AN INVESTIGATION INTO THE FUNCTION AND SIGNALLING OF THE ENDOTHELIAL RECEPTOR TYROSINE KINASE, TIE 1

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by

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Abstract

AN INVESTIGATION INTO THE FUNCTION AND SIGNALLING OF THE ENDOTHELIAL RECEPTOR TYROSINE KINASE, TIE 1

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The receptor tyrosine kinase Tie1 is expressed in endothelial cells. Genetic studies have shown that this receptor is essential for development of the vascular system. No ligand for Tie1 has been identified and little is known about the signalling pathways used by Tie1. The receptor has previously been found to have low kinase activity and poor ability to undergo autophosphorylation. The aim of this study is to define the function and signalling mechanisms used by Tie1.

This study demonstrates that the Tie1 endodomain can undergo tyrosine phosphorylation via its own kinase activity and that this is can be regulated by Tie2. Furthermore, phosphorylation is mainly confined to a carboxy-terminal fragment of the endodomain, containing the kinase domain and thus suggests possible inhibitory sequences may be present in the transmembrane and juxtamembrane regions of the Tie1.

This study also demonstrates that a recombinant Ang1 protein, COMP-Ang1, can stimulate tyrosine phosphorylation of full-length and truncated Tie1 in endothelial cells. Whether this event occurs through Tie1 alone or through Tie2 remains to be answered. This work also shows that following COMP-Ang1 stimulation, Tie1 truncation is not affected. For the first time, it has been shown that COMP-Ang1 stimulation gives rise to a heavily phosphorylated 42kDa protein, thought not to be truncated Tie1 but rather an unknown protein associated with Tie1 in endothelial cells.

Studies to define the function of Tie1 in endothelial cells show that Tie1 protects endothelial cells against apoptosis. Attempts to define whether this effect occurs in non-endothelial cells suggest that the cytoprotective function of Tie1 may be endothelial cell specific.

Work presented in this thesis demonstrates novel findings into the signalling mechanisms and function of Tie1.

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Dedication

I dedicate this work to my family, whose support and encouragement over the years has been so important to my success.

Finally, I dedicate this work to my husband, Asif. His love, patience, support and belief in me throughout this project and beyond has never faulted. He has been a constant source of strength and to whom I am eternally indebted.

Abbreviations

ABIN	A20-binding inhibitor of nuclear factor kappa-B
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
aHGF	acidic
Ang1	Angiopoietin-1
Ang2	Angiopoietin-2
Ang3	Angiopoietin-3
Ang4	Angiopoietin-4
Arg	Arginine
ATP	Adenosine 5'-triphosphate
bFGF	basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
cDNA	complementary Deoxyribonucleic acid
CD31	Cluster of differentiation molecule 31
CD34	Cluster of differentiation molecule 34
CHO	Chinese Hamster Ovary cell line
COMP-Ang1	Cartilage Oligomeric Matrix Protein
ddH_2O	double-distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECCAC	European Collection of Cell Culture
ECGS	Endothelial Cell Growth Supplement
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
Eph	Ephrin
ERK1	Extracellular signal-regulated kinase-1
ERK2	Extracellular signal-regulated kinase-2
FCS	Foetal Calf Serum

FGF2	Fibroblast Growth Factor 2
Flt-1	fms-related tyrosine kinase 1
FOXO-1	Forkhead transcription factor
g	Grams
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI-linked	Glycosylphosphatidyl-inositol-linked
HCl	Hydrogen Chloride
HGF	Hepatocyte Growth Factor
HIF-1	Hypoxia Inducible Factor-1
HMEC-1	Human microvascular endothelial cell line
HRP	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cell
IB	Immunoblot
IDV	Individual Density Value
Ig	Immunoglobulin
IGF-1	Insulin-like Growth Factor-1
IP	Immunoprecipitation
kb	Kilobase
KCl	Potassium Chloride
kDa	KiloDalton
KDR/Flk-1	Kinase-insert domain containing receptor/fetal liver kinase-1
KH_2PO_4	Potassium dihydrogen phosphate
KID	Kiase Insert Domain
LB	Luria Bertani
Lys	Lysine
MAPK	Mitogen-activated protein kinase
mM	Milli-molar
β-ME	2-Mercaptoethanol
ml	Milli-Litre
MMP	Matrix metalloprotease
MMP-9	Matrix metalloprotease-9
$MnCl_2$	Manganese Chloride

mRNA	Messenger Ribonucleic acid
NaCl	Sodium Chloride
$NaPO_4$	Sodium Phosphate
Na_2VO_4	Sodium Orthavanadate
NFκB	Nuclear Factor-kappa B
NGF	Nerve Growth Factor
NO	Nitric Oxide
OD	Optical Density
Oligo	Oligonucleotide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet Derived Growth Factor Receptor
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PIGF	Placental Growth Factor
PI3K	Phosphoinositide-3 kinase
PLC-γ	Phospholipase C-gamma
PTB	Phosphotyrosine-binding domain
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
RasGap	Ras GTPase activating protein
RNA	Ribonucleic acid
RT	Reverse Transcription
SAM	Sterile Alpha Motif
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH2	Src Homology 2
SIP1	Smad-interacting protein-1
TAE	Tris Acetate
TAPI	TNF-a protease inhibitor
TBS	Tris Buffered Saline
TBS-TX100	Tris Buffered Saline-Triton X100
TEMED	Tetramethylethylenediamine
TGF-β	Tumor Growth Factor-beta

TICD	Tie1 Intracellular Domain
Tie1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1
Tie2	tyrosine kinase with immunoglobulin-like and EGF-like domains 2
V	Volts
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cell
vWF	von Willebrand Factor
WCL	Whole Cell Lysate
°C	Degrees centigrade
%	Percentage

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Chapter One

Introduction

1.0 Introduction

The developing embryo from the earliest stages receives nutrition by simple diffusion. By the third week of development the human embryo can no longer nourish itself by the process of diffusion, thus the development of a primitive vascular network begins (Noden,1989).

Blood vessels provide the growing embryo with essential nutrients and oxygen. The development of the blood vascular system begins with the assembly of embryonic progenitor cells to produce a primitive vascular plexus in a process known as vasculogenesis. Subsequent to the formation of this vascular plexus, the vascular network expands by sprouting, remodelling and regression, this process is known as angiogenesis. The process of angiogenesis occurs throughout the growth of the foetus, particularly in the development of organs and ultimately leads to the formation of mature blood vessels. Vasculogenesis and angiogenesis are downregulated in the healthy adult, with the exception of wound healing and the female reproductive cycle (Folkman et al., 1992; Cockerill et al., 1995). The process of angiogenesis is associated or induced with pathological processes that include the progression and metastases of tumours, diabetic retinopathy, psoriasis, thrombosis and inflammatory disorders such as rheumatoid arthritis and atherosclerosis (Folkman, 1995). Angiogenesis features in obesity, diabetes, asthma, infections and endometriosis; insufficient angiogenesis is involved in ischaemic heart disease and pre-eclampsia. Although the diseases in which the process of angiogenesis occurs are vast and differing, most of the diseases are restricted to specific vascular beds and organs.

Vasculogenesis

The first signs of blood vessel formation appear on the yolk sac, as focal aggregations of mesenchymal cells, known as blood islands, within the mesoderm adjacent to the extra embryonic endoderm. The outer cells of the blood islands differentiate into endothelial cells, known as angioblasts and the inner cells into haemopoietic precursors. The earliest markers common to endothelial and haemopoietic precursors identified include CD31, CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2) (Yamaguchi et al., 1993). VEGFR-2 gene inactivation, in mice, results in embryonic lethality, with lack of development of both endothelial cell and haemopoietic cell lineages; this observation therefore highlights the critical importance of this receptor tyrosine kinase in the regulation of differentiation of cells into either endothelial or haemopoietic cells (Shalaby et al., 1995).

At around day 7.5 post-coitum, as the yolk sac vasculature begins to form, angioblasts begin to aggregate, proliferate and differentiate to form a plexus with endocardial tubes. Subsequently, the dorsal aortae, cardinal veins and the embryonic stems of the yolk sac arteries and veins are formed (Carmeliet et al., 2000). The precise mechanism by which angioblasts identify where and when vasculogenesis should be initiated is not precisely known, however several growth factors and receptors thought to be involved include, vascular growth factor receptor (VEGF); granulocyte monocyte-colony stimulating factor (GM-CSF) and other cytokines have been implicated (Asahara et al., 1999;Takashashi et al., 1999; Rafii, 2000). Differentiation of angioblasts is thought to be mediated by VEGF,

VEGFR-2 and basic fibroblast growth factor (bFGF) (Carmeliet et al., 1996; Ferrara et al., 1996: Ferrara 1999) conversely, VEGF receptor 1(VEGFR-1) suppresses angioblast differentiation (Shalabay et al., 1997).

Angiogenesis

The process of angiogenesis can be divided into four phases:

(i) Increased permeability known as the initiation phase

Vasodilation of existing vessels occurs in early angiogenesis, predominately in response to nitric oxide (NO), which upregulates the transcription of VEGF (Kimura et al., 2000), along with a redistribution of intercellular adhesion molecules, vascular endothelial (VE)-cadherin and platelet endothelial cell adhesion molecule (PECAM)-1 which results in increased permeability of blood vessels. Tight regulation of vessel permeability is critical to ensure normal vessel development, excessive permeability results in pathologies such as circulatory collapse. Angiopoietin-1 (Ang1) is a natural anti-permeability factor that provides protection against excessive permeability (Thurston et al., 2000).

(ii) 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, this is known as the progression phase; degradation of the extracellular matrix occurs by the production of matrix metalloproteinases (MMPs), which in addition to enabling endothelial cells to migrate, results in the release of basic fibroblast growth factor (bFGF), VEGF and insulin-like growth factor-1 (IGF-1) from the extracellular matrix (ECM) (Nelson et al., 2000).

(iii) Differentiation into new vessels

Angiopoietin -2 (Ang2) an inhibitor of Tie2 signalling and a natural antagonist of Ang1 is involved in the process of endothelial sprouting. Ang2 occurs at sites of vascular remodelling and is involved in detaching smooth muscle cells and loosening underlying matrix (Thurston et al., 2000).

(iv) Stabilisation and maturation phase.

Vessels are stabilised by the recruitment of mural cells and by generating an extracellular matrix (ECM). Growth factor signalling pathways involved in the regulation of this process include platelet-derived growth factor (PDGF)-B/PDGF receptor (PDGFR)-β; sphingosine-1-phosphate-1(S1P1)/endothelial differentiation sphingoloid G-protein-coupled receptor-1 (EDG1); Ang1/Tie2 and transforming growth factor (TGF)-β (Pepper 1997; Lindahl et al., 1999). The extracellular matrix provides a site for storage of growth factors and pro-enzymes like matrix metalloproteinases (MMPs), in addition the ECM provides binding sites and targets for endothelial and mesenchymal cell-derived integrins and growth factors, such as integrin $\alpha_v\beta_3$ and VEGF (Brooks et al., 1994).

A model for blood vessel formation and development is shown in Figure 1.1.

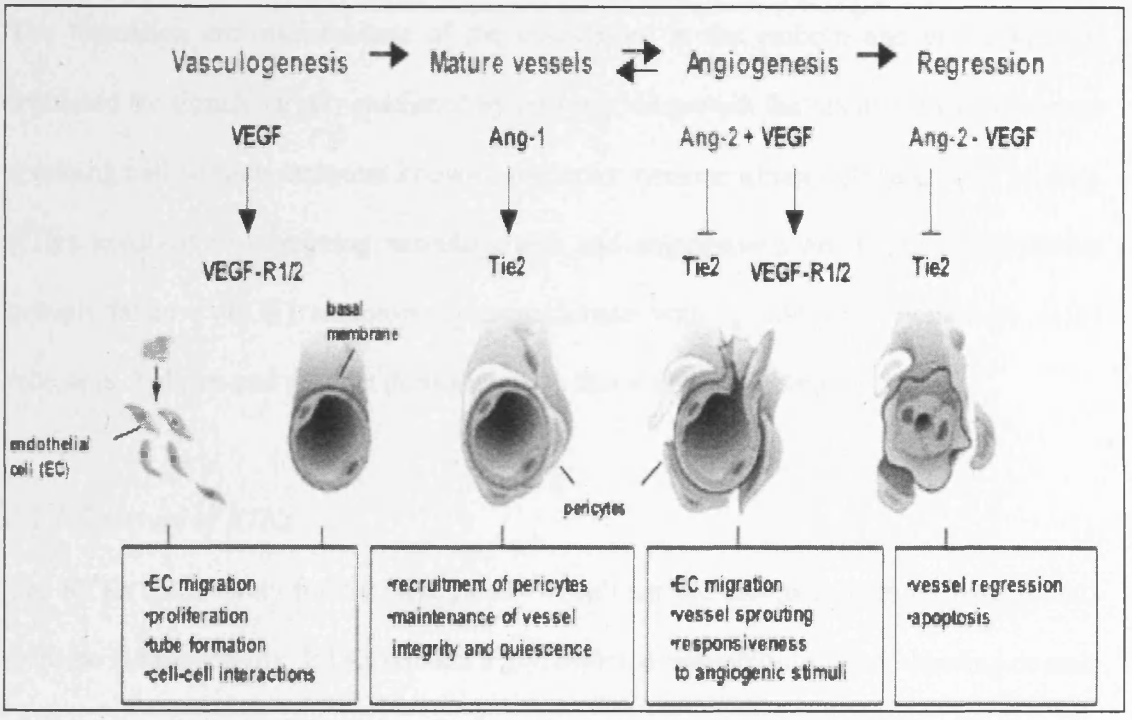


Figure 1.1 Model of Vasculogenesis and Angiogenesis

Vasculogenesis, the formation of a vascular network includes focal aggregations of mesenchymal cells, known as blood islands, subsequently the outer cells of the blood islands differentiate into endothelial cells, known as angioblasts and the inner cells into haemopoietic precursors. Endothelial cell migration and proliferation leads to the assembly of the basement membrane and marks initial tube formation and cell-cell interactions. Vascular endothelial growth factor (VEGF) is a major stimulator of vasculogenesis. Endothelial cell migration and vessel sprouting, in response to angiogenic stimuli, results in the remodelling and maturation of early blood vessels. This process is known as angiogenesis. (Adapted from Conway et al., 2001).

