M1) or 10 minutes (M2) and phosphorylation of tie-1 receptor was compared to control cells that had only been quiesced (C). Immunoprecipitation (Ip) using anti-phosphotyrosine antibody (αPy). Western blot (A) was probed with

anti-tie-1 antibody (α tie-1) then stripped and reprobed (B) with an anti-phosphotyrosine antibody (αPy). The results for five independent experiments are shown (C), these values represent the mean and SEM for the percentage increase in phosphorylation against control cells after incubation of HUVE cells for 10 minutes with SK-Mel 24 conditioned medium (M). * p< 0.06 (paired t-test) versus non-treated control cells.

If the component of melanoma conditioned medium that induced tie-1 tyrosine phosphorylation was a ligand for tie-1, it would be anticipated that tie-1 ED-myc/His fusion protein could be used to deplete the medium of this component. To determine whether this was the case, tie-1 ED-myc/His fusion protein was bound to the Probond resin, as previously described, and then incubated with conditioned SK-Mel 24 media for 1 hour at 4^0C. The slurry was then centrifuged and the supernatant assessed for its ability to activate tie-1 phosphorylation. Figure 3.16 shows that again Sk-Mel 24 conditioned media caused phosphorylation of the tie-1 receptor at 135 kDa, however, there was a small decrease in phosphorylation following incubation with tie-1 ED-myc/His. It was unlikely that this decrease was due to non-specific binding or reaction of the activating factor with Probond resin, as medium treated with trkA- myc/His/ Probond resin did not show such a large decrease. The blot was stripped and probed with anti-tie-1 antibody which confirmed a tie-1 immunoreactive band at 135 kDa which corresponds to the phosphorylated band in Figure 3.16. The ability of tie-1 ED resin to deplete the SK-Mel 24 conditioned medium of activity was variable and small. Although this suggested the activating factor did not bind tie-1 ED, it is also possible that proteolysis of tie-1 ED on the resin may have resulted in incomplete depletion of the activity from the medium. This finding is discussed in more detail in Chapter 5 and later in this chapter. As discussed in chapter 1, tie-2 ligands, angiopoietin-1 and -2 are glycoproteins. It is possible that the activating ligand for tie-1 is a glycoprotein. To determine whether this was the case, conditioned medium from melanoma cells was depleted of glycoprotein by incubation with immobilised wheat germ agglutinin and the ability of this depleted medium to activate tie-1 autophosphorylation was determined (Figure 3.16). As can

be seen in Figure 3.16, depletion by wheat germ agglutinin decreased the ability of the conditioned medium to activate tie-1 autophosphorylation. This is consistent with the activating ligand binding to wheat germ agglutinin. To confirm this glycoproteins could be eluted from the WGA by competition with N-acetyl glucosamine and tested for their ability to stimulate tie-1 phosphorylation. A further test for of the specificity of binding to WGA would have been to examine the effect of incubating medium with sepharose. The apparent increase in phosphorylation observed when cells were treated with non-conditioned medium that had been incubated with WGA-sepharose was suprising. The reason for this is not known. It is possible that not all of the WGA-sepharose was removed from the medium prior to addition to HUVE cells or there may have been some free WGA released in the medium. If this were the case it would be likely to cross link glycoproteins such as tie-1 at the cells surface leading to artifactual stimulation of phosphorylation.

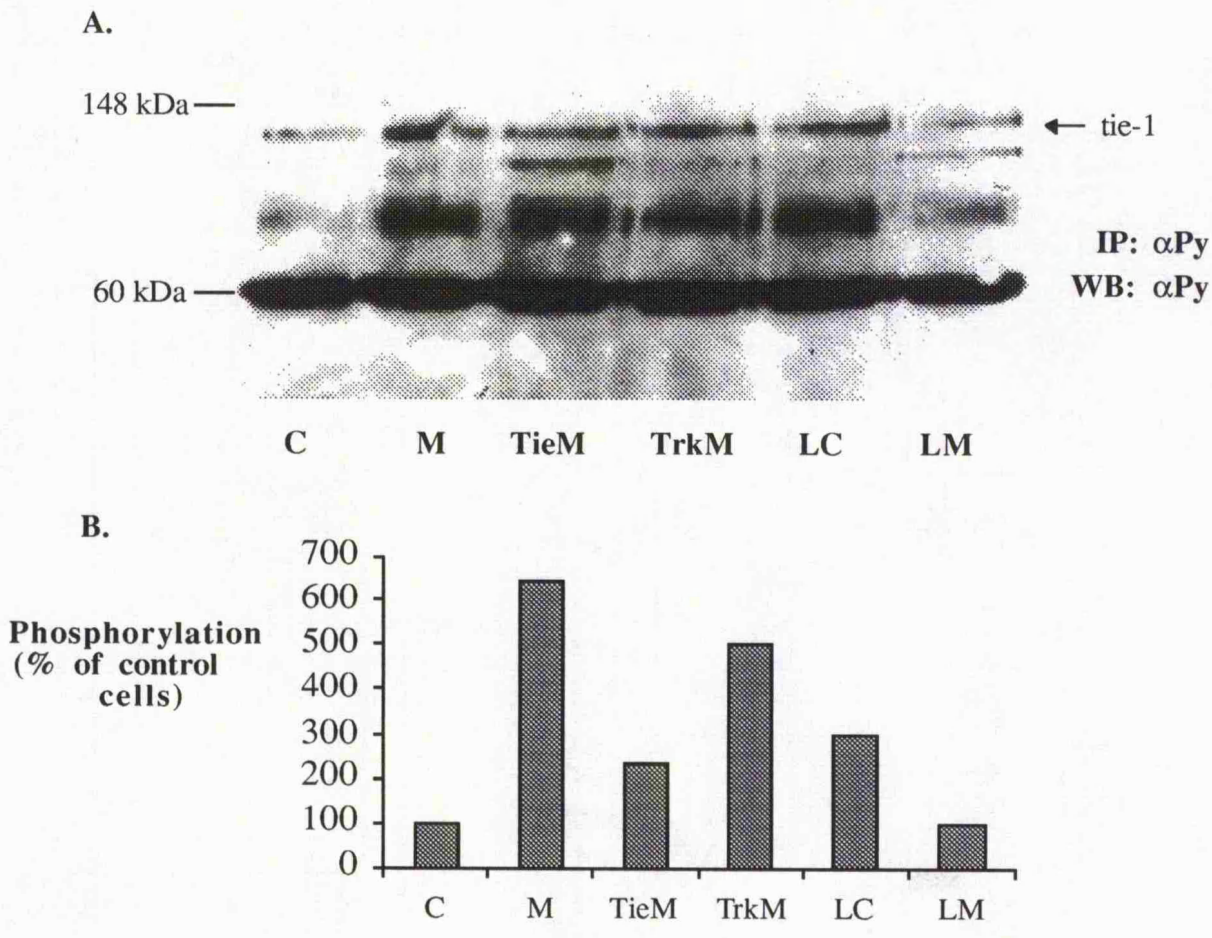


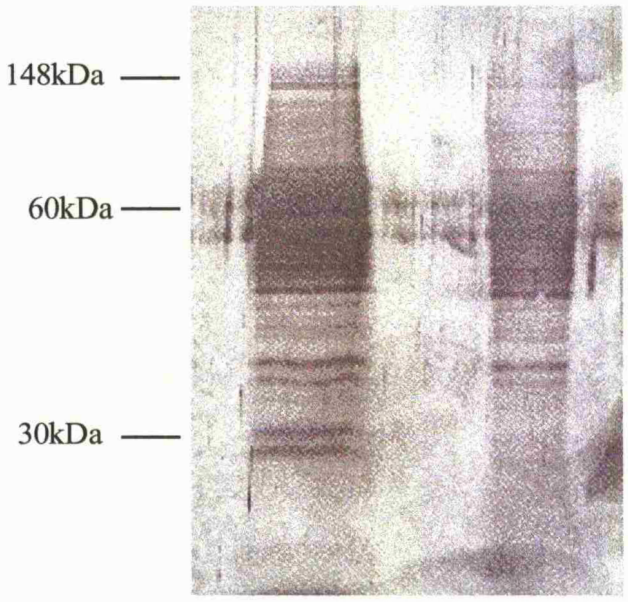
Figure 3.16 Tie-1 receptor phosphorylation in HUVE cells after addition of Sk-Mel 24 conditioned medium. Sk-Mel 24 conditioned medium (M) was

incubated with tie-1ED-myc/His bound to Probond Resin (TieM), Trk A ED-myc/His bound to Probond Resin (TrkM) or WGA-sepharose (LM) for 3 hours at 4° C. Sk-Mel serum-free medium that had not been in contact with cells was incubated with lectin-Sepharose as a control (LC). All media were incubated with quiesced HUVE cells for 10 minutes at 37°C, followed by washing with PBS. Cell lysates were incubated with anti-phosphotyrosine antibody on agarose for 3 hours for 4°C. The immunoprecipitates (IP) were centrifuged, washed with PBS and resolved by SDS-PAGE. Tie-1 phosphorylated proteins were analysed by (WB) Western blotting (A). The blot was probed with an anti-phosphotyrosine antibody (αPy), the blot was then stripped and reprobed with an anti-tie-1 antibody and the position of tie-1 is indicated. The optical densities of immunoreactive bands were measured and the values shown in B. are the percentage increases in phosphorylation compared to control cells (C) treated with unconditioned medium. The blot and histogram are from a single experiment representative of two independent experiments.

3.3.2.4 Attempted isolation of the tie-1 ligand

The experiments described above indicated that media conditioned by melanoma Sk-Mel 24 cells contained a component or components that can bind tie-1 ED-myc/His and induce tyrosine phosphorylation of tie-1. The simplest interpretation of these data was that the medium contained a ligand(s) for tie-1. In order to isolate the tie-1 ligand from SK-Mel conditioned media, the media was incubated with tie-1 ED-myc/His fusion-protein following which any ligand that was bound to the fusion-protein was eluted off using the pH elution method (section 2.5.2). The TrkA ED myc/His fusion protein was used as a control. In order to visualise the proteins following transfer onto nitrocellulose a sensitive protein stain was used, Protogold (British Biocell, Cardiff) followed by Silver enhancement.

A.



B.

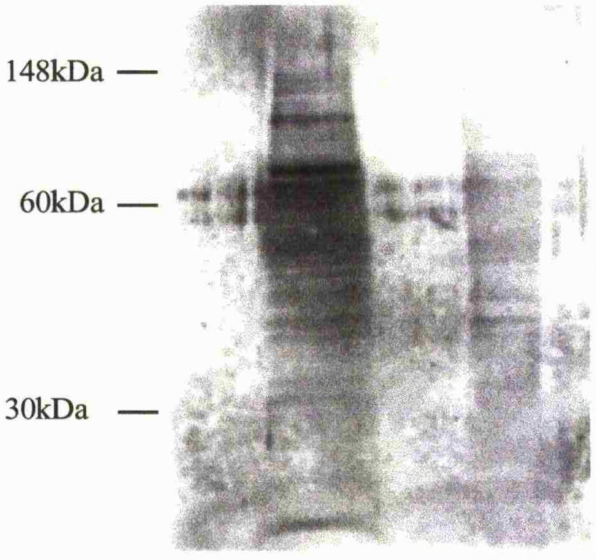


Figure 3.17 Protein analysis of conditioned Sk-Mel 24 medium. Protogold staining with silver enhancement (A) of the eluates of conditioned Sk-Mel medium incubated with tie-1ED myc/His or TrkA ED-myc/His fusion proteins bound to Probond Resins. Proteins bound to the fusion-proteins were eluted by pH elution method, as described in section 2.5.2. The

membrane was then incubated with biotinylated-wheat germ agglutinin to identify any glycoproteins(B). The membrane was blocked with 5% BSA in TBS-T for 1 hour at 37°C followed by incubation with 10μg/ml of biotinylated lectin (from Tritcum Vulgaris) for 1 hour at 37°C. Lectin was detected by Streptavidin and the developed with ECL reagents. This represents a single experiment. Concentrated medium from eight x 80 cm² flasks of confluent SK-Mel 24 cells was used for incubating with tie-1 ED or trkA ED.

Figure 3.17 A. is the Protogold and silver enhanced Western blot of the eluates from tie-1 ED and TrkA fusion proteins bound to the Probond Resin. There were numerous proteins eluted from both tie-1 and trkA fusion protein columns. The result of this pilot experiment indicated more stringent washing conditions and additional purification steps would probably be required.

3.3.2.5 Incubation of SK-Mel 24 media with Wheat Germ Agglutinin

The ligands for tie-2, Angiopoietin-1 and 2, are glycoproteins and it is possible that the tie-1ED ligand is also glycosylated. This was supported by the data shown in figure 3.16 that showed incubation of Sk-Mel 24 conditioned medium with lectin-Sepharose removed a component from the medium that activated tie-1 phosphorylation. To establish if any proteins transferred onto the nitrocellulose membrane in Figure 3.17 A. were glycoproteins the blot was probed with biotinylated WGA. Figure 3.17 B. shows a glycoprotein of approximately 80-90kDa in size that was present in the tie-1 ED eluate but not the TrkA eluate lanes. If the ligand is a glycoprotein capable of binding WGA, it may be possible to use WGA binding as an initial stage in purification prior to the tie-1 ED affinity resin.

The number of proteins secreted by SK-Mel 24 cells that bound to WGA and could be detected by protogold staining was examined.

Figure 3.18 shows that the SK-Mel 24 cells produced and secreted several proteins that could bind WGA. It was considered that initial fractionation by WGA binding followed by affinity isolation of the tie-1 ED binding proteins eluted from the lectin may improve purification. Furthermore, at this time other experiments (see chapter 5) indicated that the tie-1 ED-myc/His is subject to cleavage which could be inhibited by the metalloprotease inhibitor, marimastat. Therefore the purification steps were undertaken in the presence of marimastat. Glycoproteins from SK-Mel 24 conditioned medium were bound to lectin-Sepharose (WGA) and eluted with N-acetyl glucosamine (186). The resultant supernatant was then incubated with tie-1 ED-myc/His fusion protein in the presence of marimastat, in order to further isolate the possible tie-1 ligand. Trk A ED fusion protein was used as a control.

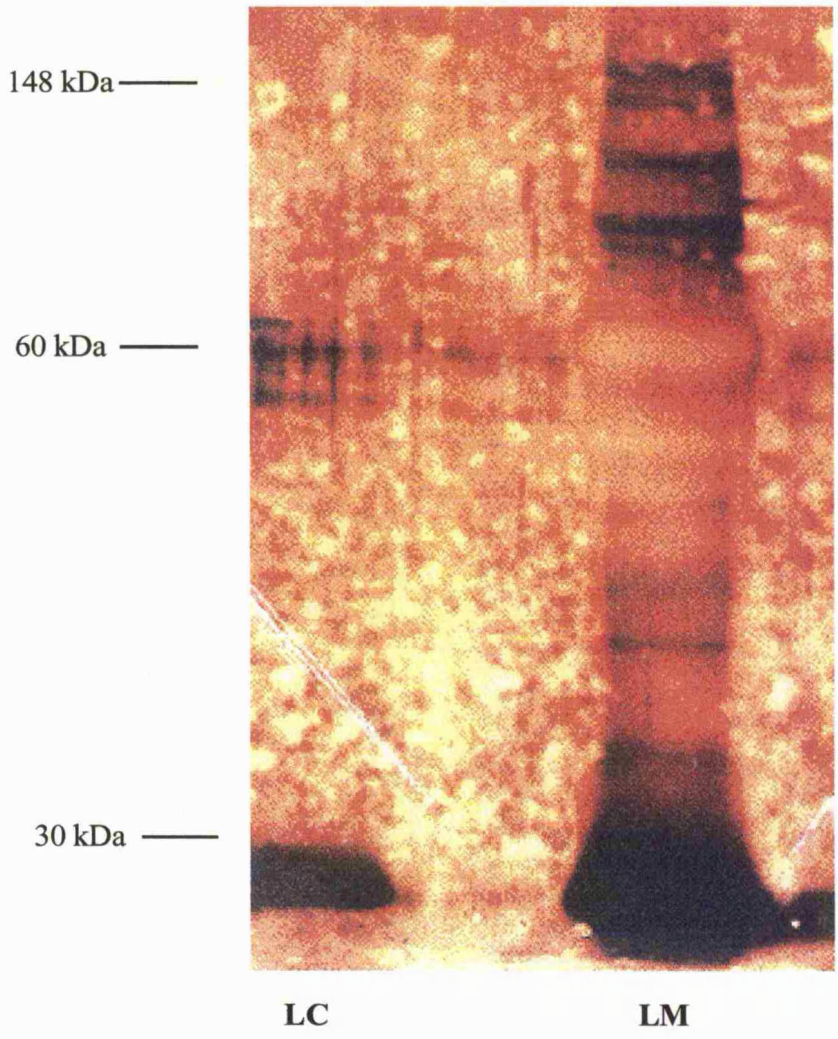


Figure 3.18 WGA binding of glycoproteins from conditioned (LM) and unconditioned (LC) Sk-Mel 24 media. Glycoproteins were bound to WGA from concentrated medium conditioned by ten 80cm^2 flasks of SK-Mel 24 cells and the same volume of unconditioned Sk-Mel 24 medium as described in the text and resolved by SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and stained with Protogold with Silver enhancement. This is representative of two experiments.

Figure 3.19 clearly shows several bands in the tie-1 ED fusion protein lane that are not present in the TrkA fusion protein lane. These bands may be possible candidates for the tie-1

ligand, especially the prominent band at approximately 80kDa which was present in the other blots of Figures 3.17 and 3.18. Tie-1 ED-myc/His fusion protein appeared as a faint band at approximately 90kDa. Further purification and the direct testing of each of these proteins for their ability to bind tie-1 ED and to activate receptor phosphorylation will be required before any of the proteins can be designated as putative ligands.

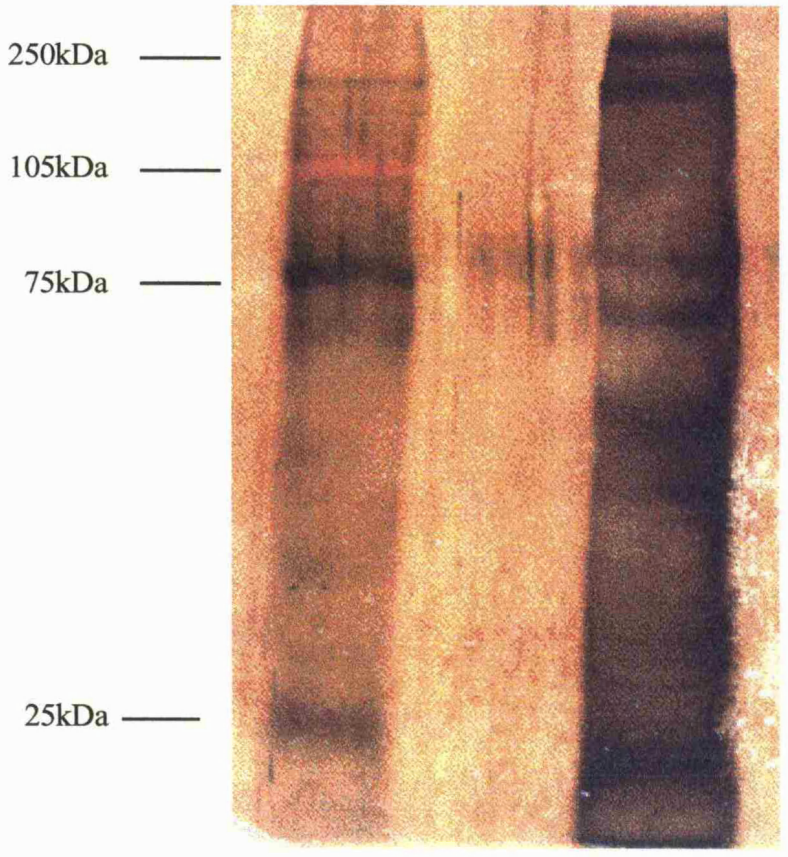


Figure 3.19 Attempted isolation of tie-1 ligand from Sk-Mel 24 conditioned medium using a multistep purification process in the presence of marimastat. Glycoproteins were isolated from concentrated medium from five 80cm^2 confluent flasks of Sk-Mel 24 cells using wheat germ agglutinin affinity chromatography, followed by elution by incubating for 30 minutes at room temperature with 0.5 M N-acetyl glucosamine in PBS. The eluates were then incubated with either tie-1 ED-myc/His or TrkA ED-myc/his fusion proteins bound to Probond resin, in the presence of 1x10^{-5} M marimastat. To the

washed resin samples (tie-1 ED eluate and TrkA eluate) was added an equal volume of 2xSB-DTT which was vortexed, boiled and centrifuged. The samples were analysed using SDS-PAGE and transferred to a nitrocellulose membrane. Proteins were identified by Protogold protein stain and silver enhancement. This experiment was performed once.

3.4 Discussion

Tie-1 and tie-2 are endothelial cell tyrosine kinases receptors whose functions are essential for the later stages of blood vessel development and maturation. They possess very similar structural characteristics within the extracellular domains/ligand binding domains, containing approximately a 40-50% amino acid identity (134). Angiopoietin-1 is a 70-80 kDa glycoprotein which is a natural activating ligand for tie-2, however it does not activate tie-1 (140). Angiopoietin-2 is an antagonist for angiopoietin-1(141). Given the similarity between the tie-1 and tie-2 receptors it is possible that they bind ligands of approximately the same size and structure. Despite angiopoietin-1 being cloned, by a method termed secretion-trap cloning (140), at the time of writing a ligand for tie-1 has not been identified. This suggests that the nature of the tie-1 receptor /ligand system is different and perhaps more complex than that of tie-2.

At the beginning of the work presented in this thesis, very little was known about the tie receptor family and at that time there were no known ligands for tie-1 or tie-2. Furthermore, it was not recognised that the tie-1 receptor underwent ectodomain cleavage. Ligands for several other receptors had been isolated and cloned using a tagged receptor approach e.g. ELF-1 (174). Therefore, this method was initially adopted to help isolate the tie-1 ligand. As highlighted in Section I of this chapter, cloning of the tie-1 ectodomain involved a multistep process due to the inability of being able to amplify the entire tie-1 ED cDNA sequence by PCR. Reasons for this could partly be due to the failure to amplify large size PCR product of 2.2kb, lack of optimisation of the PCR conditions or inadequate primer design. Once tie-1 ED had been subcloned into the expression vector pSecTag, transient transfection into CHO cells allowed production of tie-1 ED-myc/His fusion protein into conditioned media. An attempt was made to isolate and culture stable transfected CHO cells by utilising the Zeocin resistance sequence within the pSecTag vector. This was unsuccessful.

Tie-1 is a receptor tyrosine kinase, therefore, on binding an activating ligand it becomes autophosphorylated. Increased tyrosine phosphorylation was used as a marker to identify the

presence of an activating tie-1 ligand in various cell lines. Malignant melanomas are very angiogenic tumours and have been shown to have increased expression of tie-1 mRNA (156) in their endothelia. Therefore, it was hypothesised that these tumours may produce tie-1 ligand. From the data presented, Sk-Mel 24 malignant melanoma cell line produced factors that increased the proliferation of endothelial cells. Furthermore, these cells appeared to produce a factor which stimulated phosphorylation of tie-1. However, the level of tyrosine phosphorylation induced by Sk-Mel conditioned media was variable, and as seen in Figure 3.15, an additional tie-1 immunoreactive band was identified at 40kDa. This band represents the tie-1 endodomain that remains within the cell membrane following tie-1 ectodomain cleavage (see Chapter 5). The variability in tyrosine phosphorylation observed may, therefore, have been due to the ectodomain being cleaved by a protease produced by the melanoma cells. This would have removed the tie-1 ligand binding domain and prevented ligand binding. Support for a tie-1 ligand being present within SK-Mel 24 conditioned medium was provided by the ability of a tie-1 ED-myc/His fusion protein to bind to Sk-Mel 24 conditioned medium immobilised onto a nitrocellulose. In order to test whether the tie-1 ED fusion protein was binding to an activating tie-1 ligand, an attempt was made to deplete Sk-Mel 24 conditioned medium of tie-1 ligand by incubating the media with tie-1 ED myc/His bound to Probond resin, collecting the media and then testing its ability to tyrosine phosphorylate tie-1 in HUVE cells. This resulted in a small variable decrease in the ability of cultured medium to induce tie-1 phosphorylation. The decrease in ability of conditioned medium to induce phosphorylation may have been due to non-specific binding of activating activity to the fusion protein. This is unlikely as incubation with trkA-fusion protein had even less effect on activating activity.

The apparent inability of tie-1 ED to deplete the melanoma medium of activating activity could be due to several reasons . It may be the activating factor was not a tie-1 ligand. It was also possible that the activating ligand present in media did bind to the tie-1 ED fusion protein but there was insufficient fusion protein to completely deplete the medium. Thirdly, it is conceivable that some tie-1 ectodomain was cleaved from the fusion protein by a protease produced by the Sk-Mel 24 cells. This proposal can only be made in retrospect as the

presence of tie-1 ectodomain cleavage produced by a protease was not fully appreciated at the time the work was performed. With extra time, it would be appropriate to repeat these experiments in the presence of a metalloprotease inhibitor. To determine whether tie-1 ED was limiting, further experiments could be performed in the presence of increasing amounts of tie-1 ED. The ability of lectin-Sepharose (wheat germ agglutinin) to deplete the conditioned medium of an activating tie-1 ligand suggested that the activating tie-1 ligand may bind to the lectin. WGA binds N-acetyl-glucosamine residues. This raised the possibility that the activating ligand was a glycoprotein.

Sk-Mel 24 conditioned medium leads to tyrosine phosphorylation of tie-1 in HUVE cells, one logical step in attempting to isolate the ligand and gain information on its molecular weight, would be to cross-link the tie-1 ligand to the tie-1 receptor in HUVE cells and then to analyse the proteins by SDS-PAGE. This was attempted by using S^{35} labelled methionine that had been incubated with Sk-Mel 24 cells and then incubating the resultant conditioned medium with HUVE cells. Cross linking of the tie-1 ligand to the tie-1 receptor was attempted using the cross linker Disuccinimidyl suberate (DSS) (187). Following incubation, immunoprecipitation of tie-1 was performed an anti tie-1 antibody and the proteins were analysed by SDS-PAGE. Even after several weeks of exposure of the gel to an autoradiogram no obvious cross linking had occurred. This was performed on one other occasion and again no band appeared on the gel. Therefore, this technique was unsuccessful and other methods were used to acquire further knowledge of the tie-1 ligand. The cross linking experiments are not presented in this thesis.

Tie-1 ligand isolation was attempted by incubating concentrated Sk-Mel 24 conditioned media with the tie-1 ED- myc/His fusion protein affinity resin or TrkA ED-myc/His as a control. Proteins that bound to the fusion proteins were then eluted by a pH elution method. As shown in Figure 3.17 B a prominent WGA reactive band was seen between 60-90kDa in the tie-1 ED eluate lane, which was not present in the TrkA eluate lane. However, whilst this was consistent with the idea that this band may be the ligand there were also many other different sized proteins ranging in size from 30-148 kDa in size. What proteins these bands

represent is not known, however, it may be that they were binding non-specifically to the fusion proteins or the Probond resin. The apparent ability of WGA to deplete tie-1 activating activity from conditioned medium suggested it may be possible to use WGA binding as an initial round of purification.

The Protogold stained blot in Figure 3.18 shows that several protein bands were precipitated from Sk-Mel 24 conditioned medium by lectin-Sepharose. Again a prominent band was seen at approximately 80-90kDa. There were also many other bands present on the nitrocellulose membrane. In an attempt to isolate tie-1 binding proteins from this fraction, proteins were eluted from the lectin-Sepharose beads and incubated with tie-1 ED-myc/His fusion protein bound to Probond resin. This step was performed in the presence of a metalloprotease inhibitor, marimastat. As will be discussed in Chapter 5, the tie-1 ectodomain is subject to proteolytic cleavage and this is inhibited by marimastat. Figure 3.19 shows proteins of 80-90kDa were recovered from conditioned medium by sequential WGA and tie-1 ED affinity isolation. Proteins of approximately 30, 55, 60, 70, 220, and 250 kDa were found to bind both tie-1 ED and trkA ED and therefore probably represented non-specifically adsorbed proteins. However, a prominent 80-90kDa protein did appear to bind specifically to tie-1 ED. This protein is therefore a candidate ligand for tie-1. However, this could not be confirmed without additional experiments. Due to time constraints these experiments were not performed. Further work will be necessary to determine the reproducibility of this purification and to identify which, if any, of the proteins recovered is a ligand for tie-1. Ultimately the purification of candidate ligands would be scaled up and the purified proteins tested directly for their ability to bind and stimulate tie-1.

Chapter Four

Regulation of expression of the tie-1 receptor protein

4.1 Introduction

The tie family of receptors are expressed predominantly on vascular endothelial cells, angioblasts and on some haematopoietic cells (63, 139, 142, 145) and play an essential role in angiogenesis. Tie-1 expression is first evident at embryonal day 8.5 in angioblasts of the head, endothelium of the dorsal aorta and yolk sac blood islands in the developing mouse (136). The expression of tie-1 is increased during the embryonal period and then decreases in adult endothelium (134, 136). Its expression in adulthood is increased during normal physiological conditions: wound healing (62) and during the female reproductive cycle (62). However, its expression is increased in adulthood during pathological situations such as tumour vascularisation (151, 156, 157, 180) and in arteriovenous malformations of the brain (155). Despite the importance of this receptor in angiogenesis and blood vessel integrity very little is known about factors that control its expression.

Two potential regulators of tie-1 expression are hypoxia and VEGF. Tissue hypoxia is known to be a stimulus for neovascularisation occurring within growing tumours and ischaemic tissues (188) and it may well be one of the initiating factors during embryonal vascular development. Expression of both VEGF and its receptor VEGFR-2 are known to be upregulated by hypoxia (106, 111, 189, 190). VEGF has a central role in angiogenesis during embryonal development and in certain situations in adult tissues (71, 191)VEGF and its receptors VEGF receptor -1 (VEGFR-1, also called Flt-1) and VEGF receptor-2 (VEGFR-2, also called Flk-1 in mice and KDR in humans) are expressed at day 7 of embryonal development in mice (169) and hence, precede expression of the tie receptors. In this chapter the effects of hypoxia and VEGF on expression of tie-1 were examined.

4.2 Results

4.2.1 The effect of hypoxia on tie-1 protein expression in endothelial cells

To test the effect of hypoxia on tie-1 expression in endothelial cells, confluent monolayers of BAEC's were subjected to hypoxia over varying time points as described in section 2.7. In these experiments BAEC's were used as preliminary experiments suggested hypoxic treatment of HUVE cells led to a high degree of cell death (data not shown). Tie-1 protein expression was analysed by Western blotting of cell lysates. Tie-1 protein expression was increased by hypoxia in a time dependent manner (Figure 4.1). This increase was apparent as early as 1 hour after exposure and was maximal at 12 hours with an almost 4-fold increase in tie-1 protein expression compared to control cells. The results shown are four independent experiments, the time course curve represents the percentage increase at each time point compared to control treated cells.

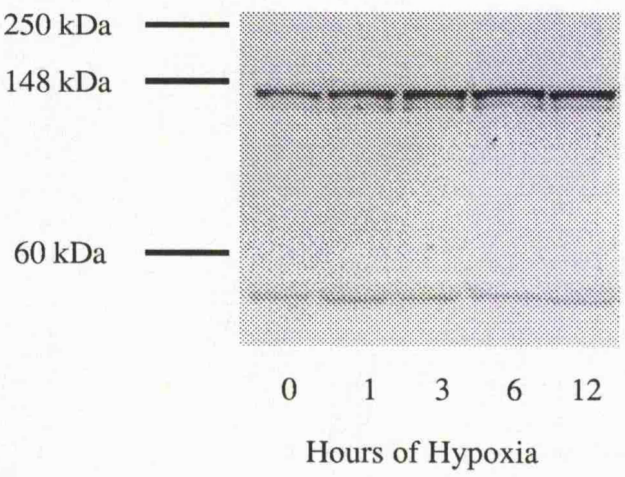
4.2.2 The effect of VEGF on tie-1 protein expression in endothelial cells

In addition to hypoxia the newly developing blood vessel is exposed to VEGF, therefore, VEGF may control the expression of the tie-1 protein. To investigate this, confluent monolayers of BAEC's were challenged with recombinant VEGF and tie-1 protein expression was analysed by immunoblotting. From previous work in this laboratory, the optimum concentration of VEGF for maximal endothelial cell growth and motility is 2.5 nM (Sarah Kemp, University of Leicester, personal communication). This was the concentration that was used in this set of experiments. Confluent monolayers of BAEC's were cultured in serum free medium for 12 hours. VEGF was added for the times indicated and cell lysates prepared as described in section 2.7.1. Tie-1 protein expression was analysed by Western blotting with an anti-tie-1 antibody. VEGF increased tie-1 protein expression in a time dependent manner, with an almost 3-fold increase by 12 hours, Figure 4.2.

Hypoxia induced a 2.9-fold increase (n=4) and VEGF a 3-fold increase (n=4) in tie-1 protein expression by 16 hours(Fig. 4.3). When hypoxia and VEGF were present together, tie-1

was increased 2.4-fold of control cells ($p<0.02$) indicating no synergistic effect between hypoxia and VEGF, in fact the increase in tie-1 was less than either stimulus present on its own, however there was no statistical difference between the combined VEGF and hypoxia treated group against using VEGF or hypoxia on their own.

A.



B.

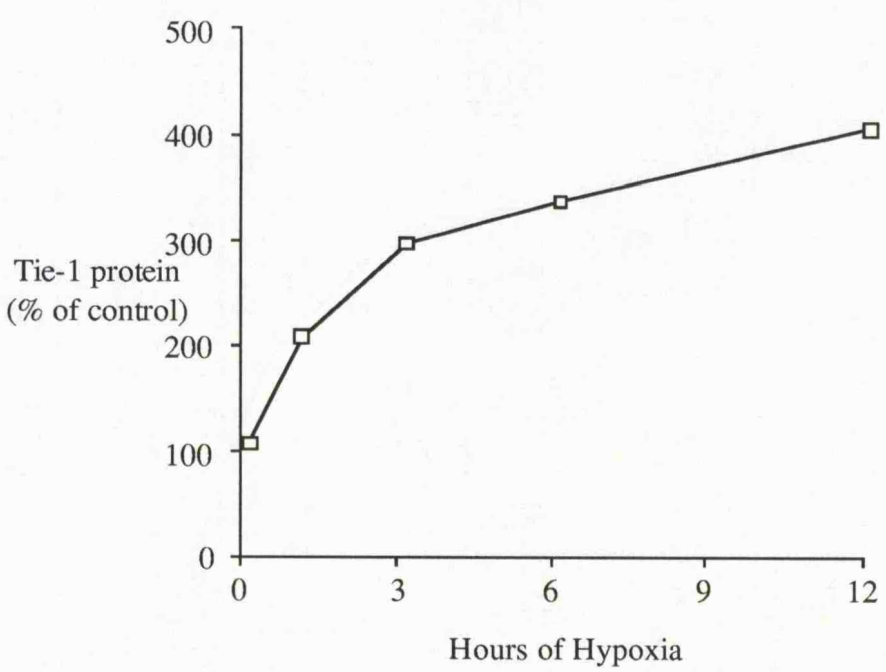
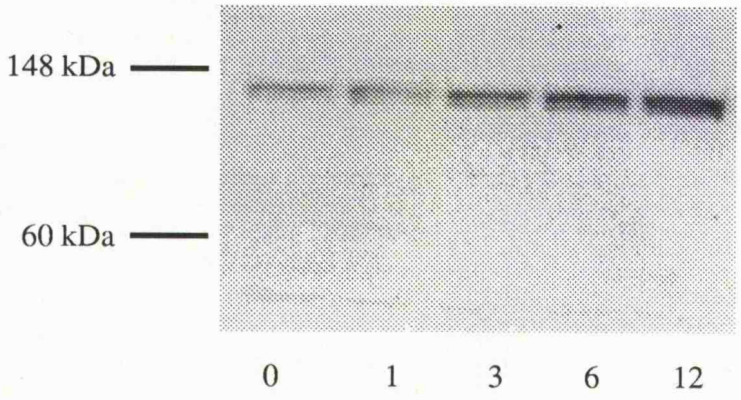


Figure 4.1 Time course of the effect of hypoxia on expression of tie-1 protein in endothelial cells. BAEC's were subjected to hypoxia (2% oxygen) for the times indicated. Equal amounts of total cell protein were resolved by SDS-PAGE and tie-1 protein determined by Western blotting (A).

Densitometric scanning of the blot in 4.1A was used to determine the level of tie-1 protein in cells treated with hypoxia relative to control cells (B). This is a representative immunoblot from four independent experiments.

A.



B.

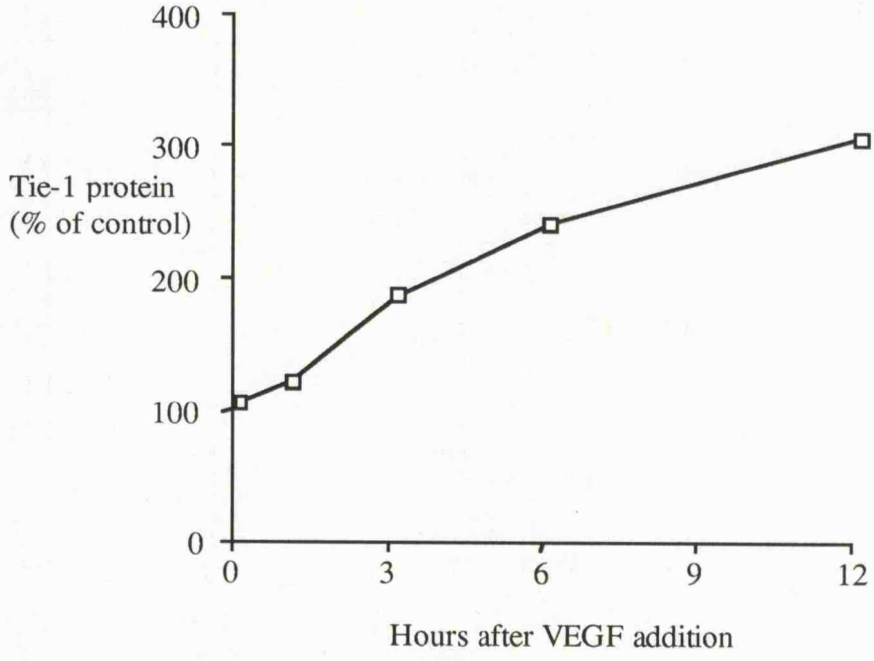
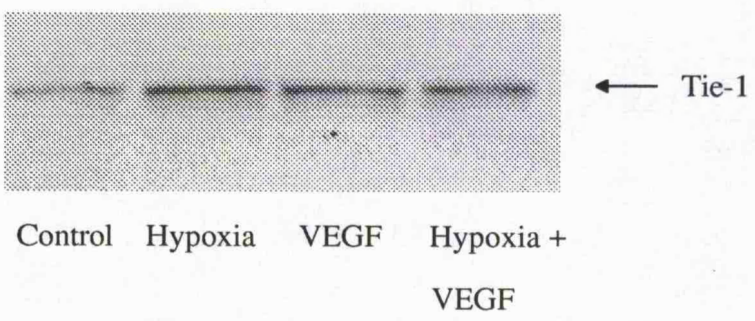


Figure 4.2 Time course of the effect of VEGF on expression of tie-1 protein in endothelial cells. Confluent monolayers of BAEC's were cultured under normoxic conditions (21% oxygen) in the presence of 2.5nM VEGF for the times indicated. Equal amounts of total cell protein were resolved by SDS-

PAGE and tie-1 protein analysed by Western blotting (A). Densitometric scanning of the blot in 4.2A was used to determine the level of tie-1 protein in cells treated with VEGF relative to controls (B). This is a representative immunoblot from four independent experiments.