1.1 Vascular endothelial receptor tyrosine kinases and their ligands

The formation and maintenance of the vasculature in the embryo and in the adult is regulated by signals largely mediated by polypeptide growth factors and their membrane spanning cell surface receptors known as receptor tyrosine kinases (RTKs). The primary RTKs involved in regulating vasculogenesis and angiogenesis are vascular endothelial growth factor (VEGF) receptors; tyrosine kinase with Ig and EGF homology (TIE) receptors. Ephrins and platelet derived growth factor (PDGF) receptors.

1.1.1 Structure of RTKs

The RTKs collectively form a large family of cell surface receptors with intrinsic protein tyrosine kinase activity. RTKs contain a glycosylated extracellular ligand-binding domain, connected to the cytoplasmic domain by a single hydrophobic transmembrane helix. The cytoplasmic domain consists of a juxtamembrane region and a conserved protein tyrosine kinase (PTK) core, containing a regulatory sequence (Schlessinger, 2000). RTKs have been classified according to their extracellular structural characteristics (Ullrich and Schlessinger, 1990; Fantl et al., 1993) (Figure 1.2).

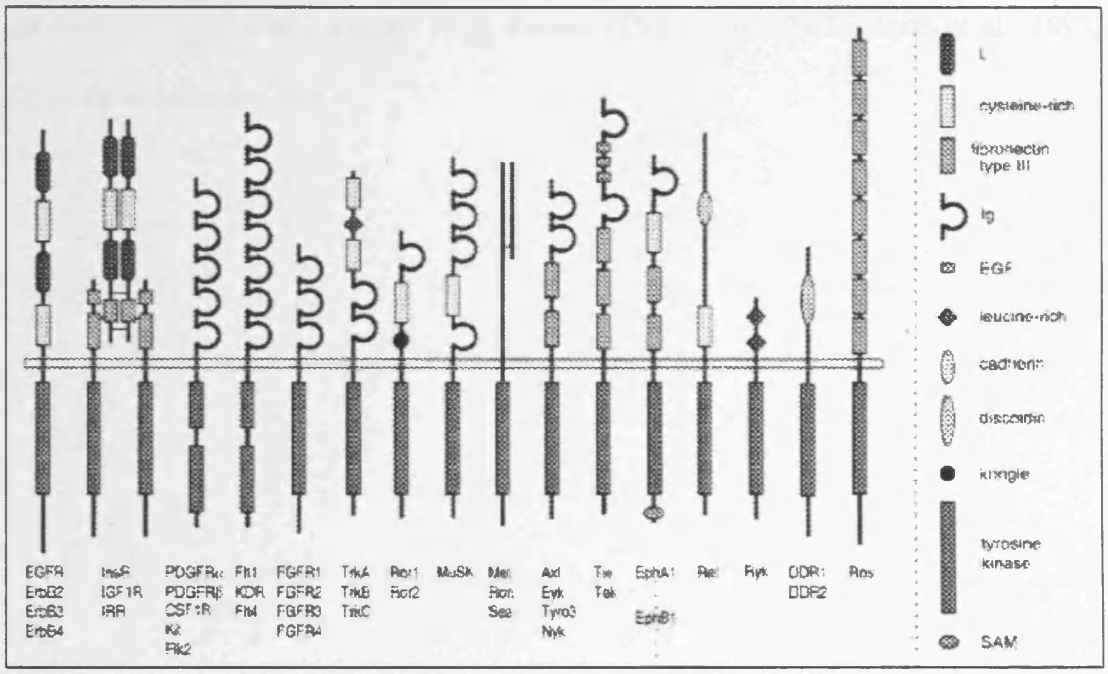


Figure 1.2 Schematic representation of structural components of the receptor tyrosine kinase subfamilies (Adapted from Hubbard and Till, 2000).

1.1.2 Paradigm for activation of RTKs

All known RTKs are monomers in the cell membrane, with the exception to the insulin receptor (IR) family of RTKs. Ligand binding induces dimerisation of receptor monomers, resulting in autophosphorylation of the cytoplasmic domains. Autophosphorylation of these regions creates docking sites for Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains of a variety of signalling proteins. SH2 domain-mediated binding of signalling protein provides a mechanism for the recruitment and assembly of signalling complexes by activated receptor tyrosine kinases (Pawson and Scott, 1997). Proteins that function as downstream signalling molecules include phosphatidylinositol 3-kinase (PI3K); phospholipase C-γ; GTPase-activating protein;

pp60-src; p21 ras; Raf-1 kinase; MAP kinases (ERK1 and ERK2) (Fantl et al., 1993; Shiojima and Walsh, 2003).

1.2 VEGF and its receptors

1.2.1 VEGF Ligands

There are six members of the VEGF ligand family, VEGF A-E as well as placental growth factor (PlGF) (Ferrara 1996; Neufeld et al., 1999) (Figure 1.3). All of the polypeptides except VEGF-E are expressed endogenously in mammals. VEGF-E is encoded by the double-stranded DNA virus, *orf*. VEGF-A is the most widely distributed form of the ligand in the body and genetic studies indicate it has a primary role in regulating angiogenesis (Ferrara 1996). Unless otherwise stated I will use the acronym VEGF to refer to VEGF-A. Alternative splicing of human VEGF mRNA from a single gene containing eight exons results in at least six different isoforms of 121, 145, 165, 183, 189 and 206 amino acid residues (Neufeld et al., 1996). In $VEGF_{121}$ and $VEGF_{165}$, exon 6 is absent and exon 7 is absent in $VEGF_{121}$ and VEGF145, VEGF121, $VEGF_{145}$ and $VEGF_{165}$ are secreted and form dimeric proteins whereas $VEGF_{189}$ and $VEGF_{206}$ are not freely secreted and are bound to the cell surface extracellular matrix (Ferrara, 1996). Human $VEGF_{165}$ is glycosylated at Asn75 and is expressed as a 46kDa homodimer of 23kDa monomers. As well as being the most abundant VEGF isoform it has also been reported to be the most biologically active. In addition, it has also been reported that $VEGF_{121}$ and $VEGF_{145}$ are biologically active in endothelial cells (Neufeld et al., 1996 and 1999).

VEGF is expressed by a wide range of cell types including vascular smooth muscle cells (VSMC) and keratinocytes. VEGF expression is regulated by hypoxia, which induces binding of the transcriptional factor, hypoxia inducible factor 1(HIF-1), to the hypoxia response element in the VEGF gene promoter region (Carmeliet et al., 1998). VEGF

mRNA expression is also regulated by various stimuli including interleukin-1β, tumour growth factor-β (TGF-β) and fibroblast growth factor (bFGF) (Brogi et al., 1994; Stavri et al., 1995; Li et al., 1995).

1.2.2 Mouse gene knockouts

The importance of VEGF in embryonic blood vessel development was demonstrated by the targeted inactivation of only a single allele of the VEGF gene in mice which resulted in lethal impairment of angiogenesis (Carmeliet et al., 1996). In transgenic homozygous mice expressing only $VEGF_{189}$ isoform coded by exons 6 and 7 die shortly after birth due to bleeding and ischaemic cardiomyopathy (Carmeliet et al., 1999). VEGF-C has been implicated to have a role as a growth factor for lymphatic vessels (Kukk et al., 1996; Jeltsch et al., 1997). In transgenic mice, overexpression of VEGF-C in keratinocytes of the skin epidermis, results in enlarged lymphatic vessels in their skin. In transgenic mice overexpression of $VEGF_{164}$ in skin epidermis developed only blood vessel hyperplasia (Jeltsch et al., 1997). In gene knockout mice for VEGF-B, animals do not die but exhibit vascular dysfunction after coronary occlusion (Bellomo et al., 2000).

1.2.3 VEGF receptors

The VEGF receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (FLT-4) form a subfamily within the platelet-derived growth factor (PDGF) receptor class. All three consist of seven immunoglobulin-homology (Ig) domains, a transmembrane sequence and an intracellular region containing a split kinase domain (Shibuya, 1995) (Figure 1.3).

Ligand specificities of the receptors differ, VEGFR-1 binds VEGF, VEGF-B and PlGF; VEGFR-2 binds VEGF, VEGF-C, VEGF-D and the *orf* virus VEGF; VEGFR-3 binds VEGF-C and VEGF-D. Ligand binding results in activation of the tyrosine kinase catalytic domains followed by autophosphorylation of the tyrosine residues. Several studies have demonstrated VEGFR Ig domains to have specific functions; Ig domains 1-3 mediate ligand binding, Ig domain 2 mediates ligand specificity and Ig domain 4 enables receptor dimerisation (Keyt et al., 1996; Weismann et al., 1997). Expression of VEGFR-1 and VEGFR-2 is predominantly restricted to the vascular endothelium (Ferrara, 1996), whilst VEGFR-3 is expressed on endothelial cells of lymphatic vessels and venules (Kaipainen et al., 1995).

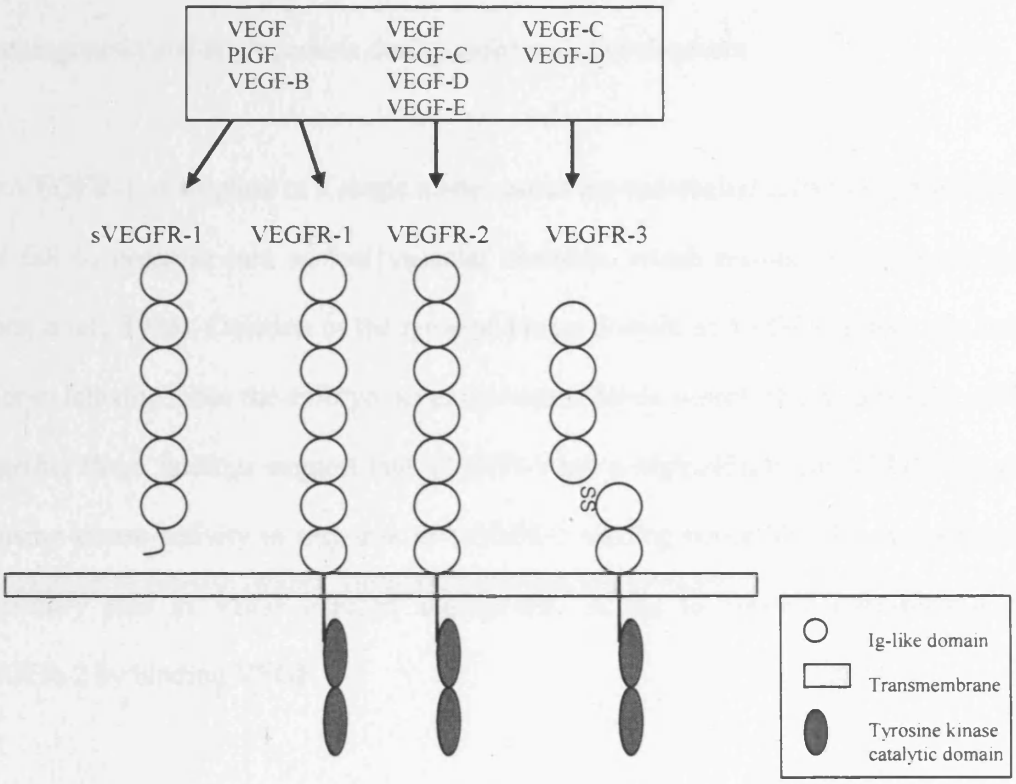


Figure 1.3 Vascular Endothelial Growth Factor Receptor (VEGFR) structure and cognate ligands. The VEGF receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (FLT-4) form a subfamily within the platelet-derived growth factor (PDGF) receptor class. All three consist of seven immunoglobulin-homology (Ig) domains, a transmembrane sequence and an intracellular region containing a split kinase domain. The six members of the VEGF ligand family, VEGF A-E as well as placental growth factor (PIGF) are shown.

1.2.4 Mouse gene knockout studies of VEGF receptors

Gene knockout studies in mice have demonstrated well the critical role of the VEGFRs in vasculogenesis and angiogenesis during embryonic development.

For VEGFR-1, disruption of a single allele causes the endothelial cells to hyperproliferate and fail to organise into normal vascular channels, which results in embryonic death (Fong et al., 1995). Deletion of the tyrosine kinase domain of VEGFR-1 does not lead to embryo lethality since the embryo develops normal blood vessels (Hiratsuka et al., 1998). Together these findings suggest that VEGFR-1 has a high affinity for VEGF, but weak tyrosine kinase activity in response to VEGFR-1 binding hence VEFR-1 has a negative regulatory role in VEGF-induced angiogenesis acting to prevent over-activation of VEGFR-2 by binding VEGF.

The VEGFR-2 knockout mouse model results in death of the embryo due to the lack of any form of vasculogenesis and displayed defects in blood island formation (Shalaby et al., 1995). This finding demonstrated the essential role VEGFR-2 plays in differentiation and proliferation of endothelial and haemotopoietic cells.

The VEGFR-3 knockout mouse results in embryo death due to a failure of remodelling of the primary vascular network and abnormal endothelial cell organisation (Dumont et al., 1998). This suggests that VEGFR-3 is necessary for the maturation of the vascular plexus into small blood vessels during the later stages of angiogenesis.

1.2.5 Biological function of VEGF

Vascular endothelial growth factor (VEGF) is a major initiator of neovascularisation during embryonic development (Carmeliet et al. 1996; Ferrara et al., 1996), cancer, rheumatoid arthritis, ocular neovascular disorders (Folkman, 1995) and cardiovascular disease (Isner and Losordo, 1999; Zachary et al., 2000). VEGF was initially recognised for its ability to produce hyperpermeability in tumour blood vessels (Senger et al., 1983). Since then, VEGF has been shown to regulate multiple biological functions including endothelial cell survival. VEGF inhibits HUVEC apoptosis by activating the anti-apoptotic kinase Akt/PKB, via a PI3K-dependent pathway (Thakker et al., 1999). Endothelial cell survival is also promoted by VEGF through the induction of anti-apoptotic proteins Bcl-2 and A1, which results in the inhibition of upstream caspase activation (Gerber et al., 1998). VEGF stimulates endothelial cell proliferation by inducing the activity of extracellular signal-regulated kinases (ERKs) 1 and 2 (Wheeler-Jones et al., 1997; Abedi et al., 1997), c-Jun N-terminal protein kinase (JNK) (Pedram et al., 1998) and PLC-γ tyrosine phosphorylation (Wellner et al., 1999; Higaki et al., 1999). VEGF also augments endothelial cell migration through the $\alpha_v\beta_5$ integrin (Friedlander et al.,1995), induces the expression of matrix-metalloproteinases (Lamoreaux et al., 1998) and induces tyrosine phosphorylation of FAK (Abedi and Zachary, 1997). VEGF also stimulates endothelial production of nitric oxide (NO) and prostacyclin (PGI_2) (Wheeler-Jones et al., 1997; Murohara et al., 1998) and these intracellular mediators have been implicated in angiogenesis, inhibition of neointimal vascular smooth muscle cell (VSMC) hyperplasia and increased vasopermeability (Zachary et al., 2000).

1.3 Tie receptor tyrosine kinases

The second subfamily of receptor tyrosine kinases involved in blood vessel formation is the Tie family. There are two members of this family, Tie1 and Tie2. Structurally, the two receptors share similarities; the extracellular domains consist of two immunoglobulin-like loops, three EGF-like repeats and three-fibronectin type III-like repeats. However, recently it has been reported that both Tie1 and Tie2 posses a third immunoglobulin-like domain in the N-terminus of their extracellular domain (Macdonald et al., 2006). The intracellular domains contain a characteristic kinase domain with kinase insert. The overall amino acid identity between Tie1 and Tie2 is 44% homology at the amino acid level and 76% homology in the intracellular domain (Wilks, 1989; Partanen et al., 1992; Ziegler et al., 1993). The structure and features of Tie1 and Tie2 are given in Figure 1.4. A family of ligands binding to Tie2 and activating the receptor have been defined, the angiopoietins (Davis et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999, Lee et al. 2004). There are four members of the Angiopoietin family, Ang1-4. Although these all interact with Tie2, none have been found to bind to Tie1 extracellular domain and a ligand for Tie1 is yet to be identified.

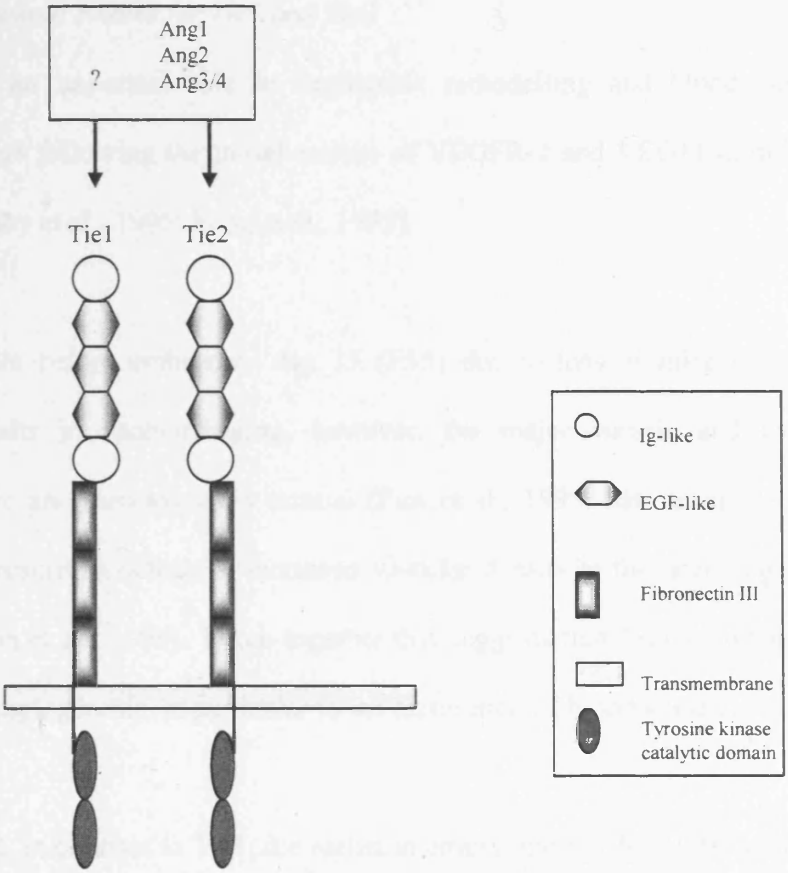


Figure 1.4 Tie1 and Tie2 receptor tyrosine kinase structure and ligands.
The extracellular region of the Tie receptors consists of one complete and one incomplete immunoglobulin (Ig)-like domain that are separated by three tandem epidermal growth factor (EGF)-like cysteine repeats and are followed by three fibronectin type III homology domains.

1.3.1 Mouse gene knockout studies for Tie1 and Tie2

Tie1 and Tie2 play an important role in angiogenic remodelling and blood vessel stabilisation that occurs following the initial actions of VEGFR-1 and VEGFR-2 during vasculogenesis (Shalaby et al., 1995; Fong et al., 1995).

Mice lacking Tie1 die before embryonic day 15 (E15) due to loss of microvascular integrity, which results in haemorrhaging, however, the major vessels and extra-embryonic vasculature are phenotypically normal (Puri et al., 1995; Sato et al., 1995). Tie1 deficiency also results in defects in increased vascular density in the later stages of angiogenesis (Partanen et al., 1996). Taken together this suggests that Tie1 is important in the later stages of angiogenesis, in particular to the maturation of blood vessels.

Embryos lacking Tie2, in contrast to Tie1, die earlier at embryonic day 9.5 (E 9.5) due to loss of vascular integrity and reduction in endothelial cell numbers, which results in widespread vascular haemorrhage and a simplified vasculature lacking in vessel branching (Dumont et al., 1994; Sato et al., 1995). In Tie2 deficient mice, animals have poorly developed hearts with occlusions, rounded endothelial cells and a lack of surrounding peri-endothelial cells with mesenchymal cells located at a distance from the endothelial cells (Patan, 1998). These findings indicate a role for Tie2 in mediating endothelial cell stretch and motility, endothelial cell and ECM interactions during the differentiation stage of angiogenesis.

The function of Tie2 thus appears to be counteracted by Tie1; whilst Tie2 promotes vascular network growth and remodelling through intussuceptive vessel growth, Tie1 inhibits these processes and functions to bring about vessel stabilisation.

1.3.2 Function of Tie1 RTK

Since a ligand has yet to be identified for Tie1, knowledge of how this receptor functions and its signalling intermediates and pathways remains to be elucidated. However, *in vivo* studies indicate an essential role for Tie1 in promoting endothelial cell survival and integrity in vascular development (Sato et al., 1995; Puri et al., 1995; Patan, 1998).

Studies in endothelial cells with overexpresed Tie1 or chimeric forms of the receptor show Tie1 to be unphosphorylated and the receptor failed to induce tyrosine phosphorylation of cellular proteins, suggesting that Tie1 function is not related to ligand-induced kinase activity (Marron et al., 2000a; 2000b). Studies have revealed the extracellular domain of Tie1 can be proteolytically cleaved following treatment of cultured endothelial cells with phorbol myristate acetate (PMA), vascular endothelial growth factor (VEGF), protein kinase C, and tumour necrosis factor alpha (TNF-α), which results in the release of soluble Tie1 into the tissue culture supernatant. Additionally, exposure to shear stress within blood vessels also contributes to Tie1 cleavage (Yabkowitz et al., 1997a, 1999b; McCarthy et al., 1999; Tsiamis et al., 2002; Chen-Konak et al., 2003). Subsequent to the cleavage of the extracellular domain, a membrane-bound Tie1 endodomain is generated which contains the intracellular kinase and transmembrane domains. Marron et al. (2000a), found that in endothelial cells

expressing both Tie1 and Tie2, the endodomain of Tie1 coimmunoprecipitates with Tie2. However, the study used a chimeric receptor which lacked intrinsic tyrosine activity and ligand–induced autophosphorylation was negligible. These findings suggested that Tie1 might function to modulate the signalling and function of Tie2. Despite only poor tyrosine phosphorylation induced by the Tie1 endodomain, it was found to coimmunoprecipitate with the protein tyrosine phosphatase SHP2, suggesting that Tie1 might signal in a ligand-independent manner (Marron et al., 2000b). These findings were of major significance into our understanding of how Tie1 might function and hence subsequent studies aimed to unravel the possible signalling mechanism(s) of the receptor were performed.

Experiments with a chimeric receptor consisting of the Tie1 cytoplasmic domain and colony–stimulating factor 1 (CSF-1), resulted in Tie1-induced tyrosine autophosphorylation (Kontos et al., 2002). In the same study, it was demonstrated that Tie1 was able to induce activation of the phosphatidylinositol-3 kinase (PI3K) and Akt pathway and this protected cells from apoptosis (Kontos et al., 1998), suggesting that Tie1 is capable of signalling and that Tie1 may share similar signalling pathways as Tie2. Following ligand activation, Tie1 may have some kinase activity; in a recent study, it was shown that Tie1 becomes activated by an Angiopoietin-1 (Ang1) chimeric protein (COMP-Ang1) and to a lesser extent by the native angiopoietins, Ang1 and Ang4 (Saharinen et al., 2005). It was also found that Tie2 significantly enhanced Tie1 activation by COMP-Ang1 (Saharinen et al., 2005). This finding is consistent with the suggestion that Tie1 and Tie2 form heterodimeric complexes (Marron et al., 2000a;

Tsiamis et al., 2002; Chen-Konak et al., 2003). It was initially proposed that the formation of heteromeric complexes between Tie1 and Tie2 allows Tie1 to modulate signalling through Tie2 and hence the recent finding that Tie2 enhances Tie1 activation, demonstrates cross-talk between the two receptors.

1.3.3 Function and signalling of Tie2 RTK

Common to other transmembrane tyrosine kinase receptors, upon ligand binding to the extracellular domain of Tie2 oligomerisation occurs allowing the kinase domain to become activated and subsequently specific tyrosine residues to become autophosphorylated. Phosphorylation of specific tyrosine residues act as docking sites for a number of signalling intermediates which contain motifs that recognise specific phosphorylated tyrosine residues.

Angiopoietin-1 (Ang1) is a specific ligand for Tie2 and results in autophosphorylation of the receptor (Davis et al., 1996). The signal transduction pathways involving Ang1/Tie2 have been studied extensively which has enabled numerous cell signalling cascades and downstream targets to be identified. Ultimately, Ang1/Tie2 signal transduction results in cellular effects that include endothelial cell survival; migration, sprouting, motility and even leukocyte adhesion. The details of the cell signalling cascades and downstream targets which result in cellular effects will be briefly discussed.