A.



B.

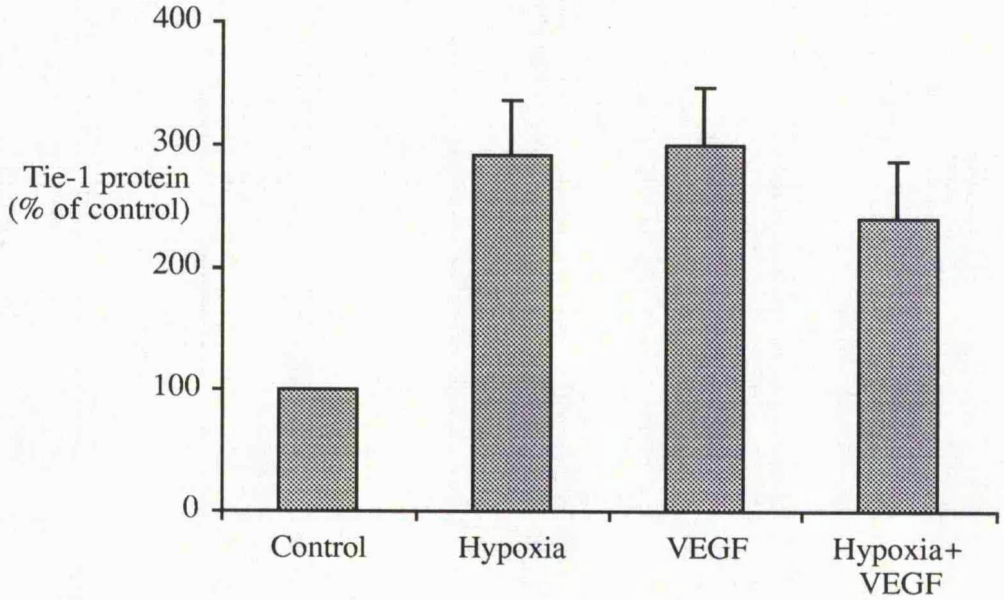


Figure 4.3 The effect of hypoxia and VEGF on tie-1 protein expression in endothelial cells. Confluent endothelial cells were incubated under hypoxic (2% oxygen) or normoxic (21% oxygen) conditions alone or in the presence of 100ng/ml of VEGF for 16 hours as indicated. Equal amounts of cellular protein were resolved on SDS-PAGE and tie-1 determined by Western

blotting (A). Levels of tie-1 protein relative to controls were determined by densitometric scanning of blots (B). Results presented are for four independent experiments as mean and standard error of the mean .

4.2.3 VEGF and hypoxia control tie-1 expression at the transcriptional level

The expression of VEGFR-2 protein is increased by hypoxia and Waltenberger et al (111) report that this does not involve increased transcription. This finding led to the suggestion that the regulation of receptor tyrosine kinases by hypoxia occurred at the post-transcriptional level (111). Therefore, to establish whether the increased expression of tie-1 by hypoxia and VEGF had a transcriptional element, BAEC's were pre-treated with actinomycin D, a known transcriptional inhibitor (192, 193). To find the optimum dose, BAEC's covering a 6-well plate were exposed to varying doses of actinomycin D and the cells were constantly viewed at 30 minute-1 hourly intervals under a x100 microscopic field to assess cell viability. Cell death was judged to have occurred when the cells had become detached from the well. The doses used are shown in the table below.

Dose of Actinomycin D	Culture observation
0	cells grew as normal
10ng/ml	minimal cell death after 24 hours
20ng/ml	minimal cell death after 24 hours
100ng/ml	all cells died within 12 hours
500ng/ml	all cells died within 8 hours
1μg/ml	all cells died within 6 hours

As a result 20ng/ml of actinomycin D was used as the dose of choice to inhibit transcription without causing cell death.

Actinomycin D at a concentration of 20ng/ml, or control vehicle, was added to cells 30 minutes before activation with hypoxia and VEGF as previously described. Tie-1 protein was not increased by either hypoxia or VEGF in cells that had been pre-treated with actinomycin D, whereas both stimuli increased tie-1 in control treated cells, Figure 4.4. This suggests that the increase in tie-1 expression does involve a transcriptional component. Although the effects of actinomycin D were marked this experiment was only performed once. It would be desirable to repeat this several times experiment in order to confirm these observations.

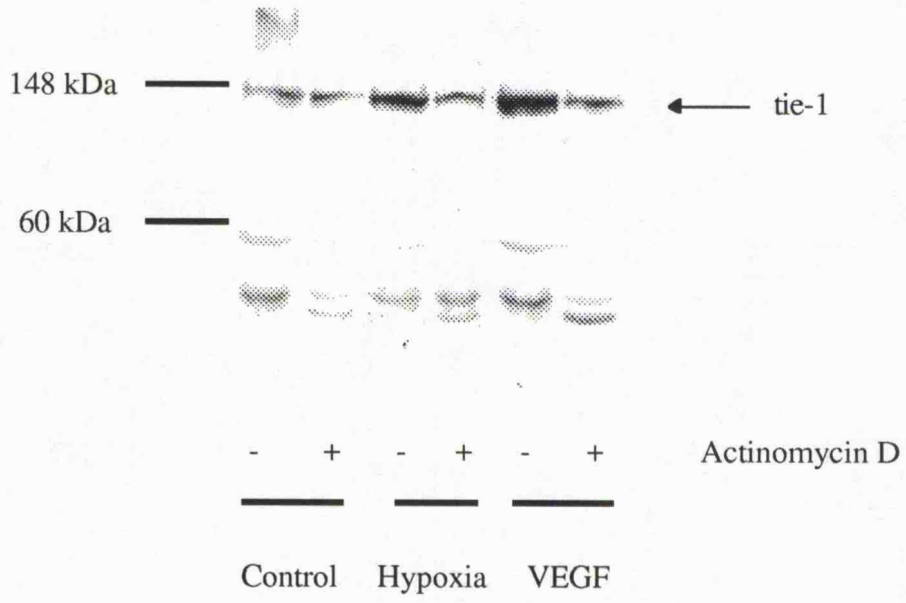


Figure 4.4 Effect of actinomycin D on hypoxia and VEGF induced tie-1 expression in endothelial cells. Confluent monolayers of endothelial cells were treated for 15 minutes with 20ng/ml of actinomycin D or control vehicle. Cells were then maintained with actinomycin D or control vehicle under normoxic (21% oxygen) and hypoxic (2% oxygen) conditions for 6 hours or treated with 100ng/ml of VEGF for 6 hours, as indicated. Tie-1 protein expression was analysed by Western blotting. Results of a single experiment.

4.2.4 The hypoxic effect on tie-1 expression was not due to a demonstrable secreted autocrine factor

It has been shown previously that VEGF expression is increased by hypoxia in HUVE cells and human dermal microvascular cells (189). The increase in tie-1 expression demonstrated with hypoxia could, therefore, be mediated by an increase in VEGF expression and secretion or another growth factor. Furthermore, this would be consistent with the finding that there was no synergy between hypoxia and VEGF (Figure 4.3) to induce tie-1 expression beyond the level seen with each stimulus alone. To examine this possibility medium was collected from BAEC's that had been cultured under hypoxic (2% oxygen) or normoxic conditions. This conditioned media was then re-oxygenated to 21% oxygen and added to previously untreated cells. These cells were then cultured for 16 hours and tie-1 expression analysed as previously described.

Tie-1 expression was not increased by treating cells with medium conditioned by hypoxic endothelial cells (Figure, 4.5). To further test whether VEGF has a role in mediating the effect of hypoxia on tie-1 a VEGF neutralising antibody was used. The increase in tie-1 expression by hypoxia was still present when a VEGF blocking antibody was added to hypoxic endothelial cells (Figure 4.6). Therefore, these findings suggest that the effects of hypoxia on tie-1 were not mediated by VEGF or any other secreted autocrine factor. However, it is not known whether this antibody completely inhibited the effect of VEGF. As only limited amounts of this immunoglobulin were available and it was not possible to perform the additional experiments necessary to characterise its ability to block VEGF action, e.g. in cell proliferation assays.

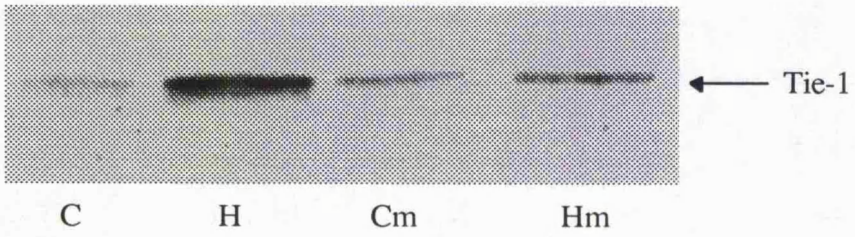


Figure 4.5 The effect of medium conditioned by hypoxic endothelial cells on tie-1 expression. Medium was conditioned by cells incubated under normoxic (21% oxygen) or hypoxic (2% oxygen) conditions 16 hours. Medium was removed, centrifuged, filtered through a 0.2 μm disc filter and made normoxic before addition to fresh endothelial cells. Cells were treated for 16 hours with medium conditioned by normoxic (Cm) or hypoxic (Hm) cells. After treatment cells were washed, lysed and tie-1 protein measured by Western blotting. Tie-1 expression in the normoxic (C) and hypoxic (H) cells used for conditioning media is also presented. A representative immunoblot of three independent experiments is shown.

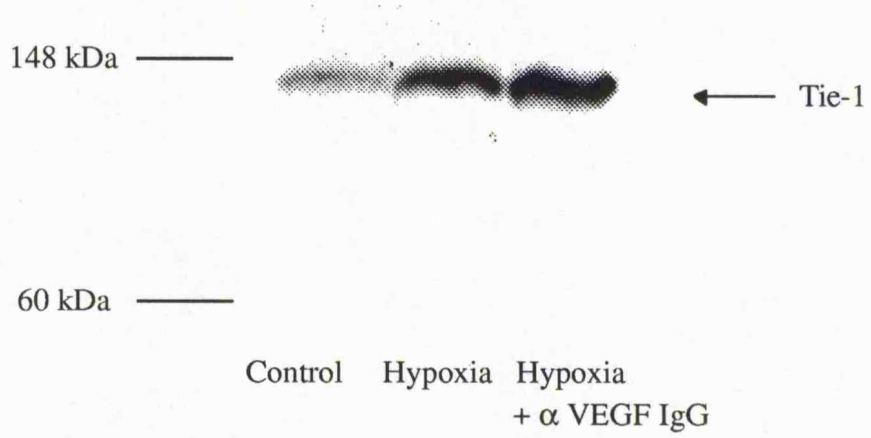


Figure 4.6 The effect of hypoxia on tie-1 expression in endothelial cells, in the presence of a neutralising VEGF antibody. Confluent monolayers of cells were cultured under hypoxic(2% oxygen) and normoxic(21% oxygen) conditions for 16 hours. To one flask of cells that were cultured under hypoxic conditions, 10ug/ml of a neutralising anti-VEGF antibody was added to the culture medium at the time it was rendered hypoxic. Cell lysates were resolved by SDS-PAGE and tie-1 protein analysis was performed by Western blotting. This is a representative immunoblot from two independent experiments.

4.2.5 The effect of hypoxia on the tie-1 ligand

Hypoxia increases the expression of VEGF (189, 194) and its receptor, VEGFR-2 (111). Therefore, because hypoxia also leads to increased expression of the tie-1 receptor it may be that the expression of the tie-1 ligand, like VEGF, is also increased by hypoxia. Within SK-Mel 24 conditioned medium there appears to be an activating ligand that causes tyrosine phosphorylation of the tie-1 receptor (chapter 3). To establish if hypoxia increased expression of this activating ligand SK-Mel 24 cells were cultured under hypoxic (2% oxygen) conditions for 16 hours in serum free medium. After this time the conditioned medium was collected, re-oxygenated to 21% oxygen, made cell free by centrifugation and concentrated ten fold as previously described. The medium was then filtered through a

0.2μm pore filter and added to quiesced confluent HUVE cells to assess for tie-1 receptor activation.

Conditioned SK Mel 24 medium led to an increase in tie-1 tyrosine phosphorylation, however, there was no additional increase in tyrosine phosphorylation in BAEC's cultured with the hypoxic conditioned medium (Figure 4.7). Indeed , levels of tie-1 phosphorylation induced by medium from hypoxic Sk-Mel 24 cells appeared similar to that seen in quiescent HUVE cells. This raised the possibility that hypoxia may even decrease the levels of tie-1 activating factor produced by melanoma cells. To examine the effects of hypoxia on factors which bind to tie-1 extracellular domain, including any putative ligands, tie-1 ligand binding was examined with a tie-1 extracellular domain fusion protein, as previously described in chapter 3, section 3.3.2.2. The hypoxic and normoxic conditioned SK Mel 24 media were immobilised on a nitrocellulose membrane using a Dot-Blot device and then incubated with the fusion protein. Tie-1 ED-myc/His fusion protein binding to tie-1 ligand was detected with an anti-myc antibody.

The fusion protein probe binds to both normoxic and hypoxic conditioned SK Mel 24 media in equal amounts (Figure 4.8). This suggests that SK Mel 24 cells secrete a tie-1 ligand under normoxic conditions but that its expression does not appear to be altered by hypoxia. The apparent decrease in ability of hypoxic medium to stimulate phosphorylation of tie-1 contrasts with the lack of effect of hypoxia on tie-1ED binding shown in Figure 4.8. This suggests hypoxia may modulate production of factors that affect tie-1 phosphorylation independently of tie-1 ligand.

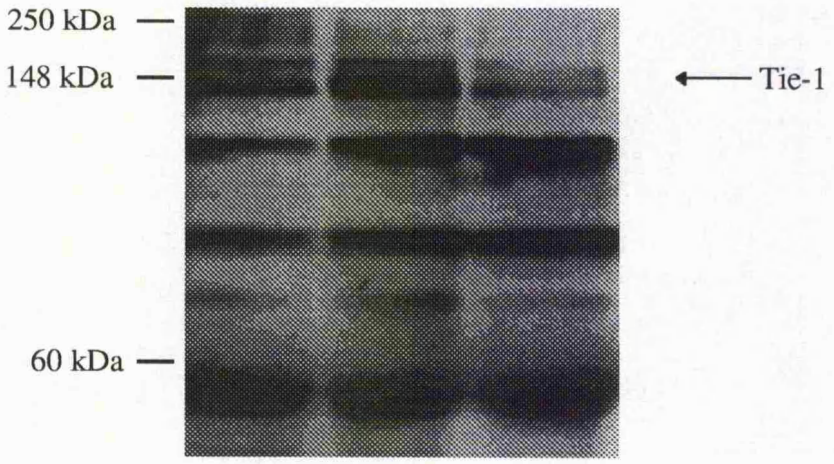


Figure 4.7 The effect of conditioned Sk-Mel 24 hypoxic medium on tie-1 protein phosphorylation in HUVE cells. 50% confluent Sk-Mel 24 cells were cultured under normoxic (21% oxygen) (M) or hypoxic (2% oxygen) (Hm) conditions. The respective media were collected, centrifuged, filtered through a 0.2 μm disc filter and added to HUVE cells that had been quiesced for 12 hours. After 10 minutes incubation at 37^{0}C the cells were washed in PBS and lysed. Immunoprecipitation (IP), with an anti-phosphotyrosine antibody on agarose beads (α Py), of the cells lysates were performed for 3 hours at 4^{0}C. The immunoprecipitates were washed and analysed by Western blotting (WB). Tie-1 phosphorylation in control quiesced HUVE cells (C) is also presented. The blot was stripped and reprobed with an anti-tie-1 antibody to identify the tie-1 protein. The bands corresponding to tie-1 are marked accordingly. This is a representative immunoblot from three independent experiments.

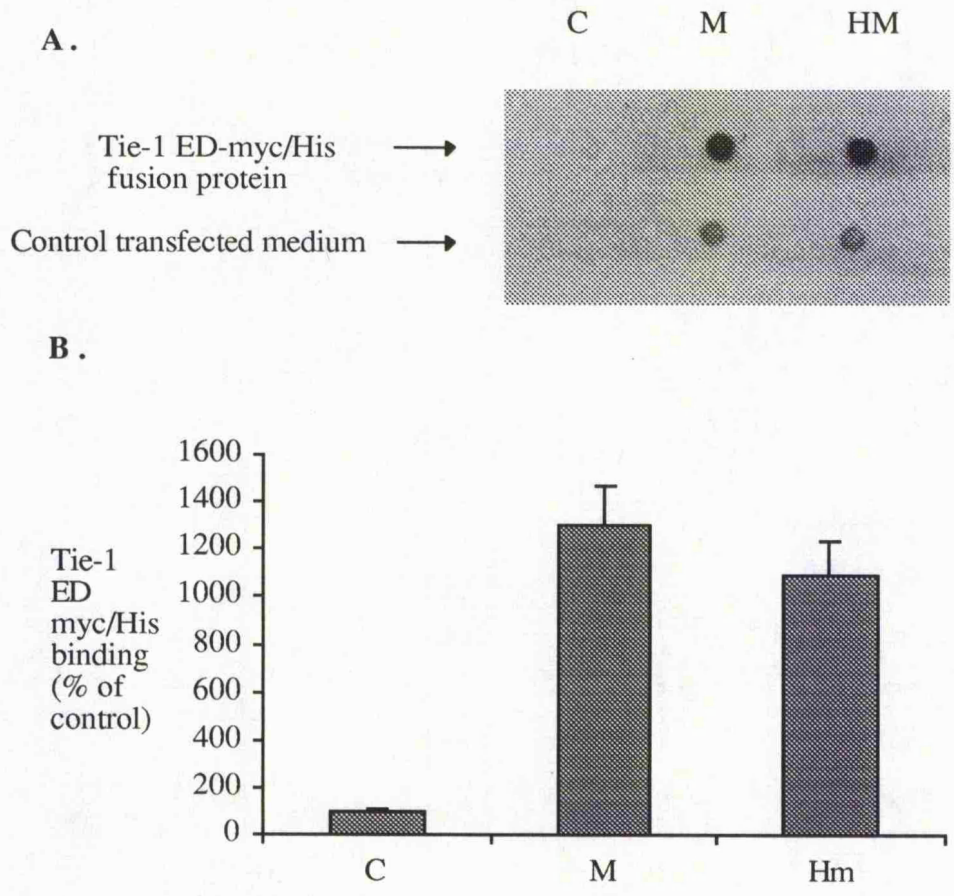


Figure 4.8 Tie-1 ED-myc/His binding to immobilised normoxic and hypoxic SK-Mel 24 conditioned media. Tie-1 ED-myc/His fusion protein was used as a probe to investigate binding to the tie-1 ligand in normoxic (M) (21% oxygen) and hypoxic (Hm) (2% oxygen) conditioned SK-Mel 24 media. Tie-1 ectodomain binding was compared to control SK-Mel 24 serum-free culture media that had not been in contact with cells (C). pSecTag transfected media was used as a control for tie-1 ED myc-His binding. The SK-Mel 24 media collected from an 80cm^2 flask of confluent cells was concentrated 10-fold and immobilised onto nitrocellulose strips using a Dot- Blot device under vacuum pressure. The strips were then incubated at 37^{0}C for 1 hour with tie-1 ED myc-His transfected media or empty pSecTag transfected media. Fusion-protein binding to the strips was analysed by Western blotting with an anti-myc antibody (A). The results of three independent experiment are

represented graphically (B). The values shown are for the percentage increase of Tie-1 ED-myc/His binding compared to control following subtraction of the comparable control transfected media optical density. Results presented are means and standard error of the means for three independent experiments.