Following Ang1 binding to Tie2, tyrosine residue 1102 (Y1102) on the intracellular domain of the receptor becomes phosphorylated (Kontos et al., 1998; Jones et al., 1999).

Phosphorylated tyrosine residue 1102 (Y1102) recruits the regulatory p85 subunit of phosphatidylinositol 3-kinase (PI3K). Activated PI3K in turn stimulates the serine/threonine kinase, Akt, (Fujikawa et al., 1999; Kim et al., 2000) which subsequently results in the phosphorylation and inhibition of the forkhead transcription factor, FKHR, in endothelial cells. FKHR is involved in the induction of apoptosis and hence this signalling pathway is important for promoting survival in endothelial cells (Daly et al., 2004). Complementary to this pathway, activated Tie2 associates with protein phosphatase SHP2 and adaptor protein Grb2 (Huang et al., 1995; Jones et al., 1999). Both of these proteins are upstream and are involved in the activation of the ras-mitogen activated protein kinase, MAPK, pathway. The MAPK pathway is involved in the survival of endothelial cells.

In addition to endothelial cell survival, Ang1/Tie2 signal transduction plays an important role in endothelial cell migration. Again PI3K plays a central role in the signalling cascade involved in endothelial cell migration, coupled with the adaptor protein, Dok-R (Master et al., 2001; Jones et al., 2003). Following the activation of Tie2 by Ang1, autophosphorylation of tyrosine 1106 (1106) on Tie2 occurs (Jones et al., 2003) which results in the adaptor protein Dok-R recruitment and phosphorylation; specifically at tyrosine residue 351 (Y351), which in turn acts as a docking site for the adaptor protein Nck (Master et al., 2001). Nck recruitment to Tie2 is followed by the recruitment of p21 activated kinase, PAK, mediated through Nck. The assembly of this Dok-R-Nck-PAK complex results in endothelial cell migration.

Ang1 mediated activation of Tie2 and the subsequent recruitment and activation of PI3K is common to a variety of signalling cascades that result in endothelial cell sprouting and motility. Such signalling cascades include (i) PI3K-dependent activation of endothelial nitric oxide synthase, eNOS (Chen et al., 2004); (ii) PI3K-dependent activation of the Rho GTPases, Rho and Rac1 (Cascone et al., 2003); (iii) PI3K-dependent activation of the adaptor proteins, ShcA and Grb7 which in turn leads to the activation of focal adhesion kinase, FAK (Kim et al., 2000).

Ang1/Tie2 signalling has also been shown to be involved in leukocyte adhesion. Following phosphorylation of Tie2, recruitment of the A20 binding inhibitor of NFκB activation-2, otherwise known as ABIN-2, occurs and this signalling intermediate serves to inhibit nuclear factor κ B (NFκB) activity. NFκB is involved in adhesion molecule expression and inhibition of NFκB is important in the inhibitory effects of Ang1 on leukocyte adhesion to endothelial cells. In endothelial cells, NFκB activity has been shown to be necessary for the prevention of apoptosis induced by tumour necrosis factor-alpha (TNFα) (Zen et al., 1999). Hence, ABIN-2 has been found to contribute to endothelial survival following Ang1/Tie2 signal transduction (Tadros et al., 2003). Thus, constitutive Tie2 phosphorylation and signalling involves various signalling cascades that are collectively anti-apoptotic and maintain the quiescent state of the resting endothelium.

Such an array of functionally relevant signalling pathways in endothelial cells mediated by Tie2 highlights the importance of understanding the mechanisms by which the receptor is activated. Understanding the mechanisms of Tie2 activation was greatly

improved following the determination of the crystal structure of Tie2 (Shewchuk et al., 2000). The novel finding that the Tie2 active loop adopts an "active-like" conformation in the absence of phosphorylation and the nucleotide binding loop of Tie2 adopts an inhibitory conformation, with the side chains of several residues occupying the ATP binding site was made. In other RTKs, the activation loop obstructs ATP or substrate binding in the inactive state. Additional to these findings, the C-terminal tail was implicated to autoinhibit Tie2 activation (Shewchuk et al., 2000). Deletion of the C-terminal tail resulted in significantly increased Tie2 autophosphorylation and kinase activity (Niu et al., 2002) thus confirming the self-inhibition of Tie2.

1.3.4 The Angiopoietins

There have been four ligands identified for Tie2, Angiopoietin1-4. The best characterised of these are Angiopoietin-1(Ang1) and Angiopoietin-2 (Ang2). Ang1 is a secreted activating ligand for Tie2 and was first isolated from COS cells using the method of secretion-trap cloning (Davis et al., 1996), in contrast, Ang2 is a natural antagonist for Tie2 (Maisonpierre et al., 1997). Ang1 is constitutively expressed by many different cell types such as, pericytes, smooth muscle cells, fibroblasts and some tumour cells (Davis et al., 1996; Sugimachi et al., 1998). Ang2 however, is almost exclusively expressed by endothelial cells although it has been detected in some tumour cells (Brown et al, 2000; Hegen et al., 2004; Fiedler et al., 2006; Yao et al., 2006). Ang1 and Ang2 are two members of the angiopoietin family and share an approximate 60% amino acid identity (Maisonpierre et al., 1997). Little is known about the remaining members of the angiopoietin family Ang3 and Ang4. Ang3 has been identified as an agonist for Tie2 in mouse endothelium and Ang4 activates Tie2 in human endothelial cells (Valenzuela et al., 1999; Lee et al., 2004).

Ang1 is a 70 kDa glycosylated protein which is unique in structure and contains an amino-terminal angiopoietin-specific domain followed by a coiled-coil domain, a linker peptide and a carboxy-terminal fibrinogen homology domain (Davis et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). The fibrinogen homology domain is responsible for receptor binding and the coiled-coil domain is required for dimerisation of angiopoietin monomers (Procopio et al., 1999). The amino-terminal angiopoietin-specific

domain is short in length, this characteristic enables the domain to form ring-like structures that cluster dimers into variable sized multimers which are required for the ligand to activate Tie2 (Davis et al., 2003).

Ang2 is a naturally occurring antagonist of Ang1 and is very similar in size and structure (Davis et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). Despite Ang1 and Ang2 binding to the same site in the extracellular domain of Tie2 with similar affinities (Fiedler et al., 2003), the binding of Ang2 does not lead to rapid Tie2 autophosphorylation and therefore inhibits the stimulatory function of Ang1 (Maisonpierre et al., 1997) which suggests that Ang2 is an antagonist ligand for Tie2. The ability of Ang2 to bind to Tie2 without inducing receptor phosphorylation is not due to insufficient clustering of Ang2 oligomers (Davis et al., 2003), nor has it been found to be due to differential receptor binding; indeed Ang1 and Ang2 have almost identical binding sites on Tie2 (Fiedler et al., 2003). The structure of the fibrinogen homology motif may be responsible for Ang2 antagonistic function (Procopio et al., 1999; Davis et al., 2003).

Importantly, Ang2 can induce phosphorylation of Tie2, when the receptor is ectopically expressed in non-endothelial cells (Maisonpierre et al., 1997). Ang2 can also stimulate Tie2 in endothelial cells when the ligand is present at high concentrations (Kim et al., 2000). Ang2 is also capable of inducing tube formation in a fibrin clot and phosphorylation of Tie2 (Teichertkuliszewka et al., 2001); stimulate tube formation and chemotaxis of endothelial cells in collagen gel assays (Mochizuki et al., 2002); stimulate expression of matrix metalloproteinase-9 (MMP-9) (Das et al., 2003); stimulate

endothelial cells to migrate and sprout in gel-embedded endothelial cell spheroids (Korff et al., 2001). Thus Ang2 has been proposed as a context-dependent agonist. Bogdanovic et al. (2006) concluded that upon Ang2 binding to Tie2, only weak activation of the receptor is achieved and may therefore be classified as a partial agonist. In support of this, Bogdanovic et al., concluded that unlike Ang1, which not only activates Tie2 but also consequently causes rapid internalisation of the receptor in endothelial cells and subsequent rapid degradation of Tie2 occurs. Ang2 weak activation of Tie2 is not sufficient to cause the receptor to become internalised in endothelial cells and furthermore mildly induces Tie2 as a target for degradation. Ang1 and Ang2 after Tie2 becomes internalised, do not themselves become internalised within endothelial cells but are released into the surrounding medium and are subsequently capable of re-binding to fresh endothelial cells. The mechanism by which the angiopoietins interact with the surface of endothelial cells and are released has yet to be elucidated at the time of writing but demonstrates Ang1 and Ang2 as emerging ligands, that are released post-receptor activation rather than being co-internalised with their cognate receptor.

The function of Ang2 in vascular remodelling is to block the agonistic actions of Ang1 stimulation of Tie2 and inhibit endothelial quiescence. However, an alternative hypothesis has recently been proposed. Daly et al. (2006) propose that Ang2 promotes vascular remodelling by compensating loss of Ang1 signalling during remodelling of vessels. During vascular remodelling, endothelial cell-cell and cell-matrix interactions are disrupted, which subsequently causes the activity of Akt to be reduced. Loss of Ang1 as a pericyte-derived stimulator of Akt, also contributes to the reduction in Akt activity.

However, when Akt activity is high (in response to Angl and Ang2) it is proposed that Ang2 expression would be shut off to prevent over stimulation of the related Tie2/ Akt signalling pathway. The latter is based on the novel finding by Daly et al. (2006), that Ang2 expression is rapidly induced in endothelial cells by the transcriptional factor, FOXO1, after inhibition of the PI3K/Akt and that Ang2 acts as a feedback inhibitor of FOXO1 function.

Expression of Angl in the embryo occurs at around day 9 to 11 when it is most prominently expressed in the heart myocardium. Towards the latter stages of embroyonic development, Angl is more widely expressed in the mesenchyme surrounding developing blood vessels in close association with endothelial cells. The importance of Angl expression in both the development of the vasculature and in the adult vasculature has been well established in transgenic mice lacking Angl.

In mice deficient of Angl (Angl +/-), animals die around embryonic day 12.5 due to under development of the vasculature. In these animals although the vasculature is formed, there is considerable reduction in vessel branching and the number of small vessels, in addition, dilated vessels lack complexity (Suri et al., 1996). In double knock-out animals (Angl -/-), vessels have fewer endothelial cells, defects in association of the endothelia with the extracellular matrix and vessel rupture (Suri et al., 1996). The role for Angl in the development of the vasculature is further illustrated in mice overexpressing Angl. Overexpression of Angl in skin results in enlarged vessels and an increase in number and branching of vessels (Suri et al., 1998; Thurston et al., 1999).

Expression of Ang2 in the embryo is similar to that of Ang1 (Davies et al., 1996), however in the adult, expression of these angiopoietins differs. Ang1 is almost universally expressed in all arterial vessels whilst the expression of Ang2 is limited to the female reproductive tract and tumours, thus predominantly at sites of vascular remodelling (Witzenbichler et al., 1998; Holash et al., 1999). Ang2 mRNA is dramatically induced at sites of endothelial-cell activation where it would normally be undetectable in the quiescent vasculature. Induction of Ang2 expression is elicited by various cytokines, including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-2 and by other factors such as hypoxia (Stratmann et al., 1998; Oh et al., 1999).

Transgenic mouse models have also been vital in demonstrating the role of Ang2 in angiogenesis. In Ang2 deficient mice (Ang2 -/-), animals die by postnatal day 14 due to a lack of ischaemia-induced neovascularisation. Animals also exhibit a failure to remodel and regress hyaloid vasculature as well as abnormal outgrowth of retinal capillaries. (Gale et al., 2002; Hackett et al., 2002). Phenotypic changes in the vascular capillaries of the kidney have also been reported due to Ang2 deficiency (Pitera et al., 2004). Overexpression of Ang2 results in death by embryonic day 9.5-10.5 with similar phenotypic changes as seen in animals deficient in Ang1 and Tie2 expression but more severe, indicative of Ang2 acting as a natural antagonist by blocking the stimulatory effects of Ang1 and Tie2. These include rounded endothelial cells, poor interaction with the extracellular matrix and endocardial defects (Maisonpierre et al., 1997).

Ang2 is produced by endothelial cells and has been found to be stored in cytoplasmic granules of endothelial cells, known as Weibel-Palade bodies. Subsequent to investigations into the expression of Ang2 in endothelial cells, it was found that Ang2 formed a characteristic granular pattern in endothelial cells, suggestive of Weibel-Palade bodies (Fiedler et al., 2004); the primary endothelial storage granules of the procoagulant von Willebrand Factor (vWF) (Ewenstein et al., 1987; Wagner et al., 1991). vWF has a primary role in vascular haemostasis hence since Ang2 is also stored in Weibel-Palade bodies, Ang2 may also have a role in vascular haemostasis. Furthermore, stored Ang2 is rapidly exported upon stimulation (PMA, thrombin, histamine) and has a long half life (>16 hours) and is rapidly regenerated upon challenge thus it is highly conceivable that Ang2 may be a potential regulator and modifier of rapid vascular responses.

1.4 Ephrin receptor tyrosine kinases

The ephrin family of receptor tyrosine kinases represents the third class of receptors primarily expressed in the endothelial lining of blood vessels from embryonic development to adulthood. However, unlike VEGF and Tie receptors, which are exclusively expressed on endothelial cells, ephrin (Eph) receptors are expressed on a range of cell types. Ephrins were initially characterised by their roles in axon guidance and neuronal patterning (Krull et al., 1997; Smith et al., 1997).

1.4.1 Eph receptor structure

The Eph family of RTKs consists of 15 members and there are two types of Eph receptor, classified as EphA and EphB which are based on structural homology and ligand specificity. The receptors consist of three distinct extracellular motifs; a globular domain, a cysteine-rich region and two fibronectin type III repeats. The globular domain of the receptor allows for ligand binding (Himanen et al., 1998). The cytoplasmic region contains a single kinase domain, several consensus binding sites for proteins with SH2 motifs, a PDZ binding region and a sterile alpha motif (SAM), (Torres et al., 1998; Thanos et al., 1999). Ligand specificity of both Eph receptors results in EphA only binding to GPI-linked ligands, conversely, EphB can only bind the transmembrane ephrins (Gale et al., 1996a).

1.4.2 Eph receptor ligands

The Eph receptor ligands are known as ephrins and are classified into two distinct groups: ephrin A and ephrin B. Both groups of ephrins must be membrane bound in order to

activate their receptors and they share between 23 and 56% identity at the amino acid level (Gale et al., 1996b). Ephrin A ligands are GPI linked and ephrin B ligands contain a single transmembrane domain and a short highly conserved cytoplasmic tail. The fact that the ephrins cannot act as soluble mediators but must be membrane-bound to activate their receptor, suggests that that Eph/ephrin signalling can only operate over short distances, that is to say from cell-to-cell which consequently results in repulsive or attractive signalling.

1.4.3 Mouse gene knockout studies

The importance of Eph/ephrin function in the development of normal vasculature has been well studied. The ephrin B2 mutant embryo has defects in both arteries and veins (Wang et al., 1998), this finding indicates that there is bi-directional signalling between ephrin B2 and its cognate receptor Eph B4; this is because ephrin B2 is only expressed in arterial endothelial cells and Eph B4 is only expressed venous vessels. In transgenic mice studies examining the functional role of Eph/ephrin B2, it was demonstrated that abnormal ephrin B2 signalling results in aberrant blood vessel projection, abnormal vascular network formation and defective recruitment of smooth muscle progenitor cells to the ascending aorta (Oike et al., 2002)

1.5 Platelet Derived Growth Factor and its receptors

In the late 1970s, a factor was purified from platelets that was found to promote proliferation of mesenchymal cells (Heldin et al., 1979; Antoniades et al., 1979; Deuel et al., 1981; Raines et al., 1982). This factor is known as platelet derived growth factor (PDGF). There have been two genes (A and B) identified for PDGF, with three biologically active forms of the PDGF protein (Heldin et al., 1979). Initially, three biological active forms of PDGF were identified and termed PDGF-AA; PDGF-AB; PDGF-BB, representative of the disulphide linked A and B chains, hence PDGF-AA and PDGF-BB, represent homodimeric combinations whilst PDGF-AB, represents the heterodimeric combination of the disulphide linked A and B chains. Later in 2000 and 2001, two additional forms of PDGF were identified; PDGF-CC (Gilbertson et al., 2001) and PDGF-DD (Bergsten et al., 2001). PDGF-CC and PDGF-DD differ from the three former members of the PDGF family, namely they are secreted as inactive forms and thus cannot activate PDGF receptors until the N-terminal CUB domain is cleared (Bergsten et al., 2001).

1.5.1 Platelet Derived Growth Factor Receptors

The receptor for platelet derived growth factor (PDGF-R) contains two receptor subunits, α-subunit and β-subunit, which are either homo-or heterodimerised by PDGF, to form a functional PDGF receptor (Heldin et al., 1998). Figure 1.6, illustrates the preferences of PDGF family members for PDGF receptors. The primary receptor type important in angiogenesis is PDGFR-β.

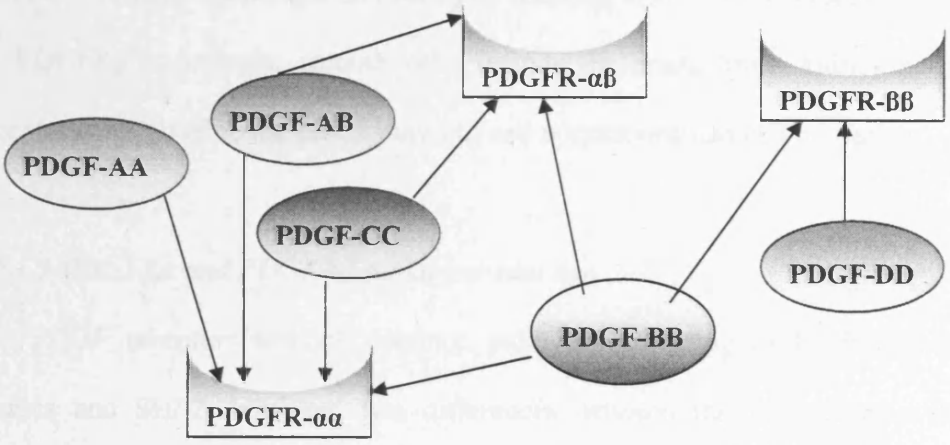


Figure 1.5 Preferences of PDGF family members for PDGF receptors.

1.5.1.1PDGFRα

PDGFRα null animals die during embryonic development. PDGFRα is involved in the correct development of a host of mesenchymal cells, including dermal (Lindahl et al., 1999); kidney (Li et al., 2000); intestinal (Lindahl et al., 1999); lung alveolar smooth muscle cells (Boström et al., 1996); non-neuronal crest (Morrison-Graham et al., 1992) and chondrocytes (Soriano 1997; Tallquist et al., 2000). Studies suggest that PDGFRα promotes proliferation of precursor populations in various cell types (Lindahl and Betsholtz, 1998).

1.5.1.2 PDGFRβ

PDGFRβ null animals also die during embryonic development. In PDGFRβ null animals smooth muscle cells are defective. In contrast, PDGFRα null animals are defective in vascular smooth muscle cells and pericytes (Lindahl et al., 1997). Vascular beds that rely on PDGFRβ in vascular smooth cells include the brain, heart, kidney and eye. The receptor is involved in the proliferation of cell populations and cell migration.

1.5.1.3 PDGFRα and PDGFRβ signal transduction

The PDGF receptors activate common pathways including PI3K, PLCγ, Src family kinases and SHP2; however, two differences between the two receptors have been identified. The adaptor protein Crk associates only with PDGFRα whilst RasGap binds to PDGFRβ only (Heldin et al., 1998).

1.6 Aims of study

The processes of vasculogenesis and angiogenesis ensure the correct development of the vasculature during embryonic development through to adulthood as well as featuring in pathophysiological states. Tie1 is essential for vascular development. Despite over 10 years of studying the receptor, our understanding into how Tie1 functions and signals remains incomplete. The aim of this work was to investigate the mechanism(s) by which Tie1 is activated with particular focus on the intracellular domain of the receptor and to examine possible cellular effects the receptor has in endothelial cells. Specifically I will determine whether the Tie1 intracellular domain can be activated alone or via Tie2, test the ability of Ang1 to modify Tie1 activation and test the cellular effects of the Tie1 intracellular domain.

Chapter Two

Materials & Methods

2.0 Materials and Methods

2.1 General Materials

All chemical reagents were obtained from Sigma Chemical Company, Fisher Scientific, Melford Laboratories or BDH Laboratories unless otherwise stated.

All plasticware was obtained from Nalge Nunc International.

Phosphate Buffered Saline (PBS)

140mM NaCl

2.7mM KCl

10mM $NaPO_4$

1.8mM KH_2PO_4, pH 7.4

Tris Buffered Saline (TBS)

25mM Tris

144mM NaCl, pH 7.4

Tris-EDTA

10mM Tris-HCl

1mM EDTA , pH7.5

2.1.1 Microbiological Media Recipes

2.1.1.1 Preparation of Luria Bertani (LB) Broth

LB broth was prepared using 10g tryptone, 5g yeast extract, 10g NaCl and distilled water to 1litre, pH 7.0. The solution was thoroughly mixed and autoclaved for 20 minutes at 120°C. Stored at room temperature until ampicillin was added before use. The final concentration of 100 μg/ml unless stated otherwise. After addition of ampicillin, broth was stored at 4°C.

2.1.1.2 Preparation of Luria Bertani (LB) agar plates

LB agar plates were prepared using 10g tryptone, 5g yeast extract, 10g NaCl, 15g agar and distilled water to 1litre, pH 7.0. The solution was thoroughly mixed and autoclaved for 20minutes at 120°C. The solution was allowed to cool to approximately 50°C before ampicillin was added to a final concentration of 100μg/ml unless otherwise stated. Approximately 25ml of agar was poured into 100mm petri dishes (Bibby Sterlin). Plates were stored at 4°C.

2.1.1.3 SOB broth

To make SOB broth; 2% (w/v) lacto-tryptone, 0.5% (w/v) yeast extract, 10mM NaCl and 2.5mM KCl were combined to a final volume of 250ml. The addition of the final two ingredients was done under sterile conditions.

2.1.1.4 SOC broth

SOC broth was prepared by adding 1ml of 2M filter-sterilised glucose solution to 100ml of SOB prior to use. SOC broth was filter-sterilised prior to use.

2.1.1.5 Transformation Buffer

10mM Pipes

55mM $MnCl_2$

15mM $CaCl_2$

250mM KCl.

1M KOH was added to aid the dissolution of 10mM Pipes.

The three solutions were combined to a final volume of 200ml.

2.2 Mammalian Cell Culture

Reagents for cell culture were obtained from Invitrogen except where stated. Foetal Calf Serum (FCS) was obtained from Harlan Sera Laboratories. All tissue culture plastics were obtained from Nalge Nunc International.

2.2.1 Cell Culture Media

For all cell culture a general Complete Medium recipe was prepared unless otherwise stated. Medium was prepared containing 10% FCS (100units/ml), 100μg/ml streptomycin and 100μg/ml L-glutamine.

2.2.2 Cell Types

Cell Type	Media	Additives
Chinese Hamster Ovary (CHO) cells	MEM	10% FCS
Human Microvascular Endothelial (HME) cells	MCDB 131	10% FCS, 1μg/ml hydrocortisone, 10ng/ml endothelial growth factor (EGF)
Human Umbilical Vein Endothelial (HUVE) cells	Medium 199	20% FCS, 5μg/ml heparin, 50μg/ml endothelial cell growth supplement (ECGS), no L-glutamine

Cells were obtained from the following sources:

CHO cells purchased from European Collection of Cell Culture (ECACC) Wiltshire, UK.

HME cells obtained from Centre for Disease Control, Atlanta, USA.

HUVE cells isolated by Joanne Jeory, Department of Cardiovascular Sciences, University of Leicester, UK. All experiments were performed with cells between passages 2 and 7.

2.2.3 Trypsinisation

When passaging cells the confluent monolayer was washed once with PBS and incubated with trypsin-EDTA (0.05% trypsin and 0.02% EDTA diluted in PBS), for approximately 1-2 minutes or until the cells lifted off the flask upon gentle

agitation. The trypsin was inactivated by the addition of Complete Medium (refer to section 2.2.1) and removed by centrifugation of the cells, 400g for 6 minutes. Cells were resuspended and seeded in an appropriate dilution of Complete Medium. All cell types were passaged in this manner unless stated.

2.2.4 Mammalian Cell Transfection

DNA transfection was carried out with two different reagents dependent upon cell type.

2.2.4.1 Superfect™ transfection

The Superfect™ transfection reagent (Qiagen) was used to transfect CHO cells. The principle of the reagent is to compact the DNA so it will bind to the cell surface and is then transported into the cell by non-specific endocytosis.

The day before the transfection, cells were seeded to 60% confluence in an $80cm^2$ flask and incubated at 37°C/5% CO_2. On the day of transfection, 6μg of DNA was mixed with 225μl of serum-free medium with no additives and 4μl of Superfect™ per μg of DNA. The mixture was incubated for 10 minutes at room temperature to allow a DNA/ Superfect™ complex to form. During this time the cells were washed twice with PBS and 2ml of Complete Medium was added to the cells. The DNA/ Superfect™ complex was then added to the cells and incubated for 2 hours and 45 minutes at 37°C/5% CO_2. The cells were then washed with PBS and incubated with 12ml of Complete Medium at 37°C/5% CO_2.

2.2.4.2 Targefect F-2 transfection

The Targefect F-2 reagent (Targeting Systems) was used for transfecting HUVE cells. Targefect F-2 is a non-lipid, cationic reagent.