4.4 Discussion

VEGF and hypoxia both lead to an increase in tie-1 receptor expression in vascular endothelial cells, whereas hypoxia does not lead to an increase in expression of the tie-1 binding activity produced by a malignant melanoma cell line (SK-Mel 24). Hypoxia and VEGF have been shown to have an integral role in angiogenesis in both pathological conditions and developmental vascularisation (188) and are, therefore, both likely to mediate the increased expression of tie-1 seen in these situations. Both stimuli lead to an increase in tie-1 receptor expression in a time-dependent manner, with maximum expression 3-fold that of controls cells. Hypoxia and certain growth factors (bFGF and TFG-β) have been shown to upregulate VEGF mRNA expression in a synergistic manner (105). However, no such synergism exists between hypoxia and VEGF in the regulation of tie-1 receptor protein expression.

Hypoxia has been shown to up-regulate the expression, *in vitro*, of VEGF and one of its tyrosine kinase receptors, VEGFR-2 in endothelial cells (111, 189, 195). The up-regulation of VEGFR-2 appears to occur either by the direct effect of hypoxia on endothelial cells (111, 195) or via a factor secreted from smooth muscle cells in response to a decreased oxygen tension (106). The direct effect of hypoxia leads to a 1.5-fold increase in VEGFR-2 expression compared to control cells (195), tie-1 expression is increased 3-fold compared to control cells. This relative increase in expression tie-1 receptor compared to the VEGFR-2 receptor could be due to an increased sensitivity of the tie-1 gene to hypoxia or alternatively be due to some other factor causing the effect. Previous observations that hypoxia increases VEGF expression in endothelial cells (189) and the effects of VEGF on tie-1 receptor expression demonstrated in this chapter, suggested that hypoxia could act to increase tie-1 receptor expression by increasing the production of VEGF or another autocrine factor in endothelial cells. However, the increase in tie-1 expression was still observed when endothelial cells were cultured under hypoxic conditions when in the presence of a neutralising antibody for VEGF suggesting that the effect of hypoxia on tie-1 was not due to an increase in VEGF expression. However, this remains unproven due to the inability to test

the full blocking ability of the VEGF neutralising antibody as previously discussed in the results section. Furthermore, the inability of conditioned medium from hypoxic cells to increase tie-1 expression in normoxic endothelial cells argues against the involvement of a secreted intermediate factor. It is possible, however, that a membrane bound form of VEGF e.g. $VEGF_{206}$, or another factor, was mediating the effect demonstrated.

Actinomycin D inhibited the stimulatory effects of hypoxia and VEGF on tie-1 expression, and thus, these stimuli affected expression of this tyrosine kinase receptor at the level of transcription. This is in contrast to the direct effects of hypoxia on VEGFR-2 which occur at the post-transcriptional level (111). However, it is possible that hypoxia and VEGF lead to the transcription of an unknown factor which works to increase tie-1 protein at a post-transcriptional level. To determine an effect on transcription it is necessary to measure mRNA. Several attempts at confirming these results with Northern blot analysis were not successful. This was due to failure of the technique due to poor hybridisation of the cDNA probe. Future work needs to investigate the transcriptional component of tie-1 expression. Is the effect of hypoxia and VEGF on tie-1 expression due to a direct increase in tie-1 mRNA levels or is it due to an increase in transcription of an unidentified factor which activates tie-1 expression at the post-transcriptional level ? An increase in tie-1 mRNA in response to hypoxia and VEGF needs to be demonstrated and this can be achieved by optimising Northern blot analysis or by RT-PCR using tie-1 specific primers. However, what is clearly demonstrated is that hypoxia and VEGF increase tie-1 protein expression and this must have some functional significance for the endothelial cell. It is possible that by increasing tie-1 receptor expression the intracellular effects following tie-1 activation are enhanced leading to an increase in endothelial cell integrity during the latter stages of the angiogenic process.

Hypoxia does not seem to increase the expression of the potential tie-1 ligand in SK-Mel 24 cells, this is in contrast to other growth factors such as VEGF which are upregulated by hypoxia (189). Work detailed in this chapter investigates the level of ligand expression in an indirect manner by studying tie-1 receptor activation and tie-1 ectodomain probe binding. These techniques allow an initial insight into the control of expression of the tie-1 ligand,

however, more definitive information on the control of tie-1 ligand expression cannot be obtained until further work has been undertaken on the isolation and cloning of the ligand. Furthermore, it is possible that these transformed cells (SK-Mel 24) already have maximal tie-1 ligand expression under normoxic conditions and, therefore, rendering these cells hypoxic may not lead to any increase in expression.

VEGF has a direct proliferative and migratory effect on endothelial cells *in vitro* (40, 70, 73, 75, 196) and initiates angiogenesis *in vivo* (41, 73). Expression of VEGF is increased under pathophysiological conditions such as wound healing (197), the ischaemic myocardium (198) and in tumour growth (103, 199). *In vitro* VEGF expression is increased in situations of low oxygen tension in both endothelial and vascular smooth muscle cells (189, 194). Conversely, in the retina, high oxygen tension inhibits VEGF expression leading to apoptosis of retinal endothelial cells and vessel progression (200). Together, these mechanisms support a model whereby vessel growth is matched to the oxygen requirement of the tissues. The ability of hypoxia and VEGF to modulate tie-1 refines this model further to include a mechanism whereby new vessels are primed to respond to tie ligands which may have a role in later stages of the angiogenic process to promote blood vessel integrity.

The effect of hypoxia on several genes, including VEGF and erythropoietin, is regulated by hypoxia-inducible factor 1 (HIF-1) or a related HIF (201). HIF-1 is a transcription factor that is a heterodimeric member of the basic helix-loop-helix family of transcription factors comprised of α and β subunits (201). HIF-1 binding sites identified to date contain the core sequence 5'-(A/G)CGTG-3'. Binding of HIF-1 subunits to these domains leads to transcriptional activation of the targeted gene. The 5' flanking region of the tie gene (159) does not reveal any *cis*- acting elements similar to those known to interact with HIF-1 in VEGF or other hypoxia sensitive genes. However, it may be that consensus HIF binding sites exist further upstream of the published tie-1 5' untranslated region. Alternatively, hypoxia could be acting indirectly or by an HIF or HIF-like transcription factor interacting with hypoxia-responsive elements distinct from those known to bind HIF-1. It is noteworthy

that several groups have recently reported cloning HIF-1 related genes that are preferentially expressed in endothelial cells and the developing vascular system and that mediate hypoxia-induced transcriptional activation (202, 203, 204). These new sequences are endothelial PAS-1 (EPAS-1), HIF1α-like factor and HIF-related factor, and appear to be present at higher levels than HIF-1 in the vasculature (202, 203, 204). The close similarity between sequences suggest they may encode the same or very closely related factors. Overexpression of EPAS-1 induces expression of a reporter construct containing 10.3 kbp of regulatory sequence from the tie-2 gene (204). Importantly this reporter gene was not stimulated by HIF-1 suggesting that EPAS-1 could interact with specific response elements not recognised by HIF-1. Therefore, it is possible that the effects of hypoxia on tie-1 may be mediated through EPAS-1, but this needs to be investigated further.

In summary the results presented in this chapter demonstrate that for endothelial cells in culture, both hypoxia and VEGF increase tie-1 protein expression in a time-dependent manner. Furthermore this effect is regulated at the transcriptional level and is not due to a secreted autocrine factor. However, preliminary data suggest potential tie-1 ligand produced by Sk-Mel 24 cells, does not appear to be regulated by hypoxia. The ability of VEGF to modulate tie-1 demonstrates that this growth factor has a function in co-ordinating the angiogenic programme in endothelial cells, allowing initiation of the early stages in vessel growth as well as upregulating factors involved in the later stages of vessel maturation. It would be of interest to examine the extent of tie-1 protein expression *in vivo* during hypoxic situations such as in the ischaemic myocardium or critically ischaemic limb. Furthermore, if VEGF is given as a therapeutic agent to promote angiogenesis in ischaemic models (39, 47, 51), this could lead to an increase in tie-1 protein expression suggesting that giving a tie-1 ligand in addition to VEGF may aid in the development of mature blood vessels.

Chapter Five

Extracellular domain cleavage of the tie-1 receptor initiates intracellular signalling and downstream events

5.1 Introduction

The extracellular domain of several membrane bound receptor tyrosine kinases (TrkA, ErbB-4, TGF-α) can be released as a soluble fragment by the action of cell surface proteolytic enzymes (205, 206, 207). Ectodomain cleavage for several receptors appears to be regulated via protein kinase C activation and it has been reported that a number of metalloproteases lead to proteolytic cleavage of receptor extracellular domains at sites close to the transmembrane region (205, 206, 207, 208). However, for the TrkA receptor, binding with its ligand, neurotrophin, also leads to ectodomain cleavage (205). The released ectodomain may act as an antagonist by binding ligand (209, 210). The retroviral oncogenes *v-erb, v-kit* and the *v-ros* code for mutated receptors that lack most of the extracellular domain and give rise to constitutively active intracellular fragments (211). Loss of the ectodomain of TrkA leads to increased catalytic activity of its intracellular portion (205), whilst in other receptors loss of the extracellular domain leads to an inhibition of intracellular signalling (207) . Clearly ectodomain cleavage is a way of regulating intracellular signals whether they are activating or inhibitory.

It has been shown that in some leukaemia cell lines tie-1 mRNA and protein expression are increased when the cells are treated with a phorbol ester, phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator (212). Yabkowitz et al have demonstrated that the tie-1 receptor is regulated by protein kinase C mediated release of the ectodomain (158). When endothelial cells were treated with the phorbol ester, PMA, tie-1 ectodomain cleavage was activated within 15 minutes, a 100kDa soluble ectodomain was isolated in cell media supernatants, while a 40kDa fragment remained cell associated. Whole receptor tie-1 usually appears as a 135 kDa and 125 kDa doublet on Western blots. Pulse chase experiments and the use of inhibitors of glycosylation have been used to demonstrate that the lower band is the immature tie-1 receptor probably situated within the golgi complex which matures by the addition of sialic acid residues before becoming incorporated into the cell membrane (158). It has been suggested that the cleavage site is situated close to the membrane portion of the receptor on the extracellular side but the protease that results in this cleavage event is

unknown. This event appears to be calcium dependent, as addition of EGTA inhibited the release of the ectodomain into the surrounding cell medium (158).

At the time of writing the significance of tie-1 ectodomain cleavage and the importance it has on cell signalling is not known. In view of the role of tie-1 in maintaining endothelial cell integrity and endothelial cell survival, cleavage of the ligand binding domain from this receptor is likely to be of significance. The loss of the tie-1 ectodomain will result in loss of ligand responsiveness and serve to inhibit ligand dependent signalling through tie-1. Furthermore, the loss of the ectodomain may activate signalling pathways by relieving ectodomain inhibiton of the intracellular tyrosine kinase domain of tie-1.

The work presented in this chapter tests the hypothesis that tie-1 ectodomain release can activate downstream signalling events and examines whether the tie-1 cleavage phenomenon occurs *in vivo* in human tissues.

5.2 Results

5.2.1 The effect of phorbol ester (PMA) treatment on endothelial cells and the expression of tie-1 protein over time

Conditions used for PMA activation were similar to those described by Yabkowitz et al (158).

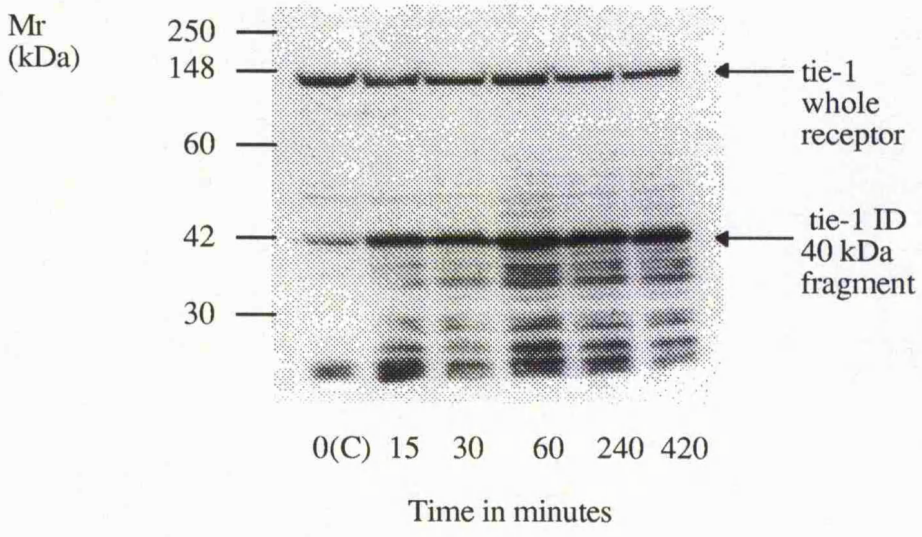


Figure 5.1 The effect of PMA on tie-1 expression in HUVE cells over time. HUVE cells were incubated with 10ng/ml of PMA or control vehicle (DMSO) over varying time points (C). Confluent HUVE cells in a 6-well plate were washed twice with basal medium (DMEM+ 0.5% Foetal Calf Serum + 1% BSA). To basal medium was added 10ng/ml of PMA (made up in DMSO) or DMSO control vehicle and endothelial cells were incubated over varying time points with PMA. After incubation the cells were washed with PBS and then lysed in 1x SB-DTT. The lysates were scraped from the wells into eppendorf tubes. Cell lysates were then sonicated for 30 seconds, boiled for 5 minutes and centrifuged at 13,000g for one minute. Samples were electrophoresed on a 12% SDS-PAGE gel followed by Western blotting. Immunoreactive bands were detected by an anti-tie-1 antibody (generated to the intracellular domain (ID) of the tie-1 receptor). Tie-1 intracellular domain (ID) appears as a 40kDa fragment. This is a representative blot of four experiments. Lysates

from equal numbers of cells corresponding to 1/10 of a well from a 6-well plate were loaded in each track.

Tie-1 ectodomain release following endothelial cell incubation with PMA is shown in Figure 5.1. PMA activation generated ectodomain release and an increase was seen in the 40kDa fragment which corresponded to the transmembrane and intracellular domains of tie-1. This fragment appeared to remain within the cell for at least 7 hours. Multiple bands below the 42 kDa marker were seen and these may have represented further proteolysis products of the tie-1 receptor. In order to determine which of the bands reacted specifically with the tie-1 antibody, further experiments were performed. HUVE cells were activated with PMA for 60 minutes and the Western blots were then probed with anti-tie-1 antibody alone or in the presence of the specific tie-1 peptide to which the antibody was raised or another peptide which differs in only 5 amino acids from the tie-1 peptide and which corresponds to the C-terminus of tie-2.

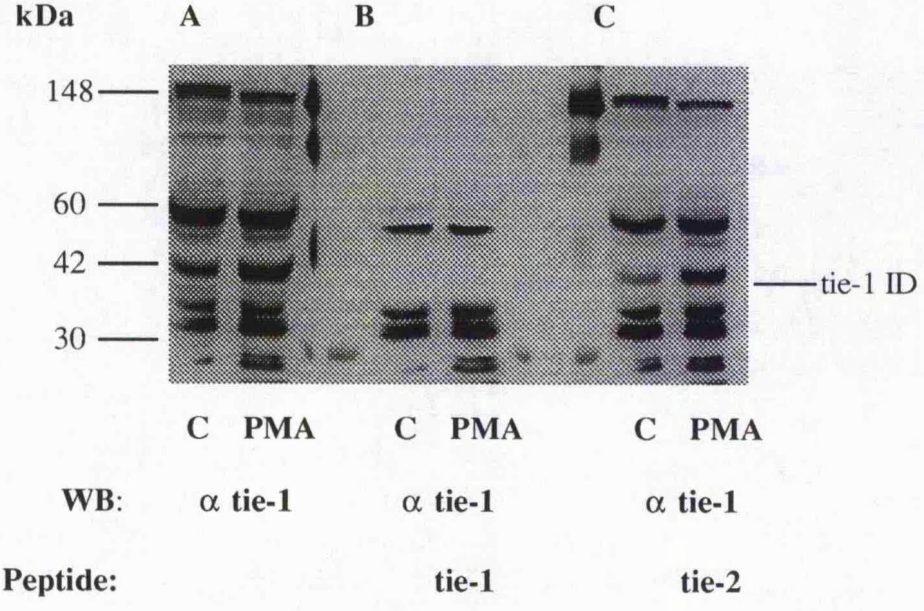


Figure 5.2 Determination of the specificty of the anti tie-1 antibody. HUVE cells were incubated with 10ng/ml of PMA or control vehicle (C) for 60 minutes. Cell lysates corresponding to 1/10 of a well from a 6-well plate were electrophoresed on a 12% SDS-PAGE gel and proteins transferred onto

a nitrocellulose membrane. Membrane A. was incubated with an anti-tie-1 antibody (α tie-1){1:1000}. Membrane B. was incubated with anti-tie-1 antibody plus tie-1 blocking peptide (α tie-1 BP) {1μg/ml} and membrane C. was incubated with anti-tie-1 antibody (1:1000) plus an tie-2 blocking peptide (α tie-2 BP) {1μg/ml}. This blot is representative of two independent experiments.

As shown in Figure 5.2 , the anti- tie-1 antibody reacted with a number of bands. Co-incubation of the antibody with tie-1 peptide during blotting prevented the antibody from recognising proteins of 135, 125 and 40kDa, whereas the staining of other major bands was unaffected by the presence of the peptide. This indicated that the 135, 125 and 40 kDa proteins contained the tie-1 carboxy terminal epitope to which the antibody was raised, the other bands represent non-specific binding. In order to confirm that the peptide block was specific for the tie-1 terminal peptide, the antibody was incubated with a peptide derived from the carboxy terminus of tie-2, in a parallel blot (Fig 5.2 C) . The tie-2 peptide was similar in amino acid sequence to the tie-1 peptide except for five amino acids. As shown in Figure 5.2, the antibody still recognised 135, 125 and 40 kDa proteins in the presence of the tie-2 blocking peptide further confirming that the proteins contain the tie-1 C-terminal sequence. Only bands of 135, 125 and 40 kDa will be considered as tie-1 specific in all subsequent experiments.

Interestingly, a 40kDa band of low intensity was seen consistently in the control lane suggesting that there was background cleavage of the ectodomain occurring in the endothelial cell during normal culture conditions. This truncation phenomenon may have a physiological role in the normal functioning of the endothelial cell. The endodomain appeared to remain in the cell for several hours. Furthermore. using subcellular fractionation, the majority of the 40kDa endodomain fragment remains within the membrane fraction of the cell, with none appearing in the cytoplasm, Figure 5.3.

The ectodomains of several receptors, in the absence of ligand, appear to have an inhibitory influence on the tyrosine kinase activity of the receptor. For example, the sevenless endodomain in ectodomain negative sevenless mutants generates downstream signalling as do several oncoproteins receptors that lack ectodomains (211). It is conceivable, therefore, that the persistent tie-1 endodomain could activate phosphorylation, recruit signalling intermediates to the membrane and lead to activation of downstream events.

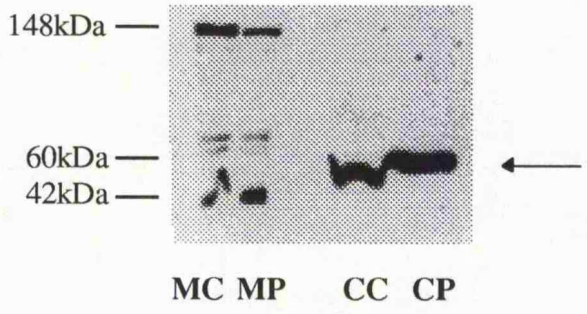


Figure 5.3 A representative immunoblot of subcellular fractionation of tie-1 following PMA activation. Following PMA activation HUVE cells were washed twice with PBS and lysed using hypotonic PBS (19:1 with water). Cells were scraped from the dish and broken up by homogenisation (10 passes of a Dounce homogeniser). Nuclei and unbroken cells were removed by centrifugation at 800g for 5 minutes, the supernatant was removed and centrifuged at 100,000g for a 45 minute period. The pellet was then solubilised by the addition of 1x SB, boiled, sonicated and centrifuged at 13,000g for 2 minutes. The supernatant was mixed with 3X SB and boiled. Equivalent amounts of cytosolic nuclear extracts were analysed by SDS-PAGE and Western blotting. Tie-1 immunoreactive bands were then identified with an anti-tie-1 antibody. MC= membrane fraction from control cells, MP= membrane fraction from PMA treated cells, CC= cytoplasmic fraction from control cells, CP= cytoplasmic fraction from PMA treated cells. The arrow corresponds to the 60 kDa non-specific band seen in Fig

5.2. This is a representative immunoblot from three independent experiments.

5.2.2 Tie-1 endodomain and possible intracellular signalling proteins

To investigate the association of known intracellular signalling molecules to the endodomain of tie-1 and the dependence of this on its phosphorylation state, immunoprecipitations were performed on extracts of confluent HUVE cells that had been treated with PMA for 1 hour and compared to control cells that had been treated with control vehicle. Immunoprecipitation with the anti- tie-1 antibody, followed by immunoblotting with an anti-tie-1 antibody as (Figure 5.4) showed the characteristic loss of ectodomain with an associated increase in the 40 kDa band from cells incubated with PMA. The large immunoreactive band at 60 kDa represented non-specific immunoglobulin binding and was not a tie-1 proteolysis product.

When the membrane was stripped and reprobed with an anti-phosphotyrosine antibody the endodomain did not appear to be tyrosine phosphorylated (B). However, several phosphorylated bands were identified at approximately 70-80kDa and 110-120kDa that appeared to have increased tyrosine phosphorylation in the PMA treated cells when compared to control cells. Therefore, within the limitations of the sensitivity of this method we could not detect phosphorylation of tie-1 endodomain. However, although tie-1 endodomain did not appear to autophosphorylate, there did appear to be increased phosphorylation of bands at 70-80 kDa and 110-120kDa that co-immunoprecipitated with tie-1. These may represent downstream signalling molecules. Possible candidates for the 70-80kDa proteins were SH-PTP2, SH-PTP1, PI3- kinase p85α, Sam 68, HSP 70 and GRP78. As part of an initial screen to examine the identity of the band seen between 70-80kDa, the membrane was stripped and reprobed with an anti SH-PTP2 antibody (Fig 5.4 C). An immunoreactive band corresponding to a polypeptide of 72kDa was seen in the PMA treated cell lane but not in the control lane, Figure 5.4C. This band corresponded to of the 72kDa band seen on the anti-phosphotyrosine probe (B) and suggests that SH-PTP2 associated with truncated tie-2 and may undergo increased phosphorylation . SH-PTP2 is a

protein tyrosine phosphatase and is a positive regulatory molecule that has been implicated in the activation of the mitogen-activated protein kinase pathway (MAPK) and is thought to modulate cell growth and differentiation (162). The nature of the association between SH-PTP2 and tie-1 was of interest as SH-PTP2 usually binds its protein partners via its SH-2 domains and, therefore, would be expected to bind in a phosphotyrosine dependent manner. Therefore, it was surprising that non-phosphorylated tie-1 should co-immunoprecipitate this protein. It is possible that the tie-1 endodomain was binding with SH-PTP2 indirectly.

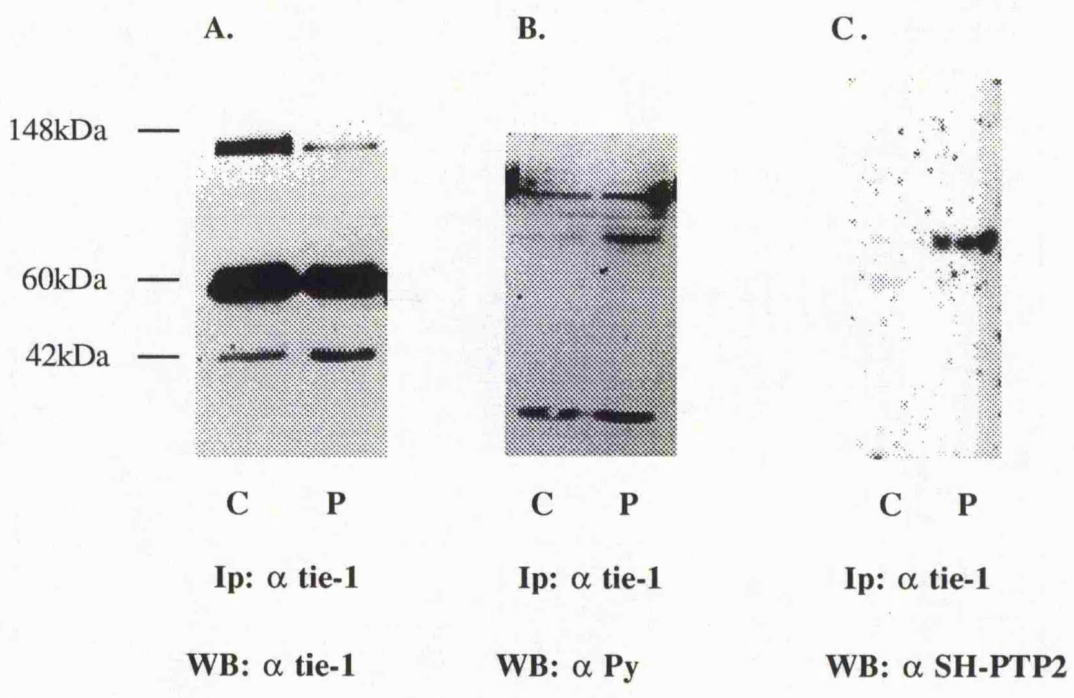


Figure 5.4 Immunoprecipitation of tie-1 following phorbol ester activation of HUVE cells.Confluent HUVE cells were treated for one hour with PMA (P) or treated with control vehicle (C) followed by cell lysis and immunoprecipitation (Ip) with anti-tie-1 antibody. Immunoprecipitates were analysed by SDS-PAGE and Western Blotting (WB). Proteins were identified by immunoblotting with an anti-tie-1 antibody (α tie-1) followed by stripping and reprobing with an anti-phosphotyrosine antibody (α Py) and an anti-SH-PTP2 antibody (α SH-PTP2). Each track shows

immunoprecipitations from lysates prepared from equal numbers of cells. This immunoblot is representative of four experiments.

These findings were confirmed when immunoprecipitating PMA treated cell extracts with an anti-phosphotyrosine antibody (Figure 5.5). Figure 5.5 A. shows an anti-tie-1 probe, and this demonstrated an immunoreactive band of 40 kDa which corresponded to the tie-1 endodomain. Reprobing with an anti-phosphotyrosine antibody confirmed tie-1 endodomain did not appear to be phosphorylated. However, phosphorylated bands did appear in the PMA treated lane at 72 kDa which corresponded to SH-PTP2, shown when the blot was reprobed with an anti-SH-PTP2 antibody. Several other bands at 120, 110, 100, 90 kDa were also phosphorylated following PMA activation. These may represent other downstream signalling molecules that were activated on PMA treatment.

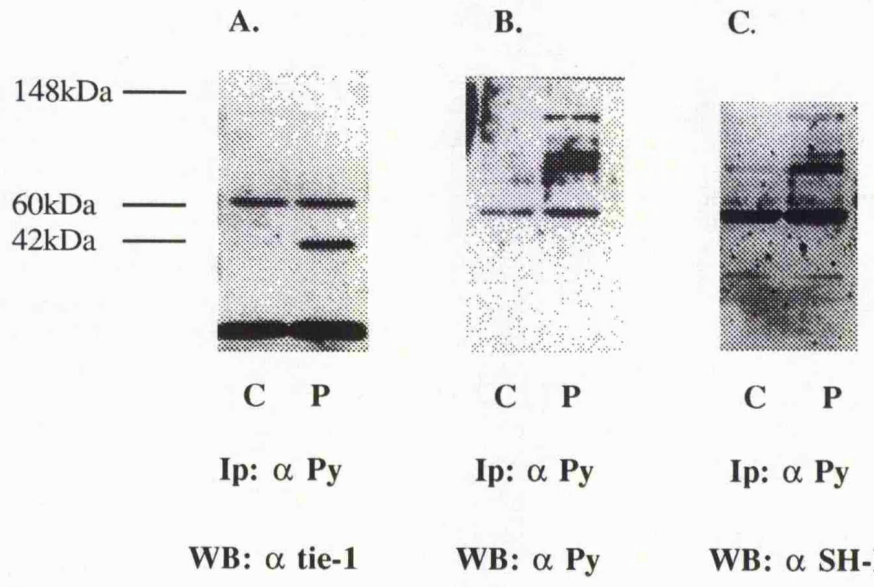


Figure 5.5 Immunoprecipitation of tyrosine phosphorylated proteins following phorbol ester activation of HUVE cells. Confluent HUVE cells were treated for one hour with PMA (P) or treated with control vehicle (C) followed by cell lysis and immunoprecipitation (Ip) with an anti-phosphotyrosine antibody immobilised on Sepharose beads.

Immunoprecipitates were analysed by SDS-PAGE and Western Blotting (WB). Proteins were identified by immunoblotting with an anti-tie-1 antibody (α tie-1). The blot was stripped and reprobed with an anti-phosphotyrosine antibody (α Py) and an anti-SH-PTP2 antibody (α SH-PTP2). Both tracks show immunoprecipitations from lysates prepared from equal numbers of cells. This immunoblot is representative of four experiments.

To determine whether SH-PTP2 associates with the tie-1 holoreceptor or the endodomain of tie-1, HUVE cells were treated with PMA and SH-PTP2 was immunoprecipitated from cell extracts using a polyclonal anti-SH-PTP2 antibody. On probing the membrane with an anti-tie-1 antibody a prominent 40kDa band was seen in PMA treated cells and was absent in the control lane, Figure 5.6 A. This band corresponded to the tie-1 endodomain. The anti-SH-PTP2 probe, Figure 5.6 C. showed SH-PTP2 was immunoprecipitated from both control and PMA treated cells. However, when probing with an anti-phosphotyrosine antibody only the SH-PTP2 in the PMA treated lane was phosphorylated (Figure 5.6 B). Thus, it appeared that the truncated tie-1 endodomain is not tyrosine phosphorylated. Furthermore, reprobing the anti-tie-1 immunoprecipitation blots with anti-phosphothreonine and anti-phosphoserine antibodies failed to show phosphorylation of these residues on the tie-1 endodomain (data not shown). However, the sensitivity and effectiveness of these antibodies was not determined. Tie-1 appears to be associated with SH-PTP2, however, it is unlikely that SH-PTP2 binds to tie-1 endodomain directly, as tie-1 endodomain was apparently not tyrosine phosphorylated. It is possible that one or more signalling intermediates that do not depend on phosphorylation to associate with tie-1 endodomain, could link SH-PTP2 to tie-1. As these intermediate proteins would be likely to be phosphorylated the blots were examined for likely candidates. In the reprobes of the blots in Figures 5.4B, 5.5B with antibodies to phosphotyrosine and on longer exposures of 5.6B, a 110-120 kDa phosphorylated protein was always present.

Proteins that could mediate tie-1:SH-PTP2 binding are SHPS-1and PECAM. SHPS-1 is a 120kDa glycoprotein that is a membrane bound receptor like protein. The extracellular domain is made up of three immunoglobulin like regions and the cytoplasmic region has four potential tyrosine phosphorylation sites for SH2 domain binding (213). Several growth factors have been shown to tyrosine phosphorylate SHPS-1, a process which then leads to association with SH-PTP2 (214, 215). It is possible that tie-1 endodomain somehow complexes with SHPS-1 and SH-PTP2 and that the 110kDa-120kDa polypeptide band seen on the previous anti-phosphotyrosine blots could be SHPS-1. At the time of this work no anti-SHPS-1 antibody was available.

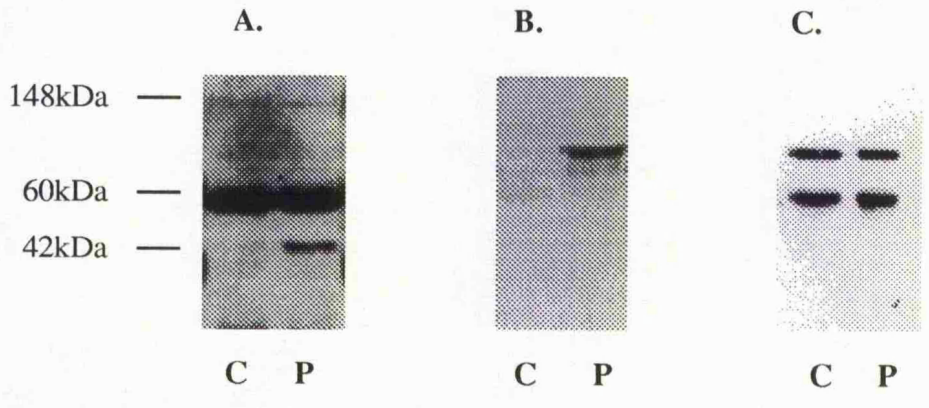


Figure 5.6 Immunoprecipitation of SH-PTP2 protein following phorbol ester activation of HUVE cells. Confluent HUVE cells were treated for one hour with PMA (P) or treated with control vehicle (C) followed by cell lysis and immunoprecipitation (Ip) with an anti-SH-PTP2 antibody. Immunoprecipitates were analysed by SDS-PAGE and Western Blotting (WB). Proteins were identified by immunoblotting with an anti-tie-1 antibody (α tie-1) followed by stripping and reprobing with an anti-phosphotyrosine antibody (α Py) and an anti-SH-PTP2 antibody (α SH-PTP2). Each track shows immunoprecipitations from lysates prepared from

equal numbers of cells. This immunoblot is representative of four experiments.

To test whether a glycoprotein was involved in tie-1/SH-PTP2 binding, cells were treated with PMA and glycoproteins recovered on lectin-Sepharose. Triticum Vulgaris{wheat germ} has a high affinity for N-acetyl- β-D- glucosaminyl residues and N-acetyl- β-D- glucosamine oligomers and can be used to recover glycoproteins containing these residues. Figure 5.7 shows the immunoblots of proteins recovered by lectin-Sepharose following PMA treatment. Immunoblotting with an anti-tie-antibody, Figure 5.7 A., showed that the whole tie-1 receptor and the 40kDa endodomain bound to the lectin-Sepharose. Although the full length tie-1 receptor extracellular domain is glycosylated, the intracellular domain is not. The first predicted glycosylation site is at asparagine 721 of the extracellular domain and the transmembrane portion commences at amino acid 766 (calculated from Peptide structure in the GCG package, Wisconsin Molecular Biology Software Package). When this blot was reprobed with anti-phosphotyrosine antibody, Figure 5.7B., tyrosine phosphorylated bands were present at 72, 90, 110 and 120kDa in both control and PMA treated lanes. There was a tyrosine phosphorylated protein of 72kDa in the PMA treated cells. When the blot was stripped and reprobed with an anti-SH-PTP2 antibody immunoreactive bands in both control and PMA treated lanes were present at 72 kDa which corresponds to SH-PTP2 protein. When the blot was further stripped and incubated with biotin -labelled lectin (see section 3.3.2.4), glycosylated proteins were seen at 135, 120 kDa and at 85 kDa, Figure 5.7 D. However, no glycosylated bands were seen at 40 kDa. This confirmed that the tie-1 endodomain was not glycosylated and, therefore, for the endodomain to be recovered by lectin sepharose it must associate with a lectin binding glycoconjugate (binding to WGA may not be specific and any NAG containing glycoconjugate may bind to WGA-sepharose). A possible candidate for this was the 120kDa glycoprotein, SHPS-1. However, this can only be confirmed by the direct immunoprecipitation with a specific anti-SHPS-1 antibody.

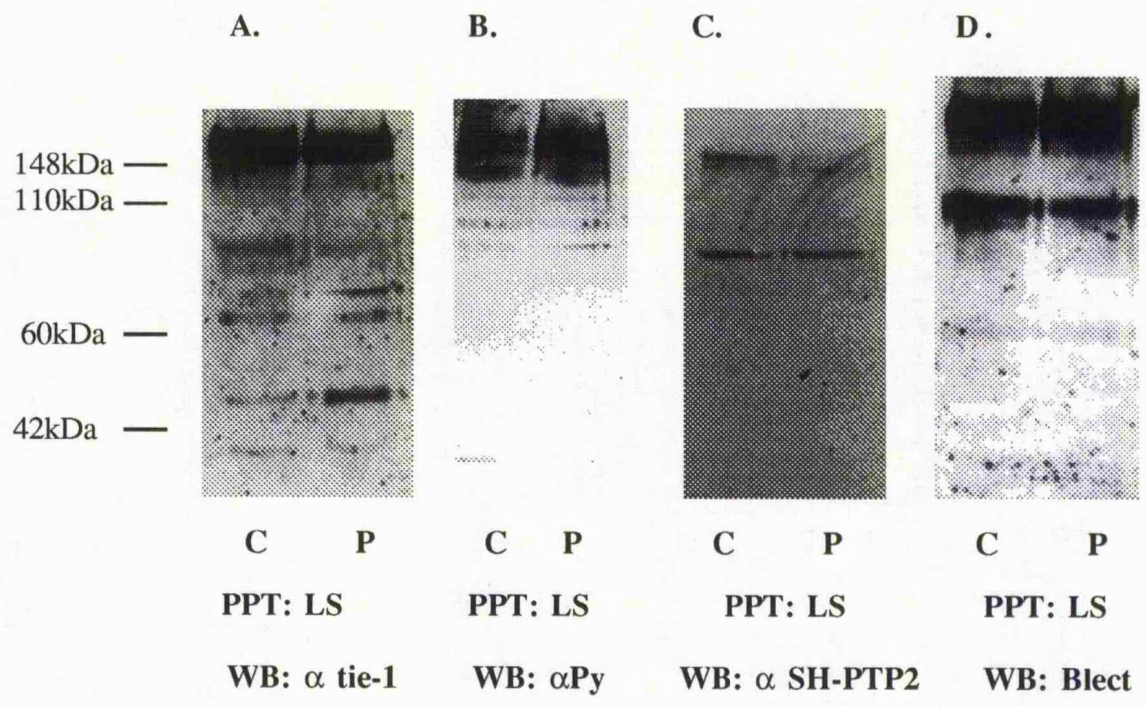


Figure 5.7 Lectin-Sepharose precipitation of glycoproteins following phorbol ester activation of HUVE cells. Confluent HUVE cells were treated for one hour with PMA (P) or treated with control vehicle (C) followed by cell lysis and glycoproteins were precipitated (PPT) with Lectin-sepharose (LS). Precipitates were analysed by SDS-PAGE and Western Blotting (WB). Proteins were identified by immunoblotting with an anti-tie-1 antibody (α tie-1) followed by stripping and reprobing with an anti-phosphotyrosine antibody (α Py) and an anti-SH-PTP2 antibody (α SH-PTP2). The blot was further stripped and probed with biotinylated lectin (Blect) for 1 hour to detect glycoproteins, washed and then probed with Streptavidin followed by development with ECL (D). Both tracks shows immunoprecipitations from lysates prepared from equal numbers of cells. This immunoblot is representative of two experiments.