HUVE cells were grown to 80-90% confluence in six-well plates or 80cm^2 flasks. 1μg (per well) or 4μg (per 80cm^2 flask) of DNA was added to 1ml or 4ml of serum-free medium and mixed thoroughly by aspiration. 5μl (for wells) or 20μl (for 80cm^2 flask) of Targefect F-2 was added and incubated at room temperature for 20 minutes. Cells were washed twice with serum-free medium (no additives) and the DNA/Targefect complex was added and then incubated at 37°C/5% CO_2 for 2 hours. The complex was then removed and Complete Medium added and incubated overnight at 37°C/5% CO_2. Due to the use of a biologically variable primary cell line, transfection efficiencies for HUVE cells were observed between 10-40%.

2.2.5 Endothelial Cell Survival Assay

HUVE cells were plated into 6 well plates and transfected with the appropriate DNA constructs and vector encoding green fluorescent protein (GFP) and allowed to recover for 18 hours at 37°C/5% CO_2. The medium was removed from the cells and used to carry out the Floating Cell Count protocol (refer to Section 2.2.6.1). Cells expressing GFP were visualised under UV light by excitation at wavelength ~385nm, emitting green colour at ~508nm. The number of cells expressing GFP were counted in selected areas of the well, which had previously been marked with a grid.

2.2.6 Apoptosis Assays

2.2.6.1 Floating Cell Count Assay

HUVE cells were plated into 6 well plates and transfected with the appropriate DNA constructs and vector encoding green fluorescent protein (GFP) and allowed to recover for 18 hours at 37°C/5% CO_2. The medium was then removed from cells and placed into fresh microcentrifuge tubes. A pellet was obtained by centrifuging the media at 13,000g for 5 minutes. The supernatant was removed and the pellet resuspended thoroughly in 20µl of PBS. Immediately prior to loading the resuspended cells into a chamber of a haemocytometer, 20µl of Trypan Blue was added to the cells and mixed. The total volume of cells loaded into a chamber of a haemocytometer was 10µl. The cells were viewed under a microscope by selecting the four outermost areas of the haemocytometer grid. Cells counted within these four areas represented the total floating cell count, an index of cell apoptosis (Levkau et al., 1998).

2.2.6.2 Cleaved Caspase Assay

A key mediator in apoptosis is the cysteine aspartic acid-specific protease (Caspase) family. During apoptosis signalling, caspase-3 and caspase-7 undergo rapid proteolytic cleavage and are activated. Thus, measurement of caspase-3 and caspase-7 activity provides an accurate way in which apoptosis can be assessed.

In order to measure apoptosis, Caspase-Glo™ 3/7 Assay was used (Promega). The principle of the kit combines a proluminescent caspase-3/7 substrate which contains the tetrapeptide sequence DEVD. Following caspase cleavage, a substrate for

luciferase (aminolucerifin) is released, resulting in the luciferase reaction and the production of light.

HUVE cells were plated into a 12 well plate and grown up in complete medium and incubated overnight at 37°C/5% CO_2. The cells were then transfected with the appropriate DNA and left to recover for 12-18 hours at 37°C/5% CO_2. The medium was completely removed and the cells gently washed twice in PBS. Complete medium was replaced with serum-free medium to enable cell starvation. Cells were incubated overnight at 37°C/5% CO_2. On the day of performing the Caspase-Glo™ 3/7 Assay, the serum-free medium was removed from the cells. The cells were washed gently twice in PBS then scraped and pipetted into fresh microcentrifuge tubes. The cells were then centrifuged at 13, 000g for 5 minutes to achieve a firm pellet. The pellet was resuspended in 25µl of PBS and 25µl of Caspase-Glo™ reagent. Resuspended cells were incubated for 30 minutes at room temperature. The entire contents of each microcentrifuge tube was pipetted into glass luminometer tubes and caspase 3/7 activity measured using a Lumat 9507 Luminometer (Berthold Technologies).

2.3 Protein Biochemistry

2.3.1 General Reagents

3x Reducing Sample Buffer

50mM Tris, pH 6.8

10% glycerol

2% w/v SDS

0.1% w/v bromophenol blue

5mM EDTA

2x Reducing Sample Buffer

300µl 3x reducing sample buffer plus 600µl double distilled water before adding 200mM DTT.

Immunoprecipitation Lysis Buffer

50mM Tris. pH 7.4

50mM NaCl

1mM Na_2VO_4

1mM NaF

1mM EGTA

Prior to use 0.1mM AEBSF and 1% Triton-X-100 were added.

Stored at 4°C.

Immunoprecipitation Lysis Wash Buffer

As Immunoprecipitaion Lysis Buffer except 0.1% Triton-X-100.

Protein Electrophoresis Running Buffer

192mM glycine

25mM Tris

0.5% w/v SDS

Protein Transfer Buffer

192mM glycine

25mM Tris

20% v/v methanol

Tris Buffered Saline

25mM Tris, pH 7.4

144mM NaCl.

2.3.2 Whole Cell Lysate Preparation

CHO, HME and HUVE cells were grown in 6 well plates (refer to relevant Results Chapter). The cells were washed gently once with sterile PBS and lysed by addition of 40µl of 2x Reducing Sample Buffer containing 200mM DTT. The lysed cells were scraped and pipetted into a microcentrifuge tube. The lysed cells were then heated to 95°C for 6 minutes, sonicated for 10 seconds and centrifuged at 13, 000g for 5 minutes. Proteins were resolved using SDS-PAGE (refer to Section 2.3.4).

2.3.3 Immunoprecipitation

2.3.3.1 Immunoprecipitation of Tie1

CHO, HME and HUVE cells were grown to confluence in 80cm^2 flasks and treated as detailed in the relevant Results Chapter. Briefly, flasks were washed twice with PBS and placed immediately on ice. The PBS was removed from the flasks and 500µl of Immunoprecipitation Lysis Buffer, pH 7.4 (refer to Section 2.3.1) was

added. The flasks were incubated with Immunoprecipitation Lysis Buffer for 5 minutes on ice, after which the cell lysates were scraped off and placed into cooled microcentrifuge tubes. Cell lysates were vortexed for 10 minutes at 4°C. Lysates were then centrifuged at 13.000g for 10 minutes at 4°C, to remove any insoluble material. 30µl of cell lysate was removed and 20µl of 2x Reducing Sample Buffer was added with 20mM DTT (refer to Section 2.3.1). The remaining supernatant was transferred to a fresh microcentrifuge tube. Tie1 (MAB619, R & D Systems) was added to a final concentration of 2µg/ml and allowed to mix with rotation for 2 hours at 4°C. 40µl of Protein G: sepharose (Sigma) which had been washed with Immunoprecipitation Lysis Buffer was added to each supernatant and allowed to mix for a further 2 hours with rotation at 4°C. The sepharose beads were collected by centrifuging at 13.000g for 1 minute and washed 3 times with Immunoprecipitation Lysis Wash Buffer (refer to Section 2.3.1). 40µl of 2x Reducing Sample Buffer containing 20mM DTT was added to the washed sepharose beads. Samples were heated at 95°C for 6 minutes, to allow dissociation of the immunocomplex from the sepharose beads to occur. Samples were then centrifuged at 13, 000g for 1 minute. 20µl aliquots of sample were loaded onto SDS-PAGE for resolution of protein (refer to Section 2.3.4).

2.3.3.2 Immunoprecipitation of Tie2

To immunoprecipitate Tie2 from CHO, HME and HUVEC cells, immunoprecipitation was achieved as described in Section 2.3.3.1, except Tie2 (R & D Systems) polyclonal antibody was used instead of Tie1.

2.3.3.3 Immunoprecipitation of tyrosine phosphorylated proteins

Immunoprecipitation of tyrosine-phosphorylated proteins was achieved as described in Section 2.3.3.1, except anti-phosphotyrosine clone PT-66 (Sigma) was used instead of Tie1. Protein G: sepharose is not required since anti-phosphotyrosine clone PT-66 is directly conjugated to agarose beads.

2.3.4 Protein separation by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the Bio-Rad Mini Gel kit.

Acrylamide gels were prepared following the recipes given below: -

	12% Resolving Gel
30% Acrylamide/0.8% Bisacrylamide	8.0ml
2M Tris, pH 8.8	3.7ml
ddH_2O	7.9ml
20% SDS	100µl
10% Ammonium persulphate	134µl
TEMED	14µl

	10% Resolving Gel
30% Acrylamide/0.8% Bisacrylamide	6.7ml
2M Tris, pH 8.8	3.7ml
ddH_2O	9.6ml
20% SDS	100µl
10% Ammonium persulphate	134µl
TEMED	14µl

	5% Stacking Gel
30% Acrylamide/0.8% Bisacrylamide	3.3ml
1M Tris, pH 6.8	2.5ml
ddH_2O	13.7ml
20% SDS	100µl
10% Ammonium persulphate	200µl
TEMED	20µl

Proteins were loaded and separated electrophoretically at 150-200V in Protein Electrophoresis Running Buffer (refer to Section 2.3.1). In addition, a rainbow molecular marker was loaded to observe the progression of protein separation (Sigma).

2.3.5 Western Blot Analysis

Proteins separated by SDS-PAGE were transferred electrophoretically from the polyacrylamide gel to nitrocellulose membrane (Hybond ECL, Amersham) using Protein Transfer Buffer (refer to Section 2.3.1). Proteins were transferred overnight at 200mA. The nitrocellulose was probed using antibodies (refer to Section 2.3.6 and Section 2.3.6.3).

2.3.6 Detection of proteins on Nitrocellulose membrane

Following overnight transfer of proteins, the nitrocellulose was removed from the Protein Transfer Buffer and washed with TBS containing 0.1% Triton X-100 (TBS-TX100). The membrane was then blocked with the appropriate blocking buffer (TBS-TX100) containing either 5% BSA or semi-skimmed milk. for 1 hour at room temperature with constant gentle agitation. The nitrocellulose membrane was then probed for 1 hour at room temperature with the relevant primary antibody (details in the relevant Results Chapter and Section 2.3.6.3). Following primary antibody incubation, the membrane was washed 3 times with TBS-TX100 for 5 minutes. Primary antibody binding was detected by the relevant HRP conjugated secondary antibody, diluted 1 in 2000 in TBS-TX100 for 1 hour at room temperature. The nitrocellulose membrane was washed 3 times with TBS-TX100 for 10 minutes at room temperature. Bound antibody was detected using the enhanced chemiluminescence protocol (Section 2.3.6.1)

2.3.6.1 Enhanced chemiluminescence (ECL) protocol

The detection of HRP conjugated antibodies or secondary antibodies, which bind to proteins on the nitrocellulose membrane, was accomplished using an enhanced chemiluminescence system. 22µl of 90mM p-Coumaric acid in DMSO, 50µl of 250mM Luminol (5-amino-2,3-dihydro-1-4-pthalazinedione) in DMSO and 3µl hydrogen peroxide were added to 10ml of 100mM Tris-HCl, pH 8.8. Immediately after the final wash in the antibody detection protocol, the nitrocellulose membrane was incubated with the above solution for 1 minute at room temperature with gentle agitation. Excess solution was drained off the nitrocellulose membrane and the membrane then wrapped in Saran wrap and exposed to biomax light film (Sigma).

2.3.6.2 Stripping of Nitrocellulose membranes

To allow the nitrocellulose membrane to be re-probed with different antibodies, the membrane was incubated with Re-Blot Plus antibody stripping solution, purchased from Chemicon International Inc. The manufacturer's protocol was followed. Briefly, the nitrocellulose membrane was incubated for 15 minutes at room temperature with gentle agitation. Excess stripping solution was removed by washing with TBS containing 0.1% Triton X-100 before being blocked with the appropriate blocking buffer.

2.3.6.3 Primary antibodies used in Western Blot Analysis

Antibody	Dilution	Blocking Buffer	Secondary Antibody	Source
FLAG® M2	1:500	3% non-fat dry milk	Anti-rabbit HRP conjugated	Sigma
Phosphotyrosine	1:1000	5% BSA	Anti-mouse HRP conjugated	Santa Cruz Biotechnology Inc.
Tie1	1:1000	5% non-fat dry milk	Anti-rabbit HRP conjugated	Calbiochem
Tie1	1:1000	5% non-fat dry milk	Anti-rabbit HRP conjugated	Santa Cruz Biotechnology
Tie2	1:1000	5% non-fat dry milk	Anti-goat	R & D Systems

2.4 Molecular Biology Techniques

2.4.1 RNA Purification

To obtain RNA required for RT-PCR the RNA purification mini kit (Qiagen) was used. HME cells were grown to confluence in $80cm^2$ and the medium aspirated and the cells washed with 1x PBS. The cells were trypsinised (0.10% in PBS); once the cells were detached they were transferred to an RNase-free polypropylene centrifuge tube and centrifuged at 300g for 5 minutes. The supernatant was completely aspirated. To the tube, 600µl of buffer containing 1% β-mecaptoethanol (β-ME) was added and the pelleted cells were loosened by thoroughly flicking the centrifuge tube. The lysate obtained from this step was homogenised by direct application to a spin column, supplied within the mini kit (Qiagen). The homogenised lysate subsequently was subjected to a series of wash and centrifugation steps to ensure the purification of the total RNA. To elute the total RNA present on the column, 30µl of RNAase-free water was added directly to the spin column and centrifuged for 1 minute at ≥8000g. Purified RNA was stored at -70°C.

2.4.1.2 Spectrophotometric Quantification of RNA

The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}) spectrophotometrically. Since the volume of total RNA eluted was 30µl, a 1/50 dilution was made, using 1µl RNA sample and the absorbance measured in a 1ml RNase-free cuvette. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A260 = 1 => 40 µg/ml). This relation is valid only for measurements in water.

An example of the calculation involved in RNA quantitation is shown below:

Volume of RNA sample = 30µl

Dilution = 1 µl of RNA sample + 49 µl distilled water (1/50 dilution).

Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free).

A260 = 0.75

Concentration of RNA sample = 40 x A_{260} x dilution factor

= 40 x 0.75 x 50

= 1500 µg/ml

Total yield = concentration x volume of sample in milliliters

= 1500 µg/ml x 0.3ml

= 450 µg = 0.45 mg RNA

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. For determination of RNA concentration, the RNA sample was diluted in water and therefore the relationship between absorbance and concentration (A_{260} reading of 1 = 40 µg/ml RNA) is based on an extinction coefficient calculated for RNA in water.

2.4.1.3 Reverse Transcription (RT)

The process of subjecting RNA to reverse transcription is often followed by the polymerase chain reaction (PCR) and is known as RT-PCR. RT-PCR was used to generate templates for *in vitro* transcription in endothelial cells. Reverse transcription was done using the RETROscript kit (Ambion (Europe) Ltd,

Cambridgeshire. UK). Reverse transcription with heat denaturation of the RNA was performed.

2 µg total RNA

2 µl Oligo(dt)

8 µl Nuclease-free water

12µl total volume

The above cocktail was mixed, microcentrifuged briefly and heated for 3 minutes at 75°C. The tube was promptly removed and placed on ice, microcentrifuged briefly and replaced on ice. The following components were then added:-

2 µl 10x RT buffer

4 µl dNTP mix

1 µl RNase inhibitor

1µl Reverse Transcriptase

20µl Final volume

This cocktail was gently mixed and microcentrifuged and incubated at 42°C for 1 hour. This step was followed by incubation at 92°C for 10 minutes to inactivate the Reverse Transcriptase.

2.4.1.4 Polymerase Chain Reaction (PCR)

PCR was performed using the cDNA obtained from Reverse Transcription. The following cocktail was prepared:-

5 µl 10x buffer

1 µl 10mM dNTPs

5 µl cDNA (template)

10 µl 1µM primer (3')

10 µl 1µM primer (5')

1.5 µl $MgCl_2$

17 µl Nuclease-free water

0.5 µl *Taq* DNA polymerase

---

50 µl total volume

The above reaction mixture was gently mixed, briefly microcentrifuged and overlaid with mineral oil. The following parameters were used to form 1 cycle of the polymerase chain reaction; annealing stage for 1 minute at 55°C, extension stage for 2 minutes at 72°C and deactivation stage for 1 minute at 94°C. A total of 34 cycles were completed.

2.4.2 Competent Cell Production (XL-1 Blues)

Epicurian coli XL-1 Blues were obtained from Stratagene. XL-1 Blue glycerol stock was streaked onto a LB-tetracycline (10µg/ml) agar plate and grown overnight at 37°C. LB broth was inoculated with a single colony and incubated at 37°C overnight in a shaking incubator at 225rpm. 250ml of SOB was inoculated and grown at 18°C; growth was monitored by optical density (OD) at 600nm. When the OD reached approximately 0.6, the culture was centrifuged at 3,500g for 8 minutes at 4°C. The

bacterial pellet was gently resuspended in 80ml of chilled Transformation Buffer (TB) and left on ice for 10 minutes. The cell suspension was centrifuged at 3,500g for 8 minutes at 4°C and the pellet was resuspended in 20ml of cooled TB buffer. Dimethyl sulphoxide (DMSO) was added to a final concentration of 7% and the cell suspension was placed on ice for 10 minutes before aliquoting. Aliquots were frozen in liquid nitrogen and stored at -80°C.

2.4.3 Storage of Bacterial Cultures

Plasmid containing *E.coli* were stored at -80°C, as described by Sambrook *et al.* (1989). Bacterial cultures were placed in a freezing vial under sterile conditions with 150µl of sterile glycerol. The mixture was mixed thoroughly before snap freezing in liquid nitrogen.

2.4.4 DNA Preparation

2.4.4.1 QIAfilter mini-prep

To obtain DNA required for cell transfections, the DNA plasmid purification mini kit (Qiagen) was used. A single colony was picked from an appropriate LB agar plate and inoculated into LB broth and allowed to grow at 37°C for 16 hours with vigorous shaking (300rpm). The bacterial culture was centrifuged at 6,000g for 15 minutes at 4°C, to harvest bacterial cells. The manufacturer's protocol was then followed. The DNA pellet was resuspended in 20µl of nuclease-free water and the DNA concentration determined by measuring the OD_{260}.

2.4.4.2 Spectrophotometric analysis of DNA

DNA concentration was determined by diluting 1µl of DNA solution into 500µl of sterile distilled water. DNA concentration was measured using a spectrophotometer by measuring the absorbance at 260nm. DNA concentration was calculated assuming 50µg/ml of DNA measured in a cuvette with a 1cm path length will have an absorbance of 1. Pure DNA has an A_{260}/A_{280} ratio of 1.8-2.0, as described by Sambrook *et al.* (1989).

2.4.4.3 Ethanol precipitation of DNA

Precipitation of DNA was performed using a 1 in 10 dilution of 3M Sodium Acetate, pH 5.2 and 2.5 volumes of 100% ethanol. The precipitate mixture was incubated at -80°C for 1 hour. The precipitate was centrifuged at 13,000g for 15 minutes. The supernatant was carefully removed and the pellet was washed with 70% ethanol by centrifugation (13,000g for 5 minutes). The pellet was air-dried for 5 minutes and resuspended in 20µl nuclease free water.

2.4.5 Subcloning of Tie1 minus transmembrane cDNA into pFLAG CMV vector b

The previously cloned Tie1 receptor DNA sequence within the vector pPCR-Script Amp SK (+), (pPCR $_3$ Script Tie E/B), was a kind gift from Dr M.B.Marron (University of Sheffield). Tie1 minus its transmembrane domain (Tie-TM DNA) was excised out of pPCR $_3$ Script Tie E/B using restriction enzyme digestion. The DNA was digested with Bam H I and Cla I restriction enzymes. Concomitantly, the expression vector pFLAG CMV-6b (Sigma) was digested with the restriction

enzymes Bgl II and Cla I for the subsequent subcloning of the newly Tie-TM DNA into this expression vector. As described in Section 2.4.6, analysis following agarose gel electrophoresis revealed the desired sizes of DNA (Tie-TM DNA, ~1Kb) and vector (pFLAG CMV-6b, 4.7 Kb). Extraction of the DNA and vector from the agarose gel using Qiagen DNA Extraction Kit was performed, as described in Section 2.4.8.

2.4.6 Agarose Gel Electrophoresis

DNA was separated on 1% to 2% agarose gels as described by Sambrook *et al.* (1989). Agarose was melted in TAE buffer in a microwave. In a fume cupboard, ethidium bromide was added to the melted agarose to a final concentration of 1μg/ml. The gel was poured into an appropriate tray and a comb was inserted immediately, to create wells for DNA loading. Once the gel had set the comb was removed and DNA was loaded into the wells. Gels were run at 100V in TAE buffer until sufficient fragment separation had occurred. The DNA fragments were visualised on an ultraviolet light transilluminator and images were captured using a Multimage light cabinet. DNA fragment sizes were compared with 1Kb ladder of DNA molecular markers (Invitrogen) run on the same gel.

2.4.7 Restriction Enzyme Digest

Restriction enzyme digest were performed using Roche restriction endonucleases using the manufacturer's recommended buffer. Briefly, 1μg of DNA was resuspended in digest buffer. 10 units of restriction endonuclease was added to a

final volume of 10µl. Tubes were incubated at 37°C and the reaction was allowed to proceed for 1 hour for a single digest or 2 hours for a double digest.

2.4.8 Gel Extraction of DNA

Following DNA cleavage using restriction endonucleases, the reaction mixture was resolved using agarose gel electrophoresis and visualised using an ultraviolet light transilluminator. The DNA fragment of interest was excised from the agarose gel using a scalpel blade. The newly excised fragment was placed into a fresh microcentrifuge tube. Using the QIAquick gel extraction kit (Qiagen), the agarose gel containing the DNA fragment was dissolved and the resulting solution added to a silica membrane column. The silica membrane adsorbs the DNA in the presence of high salt concentrations allowing impurities to pass through the silica membrane column by centrifugation. Any impurities remaining were washed away and the DNA eluted with 30µl of nuclease-free water.

2.4.9 Ligation

Reaction mixtures were prepared in the following way: 50ng vector DNA, 1µl T4 DNA Ligase, 1µl 10x Ligation buffer and nuclease-free water were added to a final volume of 10µl. The reaction mixture was incubated overnight at 4°C.

2.4.10 Transformation

Transformation of plasmid DNA into Epicurian XL-1 Blue super competent cells (Stratagene) was done in the following way: 50µl of thawed super competent cells were aliquoted into a pre-chilled Falcon 2059 (Becton Dickinson) tube. 1µl of

plasmid DNA was transferred into a separate aliquot of super competent cells. The transformation mixture was gently mixed and then incubated on ice for 30 minutes. The transformation mixture was then heated promptly at 42°C for 45 seconds. Immediately afterwards the transformation mixture was placed on ice for 2 minutes. 250µl of pre-heated (42°C) SOC broth (refer to Section 2.1.1.4) was added to the transformation mixture and incubated for 1 hour at 37°C with constant shaking at 225rpm. Once transformation was complete, 50µl and 200µl aliquots were spread onto LB agar plates (refer to Section 2.1.1.2) containing 100µg/ml ampicillin. LB agar plates were incubated at 37°C/5% CO_2 for 18 hours. After 18 hours, colonies were picked and used to inoculate 10ml of LB broth (refer to Section 2.1.1.1) containing 100µg/ml ampicillin. Inoculated broth was incubated overnight at 37°C with constant shaking at 225rpm. DNA was prepared as described in Section 2.4.3.

2.5 Production of Stable Transfectants

To establish a cell line that exclusively expressed the Tie1 or Tie2 receptor tyrosine kinase, geneticin or G418 disulphate, an antibiotic that inhibits protein synthesis was used.

CHO cells were grown in a six-well plate to test for geneticin selectivity. They were subsequently exposed to differing concentrations to determine the minimum inhibitory concentration to select for geneticin resistance. The concentration of 500µg/ml appeared to kill approximately 90% of a confluent layer of CHO cells.

Transfection of CHO cells with full-length Tie1 or full-length Tie2 DNA was accomplished using the Superfect™ protocol (refer to Section 2.2.4.1). After 24 hours the media was replaced with Complete Media with 500μg/ml of geneticin.

CHO cells that exhibited geneticin resistance by surviving geneticin selection were grown up to confluence. Cells were passaged and plated to allow colonies to form. Ten colonies were picked using a pipette tip and were pipetted into separate wells of a 96 well plate at an appropriate dilution to achieve a seeding density of one cell per well. Cells were grown to confluence and transferred into separate wells of a 24 well plate and then expanded.

2.6 Densitometric Analysis

Immunoblots were visualised using an Alpha Innotech Imager and then analysed in AlphaEase™ software. The band of interest was selected by using the Spot Denso Toolbox function and the sum of all the pixel values after background correction, Integrated Density Value (IDV) calculated.

2.7 Statistical Analysis

The students't' test was used to analyse results where three or more samples were available. Data is presented as mean and ± standard deviation. P values ≤ 0.05 were considered as statistically significant.

Chapter Three

Investigations into the mechanisms of Tie1 activation

3.1 Introduction

There is little known about the cellular functions controlled by Tie1 or the signalling pathways utilised by this receptor. The signalling potential of Tie1 was examined by Marron et al. (2000) using a chimeric receptor approach. This approach used chimeric receptors composed of the TrkA ectodomain (for which nerve growth factor (NGF) is a known ligand) fused to the transmembrane and intracellular domains of Tie1 or Tie2. It was found that the Tie1 chimera was unable to detectably phosphorylate cellular proteins or undergo auto-phosphorylation and the group concluded that this was due to the receptor's poor kinase activity.

Kontos et al. (2002) also employed the chimeric approach to investigate the signalling potential of Tie1. This group also observed the receptor had poor ability to auto-phosphorylate. However, they did show chimeric Tie1 was capable of recruiting phosphatidyl-inositol-3-kinase (PI3K). Kontos argued that the chimeric receptor TrkA/Tie1 used by Marron et al. (2000), lacked intrinsic kinase activity and hence auto-phosphorylation of the receptor was not detected. However despite this claim, it was found that the chimeric receptor, CSF-1R/Tie1, was also poorly auto-phosphorylated. In addition to these findings it has also been found that Tie1 inhibits endothelial apoptosis (Kontos et al., 2002).