5.2.3 Investigation into tie-1 ectodomain cleavage in human tissues

The observation that PMA activation of endothelial cells in culture can result in tie-1 ectodomain cleavage suggests that this event could occur *in vivo* and would thus, represent an important mechanism to regulate tie-1. In order to determine whether tie-1 is truncated *in vivo*, cell lysates from human breast and lung tumours, and placenta were immunoprecipitated with an anti-tie-1 antibody or polyclonal IgG. Figure 5.8 shows an immunoblot of tie-1 protein from a human full gestational term placenta. A protein at approximately 135kDa, consistent with tie-1 holoreceptor, was present in the anti-tie-1 immunoprecipitate lane. A prominent band appearing at 40 kDa was also seen. This immunoblot suggested that tie-1 may be in a truncated 40kDa form *in vivo* . Similar observations were seen in human breast and lung cancers and also human skin (data not shown). If endodomain is present *in vivo* it suggests this modification of the tie-1 receptor could have an important role in endothelial function.

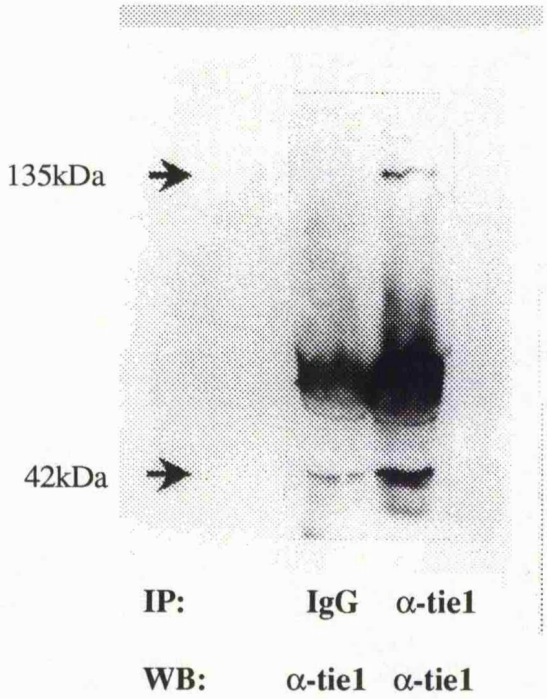


Figure 5.8 Human placental extracts contain both full-length and truncated forms of tie-1. Samples of term human placentae were frozen in liquid nitrogen and stored at -80°C. Tissue samples were crushed to powder and then lysed in buffer containing protease cocktail inhibitors. Lysates were cleared by centrifugation and supernatants immunoprecipitated with anti-tie-1 or rabbit IgG. Immunoprecipitated protein was resolved by SDS/PAGE and tie-1 detected by immunoblotting with anti-tie-1 carboxy-terminus antibody. This immunoblot is representative of ten experiments.

5.2.4 Investigation into possible initiators of tie-1 ectodomain cleavage

Neurotrophin, the natural ligand for tyrosine kinase receptor TrkA, not only activates the receptor but leads to ectodomain cleavage of TrkA, which generates a cell-bound fragment with increased tyrosine phosphorylation (205). A similar event may well occur with tie-1 and its ligand. At the time of writing no ligand has been identified for tie-1, although as shown in Chapter 3, it appears that an activating ligand for tie-1 is present in a melanoma cell line, SK-Mel 24. To establish if a product in SK-Mel 24 conditioned medium could lead to tie-1 extracellular domain cleavage in HUVE cells, SK-Mel 24 conditioned medium was incubated with quiesced HUVE cells as discussed in section 3.3.2.3 for 5 or 10 minutes. Proteins were immunoprecipitated with an anti-phosphotyrosine antibody and samples analysed by SDS-PAGE and Western blotting. Immunoblots were probed with an anti-tie-1 antibody, stripped and then reprobed with an anti-phosphotyrosine and then anti-SH-PTP2 antibodies.

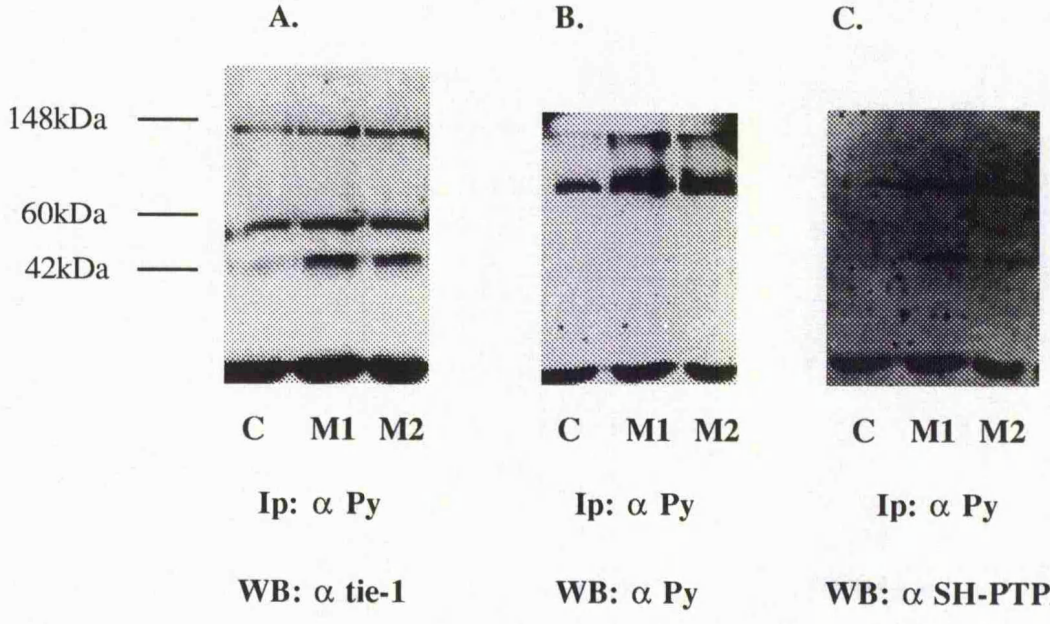


Figure 5.9 Truncation of tie-1 following addition of Sk-Mel 24 conditioned medium onto HUVE cells. Quiesced HUVE cells were placed in contact

with melanoma conditioned medium for 5 minutes (M1) or 10 minutes (M2) and phosphorylation of tie-1 receptor was compared to control cells that had only been quiesced (C). Immunoprecipitation (Ip) using anti-phosphotyrosine antibody (αPy) was used. Western blot (A) was probed with anti-tie-1 antibody (α tie-1) then stripped and reprobed (B) with an anti-phosphotyrosine antibody (αPy) the blots was further stripped and reprobed with an anti-SH-PTP2 antibody (α SH-PTP2). Each track shows immunoprecipitations from lysates prepared from equal numbers of cells. This immunoblot is representative of three experiments.

Figure 5.9 A and B. shows that SK-Mel 24 conditioned medium led to both tyrosine phosphorylation of the 135 kDa tie-1 and generation of a 40 kDa product that is not tyrosine phosphorylated. This 40kDa band corresponds to the tie-1 endodomain band suggesting that a component of SK-Mel 24 medium cleaves the tie-1 holoreceptor. The phosphotyrosine overlay, Figure 5.9 B. also demonstrated increased phosphorylation compared to control of a band at approximately 70-75kDa. These bands correspond to the 72kDa SH-PTP2 as shown in Figure 5.9 C. Increased exposure of the phosphotyrosine overlay, Figure 5.9 B., also displayed increased tyrosine phosphorylation of a 120 kDa protein in HUVE cells that had been incubated with SK-Mel 24 conditioned medium (not shown).

These data suggest that Sk-Mel 24 conditioned medium contained an activating ligand and a component that causes rapid tie-1 ectodomain cleavage. This component could be the activating ligand(s) or it could be a protease within the medium . To determine whether the component responsible for initiating ectodomain cleavage binds to tie-1 ectodomain, SK-Mel 24 conditioned medium was incubated with either tie-1 ED-myc/His or TrkA ED-myc/His suspended on Probond resin as described in section 3.3.2.4. These samples were then centrifuged at 800g for 2 minutes and the resultant supernatant examine for its ability to cause tie-1 truncation in HUVE cells (Fig 5.10). In Figure 5.10 A. there are equal amounts

of tie-1 whole receptor in both the mature and immature forms in all lanes with no disappearance of the mature upper band as seen in PMA treated cells. However, there was a tie-1 reactive band at 40 kDa in the cells treated with conditioned medium from SK-Mel 24 cells. There is little difference between medium which had been pre-incubated with tie-1 ED compared with that incubated with trkA ED. This suggested the activity responsible for inducing cleavage was not depleted by tie-1 ED. It is possible that if the activity did bind to tie-1 ED there was not enough of the fusion protein present to deplete it significantly. Further experiments will be required to test this.

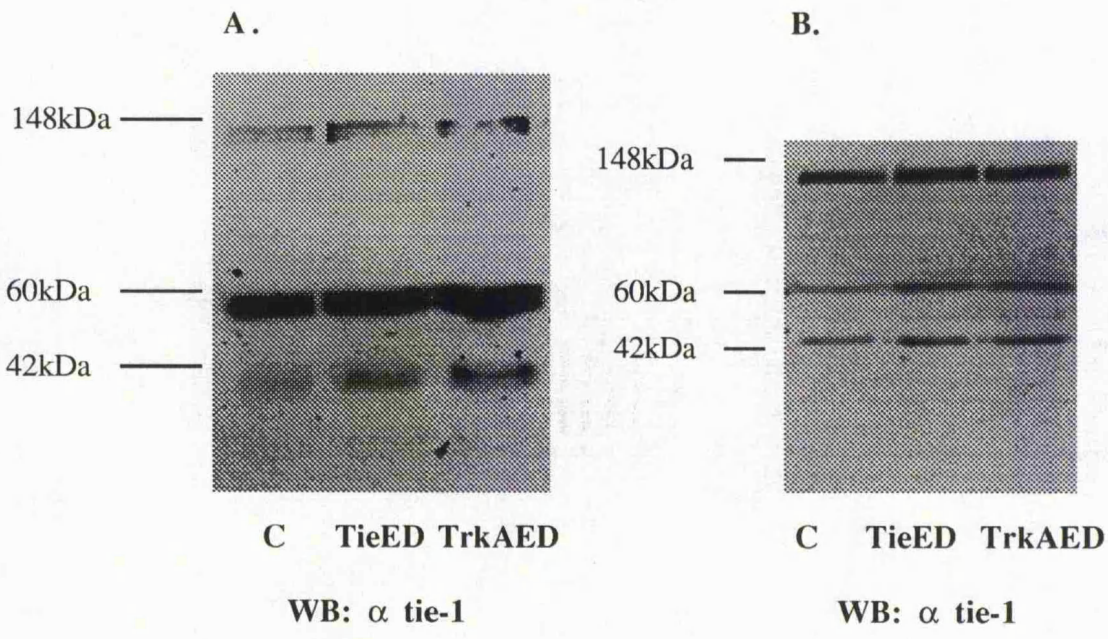


Figure 5.10 The effect of conditioned SK-Mel 24 medium, following incubation with tie-1ED-myc/His and TrkA ED-myc/His fusion proteins, on tie-1 receptor truncation. SK-Mel 24 conditioned medium was incubated with either tie-1 ED-myc/His or TrkA ED-myc/His suspended on Probond resin. These samples were then centrifuged at 800g for 2 minutes and the resultant supernatant was incubated with confluent HUVE cells on a 6-well plate for 5 minutes. Non-treated HUVE cells were used as controls (C). Cells were then lysed with 1x SB-DTT, the samples were sonicated, boiled and centrifuged for 2 minutes at 13,000g. Proteins were analysed by SDS-PAGE and Western blotting and immunoblotting performed with an anti-tie 1

antibody (α tie-1). The blots shown are two independent experiments A and B. Each track represents cell lysates from equal numbers of cells, corresponding to approximately 1/10 of each well of a 6-well plate.

The physiological activators of ectodomain cleavage are not known. It is possible that angiogenic growth factors could modulate the cleavage state. As a preliminary test of this, the ability of $VEGF_{165}$ to induce tie-1 ectodomain cleavage was tested. As shown in Figure 5.11 VEGF induced a rapid cleavage of tie-1 holoreceptor as judged by the appearance of tie-1 endodomain.

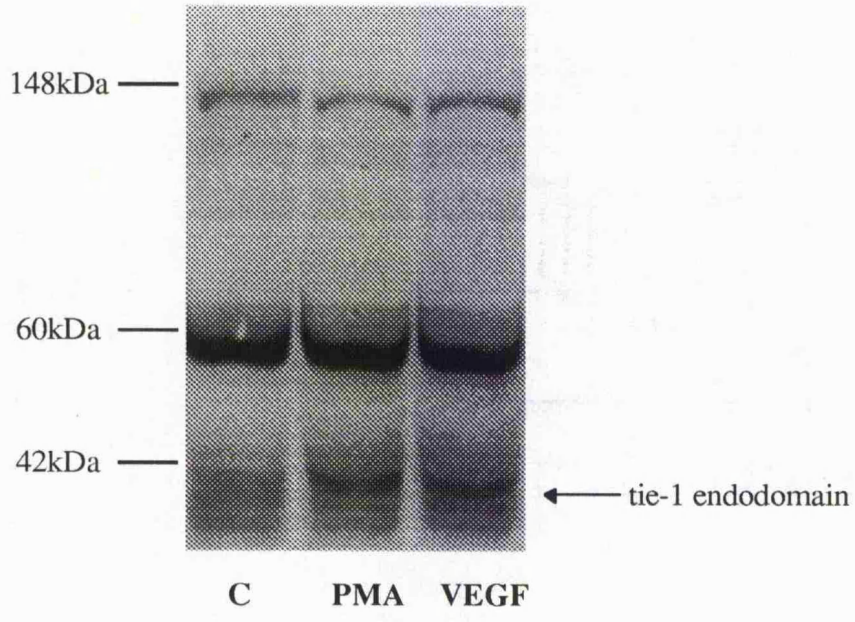


Figure 5.11 The effect of $VEGF_{165}$ on tie-1 receptor truncation. Confluent HUVE cells were incubated with PMA for 30 minutes, $VEGF_{165}$ (100ng/ml) for 10 minutes or with control vehicle(C) for 30 minutes. Cells were lysed with 1xSB-DTT, sonicated, boiled and centrifuged at 13,000g for 1 minute. Proteins were analysed by SDS-PAGE and Western blotting. Tie-1 immunoreactive bands were detected with an anti-tie-1 antibody. This immunoblot is representative of four experiments. Whole cell lysates from equal numbers of cells corresponding to approximately 1/10 of each well from a 6-well plate were loaded onto each track.

5.2.5 Function of the truncated tie-1 receptor endodomain

As described earlier tie-1 ectodomain cleavage generates a persistent 40kDa endodomain fragment that associates with SH-PTP2 and a glycoprotein. The downstream functions that could be regulated by this signalling pathway are not known. It is possible that the tie-1 endodomain may signal to maintain a state of ectodomain cleavage and may augment this by inducing the loss of tie-1 ectodomain in other cells that have not received the appropriate primary stimulus for cleavage. This effect may be due to tie-1 endodomain leading to the production of a protease. To test this a truncated form of tie-1 consisting of leader sequence-transmembrane portion and intracellular domain was expressed in endothelial cells and medium from these cells was tested for its capacity to induce cleavage of the tie-1 whole receptor in non-activated cells. This truncated form of tie-1 should approximately correspond to the endodomain seen in previous experiments, however it should be emphasised that currently the ectodomain cleavage site is not known.

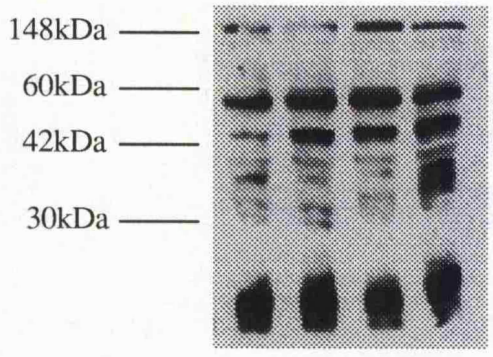


Figure 5.12 The effect of transfected tie-1 endodomain on tie-1 ectodomain cleavage. BAEC's expressing tie-1 intracellular domain (tie ID) and the tie-1 holoreceptor (tie) were cultured for 24 hours in serum free medium and this conditioned medium used to challenge parallel cultures of HUVE cells for 10 minutes at 37 ^{0} C before lysis and determination of cleavage state of tie-1 by Western blotting of whole cell lysates. DMEM media that had not been in contact with BAEC's (C) and serum-free conditioned media from BAEC's transfected with empty pFlag vector (V) were also incubated with HUVE

cells and used as controls. This is a representative immunoblot of four independent experiments. Each track represents cell lysates from equal numbers of cells corresponding to 1/10 of a well from a 6-well plate.

Figure 5.12 . shows HUVE cells treated with conditioned medium from BAEC's transfected with vector, holoreceptor and the tie-1 ID exhibit increased tie-1 endodomain compared with untreated HUVE cells. In the control lane there appeared to be a background level of endodomain generation, which was increased when cells were incubated with conditioned media from BAEC's transfected with empty vector or conditioned media from tie-1 holoreceptor transfected BAEC's. This suggests BAEC' s produced a basal cleavage activity constitutively which was not affected by holoreceptor expression or transfection with empty vector. When the BAEC's were transfected with the tie-1 endodomain the conditioned medium from these cells led to the appearance of a 35 kDa doublet tie-1 immunoreactive band. It is possible that this 35kDa tie-1 immunoreactive band is a further proteolysis product of tie-1. In order to test this lysates from cells that had been treated with medium from endodomain in expressing cells were probed with the tie-1 antibody in the presence of blocking peptide (Fig 5.13). Figure 5.13 shows that the tie-1 peptide blocked reaction of the anti-tie-1 antibody with proteins 40kDa and tie-1 doublet of 35kDa, this was not blocked with the tie-2 peptide. This indicates that the 40kDa band and 35kDa doublet contained the tie-1 C-terminal fragment. Tie-1 endodomain , tie-1 holoreceptor clones and the resultant transfected media were produced by co-workers at the Department of Surgery, University of Leicester. These data suggest conditioned medium from tie-1 ID expressing cells activates production of a 35kDa doublet proteolytic product of tie-1 in target cells. The mechanism whereby this occurs is not known. However, it is possible that the ID expressing cells produce a protease which causes this further proteolysis.

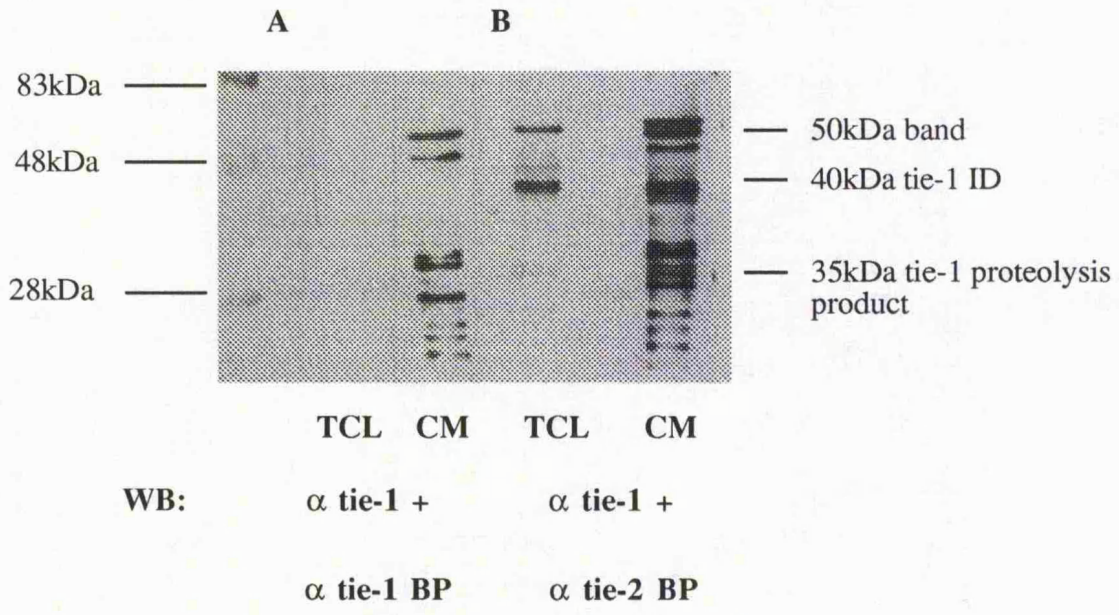


Figure 5.13 The effect of transfected tie-1 endodomain on tie-1 ectodomain cleavage: the generation of 40kDa and 35kDa proteolysis products. A. BAEC's expressing tie-1 intracellular domain (tie ID) were cultured for 24 hours in serum free medium and this conditioned medium used to challenge parallel cultures of BAEC's cells for 10 minutes at 37 ^{0}C before lysis and determination of cleavage state of tie-1 by Western blotting of whole cell lysates (CM). Whole cell lysates of the transfected BAEC's were also analysed (TCL). The membranes were incubated with an anti-tie-1 antibody and a tie-1 blocking peptide (α tie-1{1:1000} + α tie-1 BP{1μg/ml}). Identical samples of the same protein content were transferred onto another membrane (B) and incubated with anti-tie-1 antibody and a tie-2 blocking peptide (α tie-1{1:1000}+ α tie-2 BP{1μg/ml}). These are representative immunoblots from two independent experiments. Whole cell lysates from equal numbers of cells corresponding to 1/10 of a well from a 6-well plate were loaded equally onto each well.

These data suggest that in addition to cleavage producing the 40kDa product previously described, additional cleavage products are also produced. In addition to this, the tie-1 endodomain may signal for the production of a substance which leads to cleavage of tie-1 in non-activated endothelial cells. The apparent lack of effect of conditioned medium on holoreceptor levels is likely to reflect the appearance of newly synthesised holoreceptor rapidly at the cell surface. Further experiments will be required to test this hypothesis. The activity released from endodomain expressing BAEC's may be a protease or an agonist which stimulates ectodomain cleavage. To distinguish between these possibilities an *in vitro* assay of cleavage activity was established. Tie-1ED-myc/His fusion protein (90kDa) bound to Probond resin was incubated with medium from tie-1 endodomain transfected endothelial cells. If the endothelial cells produced a protease, the ectodomain would be cleaved off from the myc/His tag and released from the Probond resin. Western blotting of tie-1 ED-myc/His bound to resin would demonstrate a decrease in the amount of full length Tie-1 ED-myc/His following cleavage. Tie-1 ED-myc/His fusion protein was produced and bound to Probond resin as described in Chapter 3. Tie-1 ED-myc/His bound to Probond resin was then incubated for 45 minutes at room temperature with a) medium from tie-1 endodomain transfected BAEC's or b) medium from empty vector, pFlag, transfected BAEC's or c) medium from non-transfected BAEC's that had been incubated for 24 hours in serum free DMEM. The effect of this medium on tie-1 ED was determined by the immunoblot detailed in Figure 5.14.

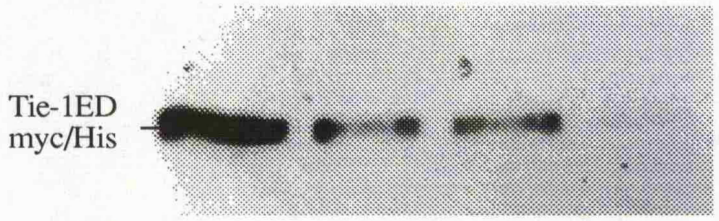


Figure 5.14 Tie-1 ED-myc/His cleavage from Probond Resin by conditioned medium from tie-1ID transfected cells. Tie-1ED-myc/His fusion protein attached to a nickel chelating Probond Resin was incubated with serum-free conditioned medium from non-transfected BAEC's(B), BAEC's transfected with empty vector pFlag(V), and BAEC's transfected with tie-1 intracellular domain (tie ID). The samples were then centrifuged at 800g for 2 minutes and the pelleted resin was washed twice with 1x PBS. An equal volume of 2x SB-DTT was added to the samples, these were then vortexed, boiled for 5 minutes and centrifuged at 13,000g for 1 minute. As a control a similar sample of tie-1 ED myc/His bound to Probond resin was not incubated with any medium and simply had an equal amount of 2x SB-DTT added to it. Proteins were then resolved by SDS/PAGE and tie-1 ED-myc/His detected by anti-myc Western blotting. Cleavage was assessed by loss of the 100kDa immobilised ectodomain by anti-myc-tag Western blotting. Tie-1ED-myc/His attached to Probond Resin that had not been in contact with media was used as a control(C). This immunoblot is representative of two experiments

Medium from tie-1 endodomain transfected BAEC's appeared to almost completely cleave tie-1 ED off the Probond resin, Figure 5.14. Quite extensive cleavage was also seen in medium from empty vector transfected cells and medium from non-transfected BAEC's. This suggested that BAEC's constitutively express and secrete a protease that led to cleavage of the tie-1 ectodomain. The presence of transfected tie-1 endodomain led to an increase secretion/activation of this protease. These data suggest endodomain generation activates release of a protease.

5.2.6 Inhibition of PMA activated tie-1 ectodomain cleavage by Marimastat

The nature of the protease responsible for PMA stimulated tie-1 cleavage is not known. To determine if this protease was a metalloprotease, HUVE cells were incubated with PMA for one hour , with or without marimastat, a known metalloprotease inhibitor. Marimastat was used at a concentration of $1x10^{-5}$ M, a concentration known to inhibit metalloprotease activity (A J Turner, University of Leeds, personal communication). Cell lysates were analysed by SDS-PAGE and Western blotting. Tie-1 reactive bands were identified with an anti-tie-1 antibody.

Figure 5.15 clearly shows that marimastat inhibited tie-1 ectodomain cleavage in the presence of PMA. This confirms that PMA activated tie ectodomain cleavage was mediated by a metalloprotease.

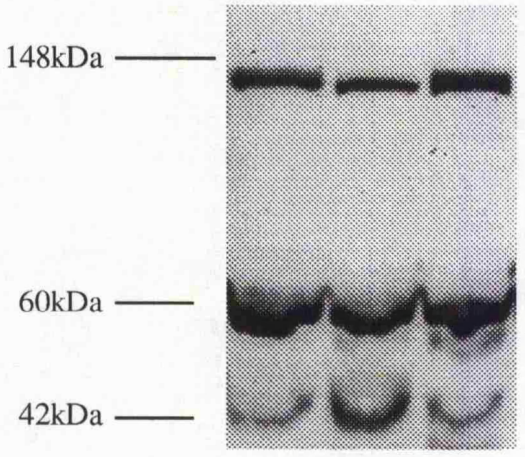


Figure 5.15 The effect of marimastat on PMA activated tie-1 ectodomain cleavage. Confluent HUVE cells were incubated with PMA (PMA) or with control vehicle- DMSO (C). One well of cells were pre-incubated with marimastat for 10 minutes before treatment with PMA (PMA+marimastat). Following treatment cells were washed with PBS and lysed with 1xSB-DTT. The samples were sonicated, centrifuged and boiled. The samples were

analysed by SDS-PAGE and the proteins transferred by Western blotting. Tie-1 immunoreactive bands were identified by immunoblotting with an anti-tie-1 antibody. This is a representative blot of four experiments. Whole cell lysates from equal numbers of cells corresponding to 1/10 of a well from a 6-well plate were loaded onto each track.

5.3 Discussion

The proteolytic cleavage of ectodomain from many cell surface receptors is known to occur (208, 217) and the biological implications of this process varies considerably from the soluble ectodomain acting as an antagonist for the ligand to the generation of cell associated endodomain that has increased catalytic activity once the ectodomain is removed (205, 217). Tie-1 ectodomain cleavage and the generation of a persistent, membrane bound tie-1 endodomain can be induced in cultured endothelial cells by phorbol ester (PMA) activation of protein kinase C (158). The membrane bound tie-1 endodomain also appears to be present in human tissues and has been demonstrated in full term placentae . This suggests that tie-1 ectodomain cleavage occurs *in vivo* and is of possible importance in the function of the tie-1 receptor.

Ectodomain cleavage of other receptor tyrosine kinases can lead to activation of signalling pathways via increased catalytic activity of the intracellular endodomain in several oncoprotein receptors (211) and in TrkA (205). Following PMA treatment of HUVE cells tie-1 endodomain remained in the cell membrane fraction for up to seven hours. This suggested that this provides an opportunity for it to bind and activate signalling intermediates and, hence, it could have signalling capability. Immunoprecipitation with an anti-tie-1 antibody and probing of the immunoblot with an anti-phosphotyrosine antibody failed to demonstrate any tyrosine phosphorylation of the tie-1 endodomain. The presence of several phosphorylated bands at 70-80kDa and at 110-120kDa in the PMA treated cells raised the possibility that that the tie-1 endodomain co-immunoprecipitated with other proteins that may be signalling intermediates. To test this hypothesis tyrosine phosphorylated proteins were immunoprecipitated with a anti- phosphotyrosine antibody following PMA treatment of HUVE cells. An anti-phosphotyrosine probe of the immunoblot demonstrated the same bands at 70-80 and 110-120kDa, however reprobing with an anti-tie-1 antibody showed the presence of the 40 kDa tie-1 endodomain which was not tyrosine phosphorylated. This suggests that one or more tyrosine phosphorylated proteins were associated with the tie-1 endodomain. Among these proteins the 72kDa signalling intermediate SH-PTP2, was

demonstrated by reprobing both of the above immunoblots with an anti-SH-PTP2 antibody. To determine whether SH-PTP2 associated with endodomain, antibody was used to immunoprecipitate proteins from cells that had been treated with PMA. The immunoblots show that SH-PTP2 is co-immunoprecipitated with tie-1 endodomain, thus confirming that tie-1 endodomain associates with SH-PTP2. SH-PTP2 has 2 SH2 domains which bind to phosphorylated tyrosine residues (162). The finding that tie-1 endodomain was not detectably tyrosine phosphorylated, raised the possibility that SH-PTP2 was not binding to tie-1 endodomain directly but was associating with another protein that was bound to tie-1 endodomain. The presence of the 120kDa phosphorylated protein on previous blots suggested that this could be a further signalling intermediate that associates with the tie-1 endodomain. SHPS-1 is a 120kDa membrane bound glycoprotein that is known to activate SH-PTP2 by tyrosine phosphorylation of its SH2 domains (213, 214, 215). It was hypothesised that this protein may have been the 110-120kDa phosphorylated protein seen on the previous blots. However, due to the lack of availability of an available SHPS-1 antibody at the time this hypothesis could not be tested. Precipitation of glycoproteins by wheat germ agglutinin precipitated a) tie-1 endodomain which is not a glycoprotein b) SH-PTP2 which is not a glycoprotein and c) several tyrosine phosphorylated glycoproteins including one of approximately 120 kDa. These data are consistent with a model in which endodomain, SH-PTP2 and a 120kDa glycoprotein (p120) form a complex. It was not possible to conclude the identity of the 120kDa glycoprotein in the present study, though the fact that SHPS-1 is a similar size and is a SH-PTP2 binding glycoprotein suggests the possibility that the protein may be SHPS-1. Whilst the blots demonstrate that SH-PTP2 and p120 were tyrosine phosphorylated it is not known whether this effect was due to the effects of PMA or the tie-1 endodomain, and only a PMA independent model of tie-1 ectodomain cleavage could answer this question. The functional significance of the complex formed between the tie-1 endodomain and SH-PTP2 is as yet not known. However, it does demonstrate for the first time possible signalling intermediates for the tie-1 receptor and, furthermore, that tie-1 ectodomain cleavage results in downstream signalling events.

The fact that the truncated endodomain is not tyrosine phosphorylated suggests that it may signal through a different pathway than that activated by the tie-1 ligand which is likely to induce tyrosine autophosphorylation of the receptor. However, it is possible that ligand binding activates tyrosine phosphorylation of tie-1 leading to activation of signalling intermediates that lead to activation of protein kinase C. This could then result in the activation/ production of a protease that would result in tie-1 ectodomain cleavage. Simultaneously, ligand binding may also result in activation of further signalling pathways.

An important finding of this study was that expression of tie-1 endodomain resulted in the production of an activity which modulated tie-1 truncation state. This activity appeared to be a protease, though further work will be required to confirm this. Whether the signalling complex of tie-1 endodomain, SH-PTP2 and other proteins participate in endodomain stimulation of protease production is not known and needs to be further investigated. Similarly, the protease responsible for tie-1 ectodomain cleavage has yet to be identified, although it is likely to be a member of the metalloprotease/disintegrin/cysteine-rich (MDC) family (208). The site of cleavage within the ectodomain of tie-1 has yet to be determined and is the subject of ongoing work. This downstream effect of tie-1 is the first cellular activity to be described that is mediated by tie-1 signalling, albeit a novel cleavage activated form of signalling.

Regulation of production of ectodomain protease could be an important factor in local control of tie-1 ligand responsiveness of vessels. Activation of endodomain signalling in one or a small group of cells by a primary stimulus could induce production of cleavage activity causing desensitisation to the tie-1 ligand in surrounding cells. At present this primary stimulus remains unknown, although it may be that activation/secretion of the protease could be protein kinase C dependent.

VEGF, which activates protein kinase C, leads to truncation of the tie-1 receptor. This may be relevant to the role of tie-1 in vessel stabilisation/destabilisation. VEGF initiates angiogenesis, therefore, in order for a mature stable endothelial cell /blood vessel to undergo

a period of proliferation and differentiation it must become destabilised and responsive. If ligand activation of tie-1 leads to blood vessel and endothelial cell stability as suggested by the studies outlined in Chapter 1, ectodomain cleavage would render the receptor unresponsive to its ligand and hence lead to destabilisation of the vessel. Further investigation into the role of tie-1 endodomain in this process needs to be explored and it will allow a greater understanding of the function of the tie-1 receptor. Therefore, it is possible that other activators of protein kinase C may also lead to tie-1 ectodomain cleavage.

From the work described it appears that the malignant melanoma Sk-Mel 24 cells produce a substance that results in cleavage of tie-1 ectodomain. From the present data it is not possible to conclude whether cleavage produced is due to a protein kinase C agonist or the presence of a protease in the medium. The *in vitro* assay of tie-1 ectodomain cleavage could be used to test if the protease was present in conditioned concentrated media from SK-Mel 24 cells. If it is present then this could be a reason for the variability seen in ectodomain binding assays used to probe for the presence of a tie-1 ligand (Chapter 3) by using the tie-1 ED-myc/His fusion protein as a probe to assess tie-1 ligand binding. The finding that broad range metalloprotease inhibitors, such as marimastat, inhibits ectodomain cleavage suggest it would be necessary to include such inhibitors for ectodomain binding assays in future work.

This work shows for the first time that ectodomain cleavage of tie-1 is associated with signalling and downstream events. The possible presence of the 40 kDa endodomain in human malignant tumours, full gestational placenta and in normal skin (data not shown) suggests that tie-1 ectodomain cleavage could play a significant role in the normal homeostatic process of the endothelial cell. However, no quantitative comparisons on the level of tie-1 ectodomain cleavage were made between these samples. Whether the amount of tie-1 ectodomain cleavage increases or decreases during angiogenesis is not known and investigation into any differences may well help to understand the true significance of this event. Tie-1 ectodomain cleavage and its augmentation by endodomain to produce local areas of ligand non-responsive cells, may be a mechanism for antagonism of positive tie-1 ligands. Isolation of the protease and the tie-1 ligand will allow a greater understanding of

the function of this receptor and the role it plays during both angiogenesis and in the normal functioning of the endothelial cell. It would be interesting to able to isolate the protease or be able to inhibit its effect as this would allow a greater understanding of the role of tie-1 ectodomain cleavage and the role it plays in endothelial cell function.

5.3.1 Future Work on ectodomain cleavage

By sequencing of N-terminal of truncated tie-1 identify the site of cleavage on the tie-1 ectodomain. This may give some insight as to which protease is causing this event.

It may also be possible to use the metalloprotease inhibitors with SK-Mel 24 conditioned medium to allow isolation and cloning of the tie-1 ligand by the methods described in chapter 3. Finally, further investigate the signalling pathway that is activated on tie-1 ectodomain cleavage, and confirm that the 120kDa protein is SHPS-1 by using immunoprecipitation with a anti- SHPS-1 antibody following phorbol ester activation of HUVE cells.

Chapter six

Concluding Remarks

6.1 Discussion

Tie-1 is an endothelial cell receptor tyrosine kinase that plays an essential role in the latter stages of vascular development. It is important in blood vessel stabilisation and may allow the differentiation of periendothelial cells (144). Histological examinations of mice deficient in the tie-1 gene demonstrates the consequences of a lack of tie-1 signalling (98, 143, 144, 147). These mice have endothelial cells that have large transcellular holes within them. Furthermore, there is an excessive increase in vascular density with many non-functional blood vessels (144). These abnormalities show the underlying importance of tie-1 in maintaining endothelial cell integrity, inhibiting further vessel remodelling and, ultimately, leading to blood vessel stabilisation. What is not known is whether tie-1 needs to signal continuously in order to maintain this stable state in adult endothelium. However, tie-1 protein is expressed throughout the adult endothelium and, therefore, could presumably signal and have a role to play in maintaining vessel stabilisation in the long term. Tie-1 receptor function could possibly be enhanced therapeutically by delivering a tie-1 ligand in conjunction with VEGF into an ischaemic limb.

At the time the work presented in this thesis was commenced there was no known ligand for tie-1, nothing was known about the factors that regulate its expression and nothing was known about downstream signalling intermediates that associate with the tie-1 receptor. Data presented in this thesis demonstrates that the expression of the tie-1 receptor protein in vascular endothelial cells is increased by both VEGF and hypoxia at the level of transcription. Whilst the addition of actinomycin D (a transcriptional inhibitor) blocked these effects, the mechanism by which VEGF and hypoxia increase tie-1 gene transcription is not known and is currently the subject of ongoing work. Both VEGF and hypoxia are known activators/initiators of angiogenesis (188) and hypoxia has been shown to increase the expression of VEGF (189) and its receptor, VEGFR-2 (106, 111, 195) in endothelial cells. By increasing the expression of tie-1, VEGF and hypoxia could, therefore, initiate the development of newly formed blood vessels and ensure that these newly developed vessels progress to a period of stabilisation. Thus, angiogenesis and the development of new blood

vessels appears to be under strict regulatory control. Strict regulation of angiogenesis is important in order to prevent hypervascularity of the ischaemic organ and to allow the development of functional, mature blood vessels. The deleterious consequences of hypervascularity are seen in diabetic retinopathy, where hyperpermeable, immature blood vessels are prone to exudates in the retina and haemorrhage. Whether diabetes mellitus results in a defect in tie-1 receptor function is unknown. It would be interesting to investigate this question as the possibility of vessel stabilisation may be a potential therapeutic option in this condition.

Understanding the mechanism by which the tie-1 receptor functions at the cellular level would be enhanced by identifying the downstream signalling pathway. This has been hampered by the absence of a known ligand for tie-1. Construction of a chimaeric form of tie-1 would allow regulated activation of downstream signalling intermediates. This method involves constructing a form of receptor in which the intracellular domain of tie-1 is fused with an extracellular domain of another tyrosine kinase receptor with a known ligand e.g. TrkA receptor and its ligand, nerve growth factor. Activation of the extracellular domain by its ligand would result in autophosphorylation of the intracellular domain tyrosine kinase residues. Co-immunoprecipitation with an anti-tie-1 antibody would allow identification of possible associated proteins. This work is currently being undertaken by others in this laboratory. However, as discussed in chapter 5, a novel signalling mechanism for tie-1 has been suggested by the data presented in this thesis. Yabkowitz et al demonstrated that the tie-1 receptor undergoes proteolytic cleavage of its extracellular domain via a protein kinase C activated mechanism (158). This effect, however, was only demonstrated on endothelial cells in culture following activation with a phorbol ester, PMA. As discussed in chapter 5, this cleavage phenomenon occurs in human endothelial cells in both physiological and pathological situations. It also appears that the membrane bound tie-1 endodomain persists in the membrane for up to several hours. Membrane bound proteins that have undergone proteolysis are usually internalised into the cytoplasm and undergo further degradation within a relatively short time span. The persistence of the tie-1 endodomain within the membrane raised the question of whether the tie-1 endodomain had a functional role and was

activating downstream intermediates. Transfection of the tie-1 endodomain into endothelial cells apparently led to the secretion of a protease into the surrounding cell medium based on the effects of this medium on tie-1 truncation *in vitro* and in naive endothelial cells. Therefore, activation of tie-1 ectodomain cleavage in one endothelial cell can result in the ectodomain cleavage of the tie-1 receptors in the surrounding cells. The protease activation/secretion may be protein kinase C dependent. Tie-1 ectodomain cleavage can occur also when endothelial cells are incubated with VEGF. VEGF is known to activate protein kinase C and, therefore, its effect on tie-1 ectodomain cleavage could be mediated via this pathway. The protease itself appears to be a metalloprotease as the effect of PMA is inhibited by marimastat, a known metalloprotease inhibitor. The protease has not been isolated and indeed its site of cleavage is not yet known. However, the cleavage of tie-1 ectodomain from the tie-1 ED fusion protein bound to Probond resin suggests that the cleavage site lies very close to the transmembrane region on the extracellular side of the cell.

SH-PTP2 and several tyrosine phosphorylated proteins which may include SHPS-1, form a complex with tie-1 endodomain following ectodomain cleavage. This association appears to be independent of tie-1 tyrosine phosphorylation and may indicate a novel signalling pathway. Whilst from the blots illustrated in chapter 5 it appears that SH-PTP2 and other proteins are tyrosine phosphorylated it is not known if this effect is due to the tie-1 endodomain kinase activity or due to other effects of PMA, this needs to be clarified further. It is not known whether association of endodomain with signalling intermediates results in the activation and secretion of the protease directly or whether it activates downstream signalling intermediates that have different functional roles. This work shows for the first time the association of tie-1 with downstream signalling intermediates and, furthermore, that proteolytic cleavage of the ectodomain can generate downstream signalling events.

The functional importance that tie-1 ectodomain cleavage has on the blood vessel and endothelial cell can only be postulated at the present time. Given that expression of the tie-1 receptor is essential for blood vessel stabilisation, inhibition of vascular remodelling and maintaining endothelial cell integrity, tie-1 ectodomain cleavage with the generation of a

membrane bound persistent tie-1 endodomain, would prevent tie-1 ligand binding. Furthermore, the secretion of a protease from the endothelial cell due to the presence of the tie-1 endodomain may result in surrounding cells becoming unresponsive to a tie-1 ligand. Thus, the endothelial cells could effectively become de-stabilised which would allow their subsequent activation by VEGF and the initiation of angiogenesis. It is interesting, therefore, that VEGF can initiate tie-1 ectodomain cleavage in endothelial cells. As previously discussed, both hypoxia and VEGF also lead to increased expression of the tie-1 holoreceptor, though over a longer time period. Therefore, it may be that VEGF, through its effects on tie-1, has a dual role such that the effects of VEGF on the cell may be self limiting. Firstly, immediate action of VEGF to activate a protease that results in tie-1 ectodomain cleavage, this renders the endothelial unresponsive to the tie-1 ligand and, hence, de-stabilises the endothelial cell. This may allow the cell to proliferate and migrate and results in the formation of new blood vessel sprouts. The long term effects of VEGF to increases the expression of the tie-1 holoreceptor protein which will presumably, once expressed, allow the endothelial cell to stabilise and maintain its integrity and halt any further blood vessel re-modelling and development.

Whilst an activating ligand has been identified for the tie-2 receptor (140), at the time of writing there is no known ligand for the tie-1 receptor. Chapter 3 discusses attempts at identifying and isolating a ligand for tie-1. The results suggest that a possible tie-1 ligand is produced by a malignant melanoma cell line which activates tyrosine phosphorylation of tie-1 in vascular endothelial cells. This activating tie-1 ligand may be a glycoprotein and SDS-PAGE of glycoproteins which bind tie-1 ectodomain demonstrates the presence of prominent bands at 80-90kDa and at approximately 25 kDa. In the case of this study, the finding that tie-1 ectodomain is subject to cleavage suggests a reason for variability in the results of ectodomain binding assays for the tie-1 ligand. The identification of marimastat as an inhibitor of this may allow a greater chance of isolating the ligand in the future. This work is ongoing and the 80-90kDa band identified in Figure 3.19 is currently undergoing N-terminal sequencing. The identification of the tie-1 ligand is important in order to allow further investigation into the function of tie-1 and also its downstream signalling pathways. This

information will enhance the knowledge of the molecular mechanisms controlling angiogenesis.

At present there is a large interest in therapeutic angiogenesis and its application in initiating the development of new blood vessels to bypass occluded vessels in both myocardial ischaemia and peripheral vascular disease (7, 51). Following on from Isner's studies on the ability of VEGF to induce angiogenesis and collateral artery development in a rabbit model of hind limb ischaemia (39, 43, 44, 53, 54, 58), the same group have now used intramuscular injections of VEGF cDNA, in human patients presenting with endstage critical lower ischaemia (7). In the preliminary phase I trial results there were no controls so the results published should be treated with caution. However, it is claimed that intramuscular injection of VEGF cDNA into ischaemic calf muscles results in significantly enhanced angiogenesis with improved tissue perfusion, relief of rest pain and healing of ischaemic ulcers. Despite the success at revascularising the limb the majority of patients developed marked tissue oedema. This is a side effect that is due to the effect of VEGF on permeability. The longevity and patency of VEGF induced neovessels are not known. It is likely that activation of the signalling pathways activating vessel maturation, at the appropriate time, would significantly enhance the ability of VEGF to produce patent vessels during therapeutic angiogenesis. At present, the tie-1 and tie-2 system would appear to be attractive targets for activation. Indeed, Asahara et al demonstrated that the ability of VEGF to produce patent vessels was increased 5-fold in the presence of angiopoietin-1 (168). This increase may well be enhanced in the future with the availability of a tie-1 ligand. Furthermore, the observation that VEGF increases tie-1 protein expression suggest that the endothelium would be primed in sites of angiogenesis to the therapeutic introduction of a stabilising tie-1 ligand.

The studies discussed in this thesis have uncovered some aspects of the biology of tie-1. Further work will be required before a complete understanding of the role and regulation of tie-1 in vascular development.

Appendix One

Sequence of cloned tie-1 extracellular domain compared to published sequence

Nucleotide Number

50
CGCTCGTCCT GGCTGGCCTG GGTCGGCCTC TGGAGTATGG TCTGGCGGGT

100
GCCCCCTTTC TTGCTCCCCA TCCTCTTCTT GGCTTCTCAT GTGGGCGCGG

150
CGGTGGACCT GACGCTGCTG GCCAACCTGC GGCTCACGGA CCCCCAGCGC
GTGGACCT GACGCTGCTG GCCAACCTGC GGCTCACGGA CCCCCAGCGC

200
TTCTTCCTGA CTTGCGTGTC TGGGGAGGCC GGGGCGGGGA GGGGCTCGGA
TTCTTCCTGA CTTGCGTGTC TGGGGAGGCC GGGGCGGGGA GGGGCTCGGA

250
CGCCTGGGGC CCGCCCCTGC TGCTGGAGAA GGACGACCGT ATCGTGCGCA
CGCCTGGGGC CCGCCCCTGC TGCTGGAGAA GGACGACCGT ATCGTGCGCA

300
CCCCGCCCGG GCCACCCCTG CGCCTGGCGC GCAACGGTTC GCACCAGGTC
CCCCGCCCGG GCCACCCCTG CGCCTGGCGC GCAACGGTTC GCACCAGGTC

350
ACGCTTCGCG GCTTCTCCAA GCCCTCGGAC CTCGTGGGCG TCTTCTCCTG
ACGCTTCGCG GCTTCTCCAA GCCCTCGGAC CTCGTGGGCG TCTTCTCCTG

400
CGTGGGCGGT GCTGGGGCGC GGCGCACGCG CGTCATCTAC GTGCACAACA
CGTGGGCGGT GCTGGGGCGC GGCGCACGCG CGTCATCTAC GTGCACAACA

450
GCCCTGGAGC CCACCTGCTT CCAGACAAGG TCACACACAC TGTGAACAAA
GCCCTGGAGC CCACCTGCTT CCAGACAAGG TCACACACAC TGTGAACAAA

500
GGTGACACCG CTGTACTTTC TGCACGTGTG CACAAGGAGA AGCAGACAGA
GGTGACACCG CTGTACTTTC TGCACGTGTG CACAAGGAGA AGCAGACAGA

550
CGTGATCTGG AAGAGCAACG GATCCTACTT CTACACCCTG GACTGGCATG
CGTGATCTGG AAGAGCAACG GATCCTACTT CTACACCCTG GACTGGCATG

600
AAGCCCAGGA TGGGCGGTTC CTGCTGCAGC TCCCAAATGT GCAGCCACCA
AAGCCCAGGA TGGGCGGTTC CTGCTGCAGC TCCCAAATGT GCAGCCACCA

650
TCGAGCGGCA TCTACAGTGC CACTTACCTG GAAGCCAGCC CCCTGGGCAG
TCGAGCGGCA TCTACAGTGC CACTTACCTG GAAGCCAGCC CCCTGGGCAG

700
CGCCTTCTTT CGGCTCATCG TGCGGGGTTG TGGGGCTGGG CGCTGGGGGC
CGCCTTCTTT CGGCTCATCG TGCGGGGTTG TGGGGCTGGG CGCTGGGGGC

750
CAGGCTGTAC CAAGGAGTGC CCAGGTTGCC TACATGGAGG TGTCTGCCAC
CAGGCTGTAC CAAGGAGTGC CCAGGTTGCC TACATGGAGG TGTCTGCCAC

800
GACCATGACG GCGAATGTGT ATGCCCCCCT GGCTTCACTG GCACCCGCTG
GACCATGACG GCGAATGTGT ATGCCCCCCT GGCTTCACTG GCACCCGCTG

850
TGAACAGGCC TGCAGAGAGG GCCGTTTTGG GCAGAGCTGC CAGGAGCAGT
TGAACAGGCC TGCAGAGAGG GCCGTTTTGG GCAGAGCTGC CAGGAGCAGT

900
GCCCAGGCAT ATCAGGCTGC CGGGGCCTCA CCTTCTGCCT CCCAGACCCC
GCCCAGGCAT ATCAGGCTGC CGGGGCCTCA CCTTCTGCCT CCCAGACCCC

950
TATGGCTGCT CTTGTGGATC TGGCTGGAGA GGAAGCCAGT GCCAAGAAGC
TATGGCTGCT CTTGTGGATC TGGCTGGAGA GGAAGCCAGT GCCAAGAAGC

1000
TTGTGCCCCT GGTCATTTTG GGGCTGATTG CCGACTCCAG TGCCAGTGTC
TTGTGCCCCT GGTCATTTTG GGGCTGATTG CCGACTCCAG TGCCAGTGTC

1050
AGAATGGTGG CACTTGTGAC CGGTTCAGTG GTTGTGTCTG CCCCTCTGGG
AGAATGGTGG CACTTGTGAC CGGTTCAGTG GTTGTGTCTG CCCCTCTGGG

1100
TGGCATGGAG TGCACTGTGA GAAGTCAGAC CGGATCCCCC AGATCCTCAA
TGGCATGGAG TGCACTGTGA GAAGTCAGAC CGGATCCCCC AGATCCTCAA

1150
CATGGCCTCA GAACTGGAGT TCAACTTAGA GACGATGCCC CGGATCAACT
CATGGCCTCA GAACTGGAGT TCAACTTAGA GACGATGCCC CGGATCAACT

1200
GTGCAGCTGC AGGGAACCCC TTCCCCGTGC GGGGCAGCAT AGAGCTACGC
GTGCAGCTGC AGGGAACCCC TTCCCCGTGC GGGGCAGCAT AGAGCTACGC

1250
AAGCCAGACG GCACTGTGCT CCTGTCCACC AAGGCCATTG TGGAGCCAGA
AAGCCAGACG GCACTGTGCT CCTGTCCACC AAGGCCATTG TGGAGCCAGA

1300
GAAGACCACA GCTGAGTTCG AGGTGCCCCG CTTGGTTCTT GCGGACAGTG
GAAGACCACA GCTGAGTTCG AGGTGCCCCG CTTGGTTCTT GCGGACAGTG

1350
GGTTCTGGGA GTGCCGTGTG TCCACATCTG GCGGCCAAGA CAGCCGGCGC
GGTTCTGGGA GTGCCGTGTG TCCACATCTG GCGGCCAAGA CAGCCGGCGC

1400
TTCAAGGTCA ATGTGAAAGT GCCCCCCGTG CCCCTGGCTG CACCTCGGCT
TTCAAGGTCA ATGTGAAAGT GCCCCCCGTG CCCCTGGCTG CACCTCGGCT

1450
CCTGACCAAG CAGAGCCGCC AGCTTGTGGT CTCCCCGCTG GTCTCGTTCT
CCTGACCAAG CAGAGCCGCC AGCTTGTGGT CTCCCCGCTG GTCTCGTTCT

1500
CTGGGGATGG ACCCATCTCC ACTGTCCGCC TGCACTACCG GCCCCAGGAC
CTGGGGATGG ACCCATCTCC ACTGTCCGCC TGCACTACCG GCCCCAGGAC

1550
AGTACCATGG ACTGGTCGAC CATTGTGGTG GACCCCAGTG AGAACGTGAC
AGTACCATGG ACTGGTCGAC CATTGTGGTG GACCCCAGTG AGAACGTGAC

1600
GTTAATGAAC CTGAGGCCAA AGACAGGATA CAGTGTTCGT GTGCAGCTGA
GTTAATGAAC CTGAGGCCAA AGACAGGATA CAGTGTTCGT GTGCAGCTGA

1650
GCCGGCCAGG GGAAGGAGGA GAGGGGGCCT GGGGGCCTCC CACCCTCATG
GCCGGCCAGG GGAAGGAGGA GAGGGGGCCT GGGGGCCTCC CACCCTCATG

1700
ACCACAGACT GTCCTGAGCC TTTGTTGCAG CCGTGGTTGG AGGGCTGGCA
ACCACAGACT GTCCTGAGCC TTTGTTGCAG CCGTGGTTGG AGGGCTGGCA

1750
TGTGGAAGGC ACTGACCGGC TGCGAGTGAG CTGGTCCTTG CCCTTGGTGC
TGTGGAAGGC ACTGACCGGC TGCGAGTGAG CTGGTCCTTG CCCTTGGTGC

1800
CCGGGCCACT GGTGGGCGAC GGTTTCCTGC TGCGCCTGTG GGACGGGACA
CCGGGCCACT GGTGGGCGAC GGTTTCCTGC TGCGCCTGTG GGACGGGACA

1850
CGGGGGCAGG AGCGGCGGGA GAACGTCTCA TCCCCCCAGG CCCGCACTGC
CGGGGGCAGG AGCGGCGGGA GAACGTCTCA TCCCCCCAGG CCCGCACTGC

1900
CCTCCTGACG GGACTCACGC CTGGCACCCA CTACCAGCTG GATGTGCAGC
CCTCCTGACG GGACTCACGC CTGGCACCCA CTACCAGCTG GATGTGCAGC

1950
TCTACCACTG CACCCTCCTG GGCCCGGCCT CGCCCCCTGC ACACGTGCTT
TCTACCACTG CACCCTCTTG GGCCCGGCCT CGCCCCCTGC ACACGTGCTT

2000
CTGCCCCCCA GTGGGCCTCC AGCCCCCCGA CACCTCCACG CCCAGGCCCT
CTGCCCCCCA GTGGGCCTCC AGCCCCCCGA CACCTCCACG CCCAGGCCCT

2050
CTCAGACTCC GAGATCCAGC TGACATGGAA GCACCCGGAG GCTCTGCCTG
CTCAGACTCC GAGATCCAGC TGACATGGAA GCACCCGGAG GCTCTGCCTG

2100
GGCCAATATC CAAGTACGTT GTGGAGGTGC AGGTGGCTGG GGGTGCAGGA
GGCCAATATC CAAGTACGTT GTGGAGGTGC AGGTGGCTGG GGGTGCAGGA

2150
GACCCACTGT GGATAGACGT GGACAGGCCT GAGGAGACAA GCACCATCAT
GACCCACTGT GGATAGACGT GGACAGGCCT GAGGAGACAA GCACCATCAT

2200
CCGTGGCCTC AACGCCAGCA CGCGCTACCT CTTCCGCATG CGGGCCAGCA
CCGTGGCCTC AACGCCAGCA CGCGCTACCT CTTCCGCATG CGGGCCAGCA

2250
TTCAGGGGCT CGGGGACTGG AGCAACACAG TAGAAGAGTC CACCCTGGGC
TTCAGGGGCT CGGGGACTGG AGCAACACAG TAGAAGAGTC CACCCTGGGC

2300
AACGGGCTGC AGGCTGAGGG CCCAGTCCAA GAGAGCCGGG CAGCTGAAGA
AACGGGCTGC AGGCTGAGGG CCCAGTCCAA GAGAGCCGGG CAGCTGAAGA

2350
GGGCCTGGAT CAGCAGCTGA TCCTGGCGGT GGTGGGCTCC GTGTCTGCCA
G

Cloned sequence of tie-1 extracellular domain compared to published sequence with one mismatch at nucleotide 1918. Cloned sequence starts at nucleotide 103 and finishes at nucleotide 2301.

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