In summary, previous studies have thus failed to define the cellular effects of Tie1 or a signalling mechanism(s) for this receptor.

The aim of the work described in this chapter is to identify the mechanism by which Tie1 is activated. Firstly, the role of protein tyrosine phosphatases (PTPs) in the control of Tie1 phosphorylation was determined by use of a broad-range PTP inhibitor. Secondly, the significance of kinase activity on Tie1 phosphorylation was investigated by employing the strategy of using a Tie1 intracellular domain construct, which either expressed kinase activity or was rendered kinase inactive (Arg→Lys) due to a single residue mutation within the kinase insert domain. Thirdly, the potential ability of Tie2 to regulate Tie1 activity was examined.

3.2 Results

3.2.1 Tie1 phosphorylation is restricted by protein tyrosine phosphatases (PTPs)

Studies have previously shown Tie1 to be poorly phosphorylated (Marron et al., 2000; Kontos et al., 2002). Reasons for this apparent impaired kinase activity may be due to the fact that the kinase activity of Tie1 is intrinsically low or that the receptor is maintained in a conformation that results in low basal kinase activity. In addition, the action of protein tyrosine phosphatases may limit the phosphorylation of Tie1. It is well established that negative control of RTK activity is the result of direct action of PTPs; specifically by the suppression of ligand-independent RTK signalling, as demonstrated in p185HER2/ neu-overexpressing human tumour cells (Jallal et al., 1992) and through the control of tyrosine phosphorylation levels of ligand-activated RTKs, as demonstrated for both EGF and PDGF receptor tyrosine kinases (Sörby and Östman, 1996). The possibility that PTPs may control Tie1 phosphorylation was therefore investigated.

To investigate the hypothesis that the Tie1 receptor is a target for tyrosine phosphatases, a construct of the Tie1 endodomain with an amino-terminal FLAG epitope tag was expressed in Chinese hamster ovary cells (CHO).

CHO cells were used, as these cells do not normally express Tie1 and therefore would not have the potentially complicating effects of a population of endogenous Tie1 receptors. These cells were transfected with cDNA corresponding to the human Tie1 endodomain comprising the entire transmembrane and intracellular domains.

The Tie1 endodomain had been subcloned into the pFLAG vector in order to incorporate an amino FLAG epitope tag (as described by Marron et al, 2000). 24 hours later, post-transfection cells were treated with pervanadate, a broad range phosphatase inhibitor (Huyer et al., 1997).

Two forms of this construct were used; *pFLAGEndoB1*^{WT}, which has wild-type tyrosine kinase activity and *pFLAGEndoB1*$^{-}$, which has a substitution of arginine for the critical lysine in the ATP-binding pocket of the kinase and is therefore kinase inactive (Marron et al., 2000). CHO cells were lysed and Tie1 immunoprecipitated using FLAG monoclonal antibody M2, an antibody specific for the FLAG amino terminal. Proteins were separated on a 12% SDS-PAGE gel and Western blotted. Blots were probed for the expression and phosphorylation of the receptor using antibodies to the carboxyl terminus of Tie1 or anti-phosphotyrosine respectively (Fig. 3.1).

Probing for the presence of the Tie1 endodomain revealed that transfection of both kinase active cDNA (*pFLAGEndoB1*^{WT}) and kinase dead cDNA (*pFLAGEndoB1*$^{-}$) had been successful (Fig.1, upper panel, lanes 3-6). CHO cells that had not been transfected with either cDNA were not expected to show Tie1 expression since Tie1 is not normally expressed in these cells. Indeed, no expression of Tie1 was detected in non-transfected cells (Fig.3.1, upper panel, lanes 1 and 2). Levels of tyrosine phosphorylation in relation to Tie1 expression as shown in Fig 3.1, were quantified using densitometric analysis (Fig 3.1b).

The Tie1 expressed corresponds to the truncated form of the receptor, previously described (Yabkowitz et al., 1997; McCarthy et al., 1999 and Marron et al., 2000). As shown in Fig. 3.1. Tie1 endodomain undergoes further cleavage to generate three cell-associated fragments (designated a-c). These further truncation products arise by proteolytic cleavage within the cell and also occur with endogenous Tie1 in endothelial cells (Marron et al., in preparation). The ability of the FLAG monoclonal antibody to immunoprecipitate truncated forms of Tie1is due to homotypic binding of Tie1 endodomain to endodomain fragments containing the previously reported oligomerisation sequence (Marron et al., 2000). Probing immunoprecipitates for tyrosine phosphorylation revealed three major phosphorylated bands in cells transfected with Tie1 endodomain. These were of molecular masses 38, 42 and 45 kDa and correspond to the endodomain fragments designated a, b and c (Fig. 3.1).

Phosphorylation of the receptor only occurred in the cells transfected with kinase active cDNA, moreover that phosphorylation was only detected in those cells that had been treated with pervanadate (Fig.3.1, lower panel, lane 3). No phosphorylation was detected in kinase-dead cDNA transfected cells despite being treated with pervanadate (Fig.3.1, lower panel, lanes 5and 6). These data indicate that Tie1 undergoes auto-phosphorylation via its own kinase activity if the action of phosphatases is suppressed.

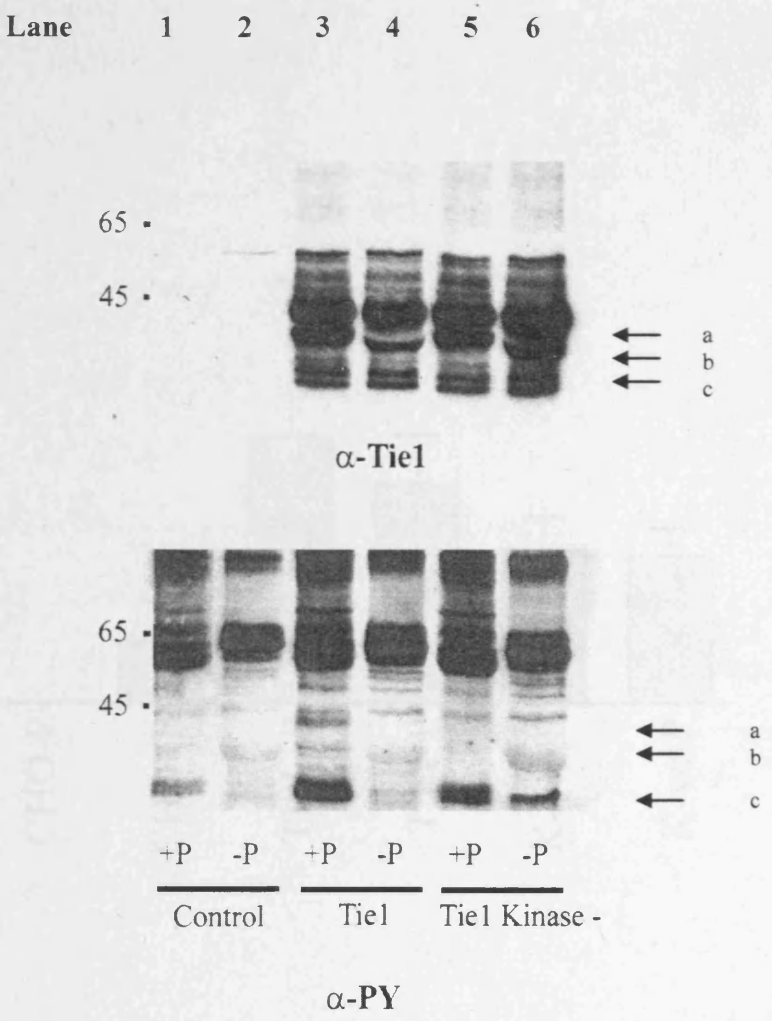


Fig. 3.1 **Tie1 phosphorylation is restricted by phosphatases.** CHO cells were transfected with kinase active cDNA (Tie1) *(lanes 3 and 4)* and kinase dead cDNA (Tie1Kinase-) *(lanes 5 and 6)* and treated with 5μg/ml pervanadate (+P) for 10 min. CHO cells were lysed and immunoprecipitated using FLAG monoclonal antibody M2. Proteins were separated by SDS-PAGE and Western blotted. Blots were probed with antibodies to the carboxyl terminus of Tie1 (*upper panel*) or anti-phosotyrosine (PY) (*lower panel*). Blots representative of at least three independent experiments are shown. The positions of the Tie1 endodomain fragments are indicated by an arrow; 45kDa (a), 42kDa (b) and 38kDa (c). Relative mobility of molecular mass markers is indicated in kDa.

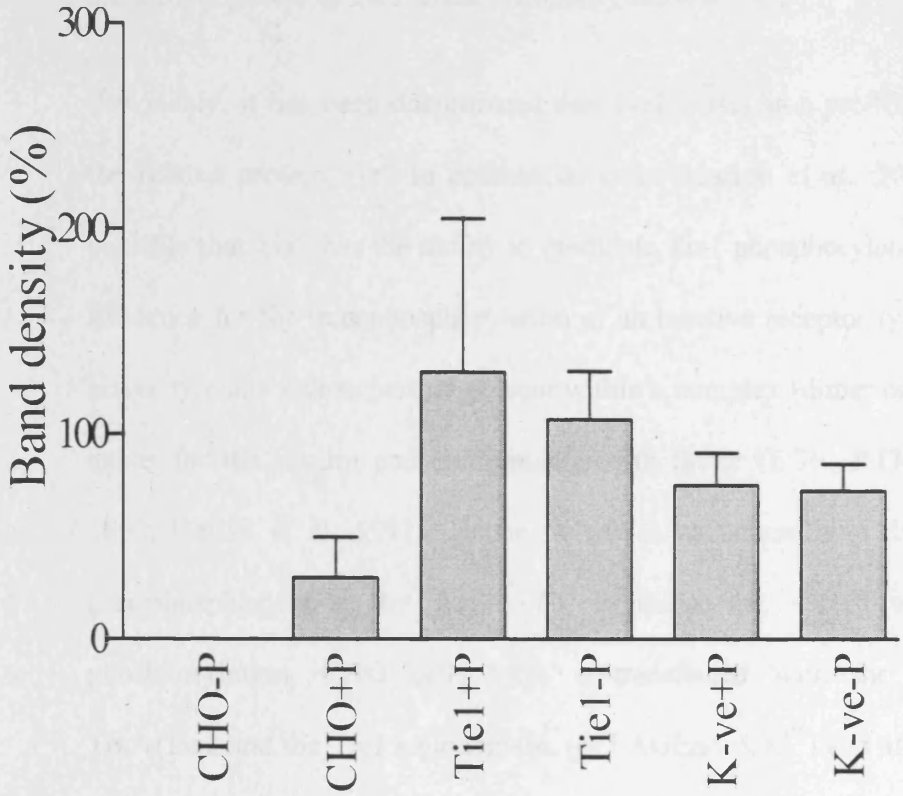


Fig 3.1b **The effect of kinase activity on Tie1 phosphorylation.** CHO cells were transfected with Tie1 endodomain alone; Tie1 kinase dead cDNA (K-ve) in the presence (+P) or absence (-P) of 5μg/ml pervanadate for 10 min. CHO cells were lysed and immunoprecipitated using FLAG monoclonal antibody M2. Proteins were separated by SDS-PAGE and Western blotted. Blots were probed with antibodies to the carboxyl terminus of Tie1 or anti-phosphotyrosine. The relative level of tyrosine phosphorylation for 45kDa Tie1 truncation product for each of the different transfected CHO cells was quantified by densitometric analysis for three independent experiments.

3.2.2 Involvement of Tie2 in the phosphorylation of Tie1

Previously, it has been documented that Tie1 exists as a pre-formed complex with the related protein Tie2 in endothelial cells (Marron et al., 2000). It is therefore possible that Tie2 has the ability to modulate Tie1 phosphorylation in this complex. Evidence for the transphosphorylation of an inactive receptor tyrosine kinase by an active tyrosine kinase partner present within a complex (dimer or oligomer), already exists for the insulin and epidermal growth factor (EGF) RTKs (Lammers et al. 1990, Herbst et al. 1991). Hence, it would be conceivable for Tie1 to undergo transphosphorylation by Tie2. To examine the effect of Tie2 on Tie1 phosphorylation, CHO cells were co-transfected with the chimeric receptor TrkA/Tie2 and the Tie1 endodomain, (pFLAGEndoB1^{WT}) and after 24 hours treated for 10 min with pervanadate. Cells were lysed and immunoprecipitated using FLAG monoclonal antibody M2. Proteins were separated on a 12% SDS-PAGE gel and Western blotted. Blots were probed for the expression and phosphorylation of the receptor using antibodies to the carboxyl terminus of Tie1 or anti-phosphotyrosine respectively (Fig. 3.2).

Probing with an antibody to the carboxyl terminus of Tie1 resulted in the detection of the truncated form of Tie1 at 45kDa (Fig. 3.2. upper panel, lanes 1-4), this observation had previously been seen in earlier experiments (Fig1, upper panel).

The anti-phosphotyrosine probe revealed again, that the Tie1 endodomain is phosphorylated in the presence of pervanadate (Fig. 3.2, lower panel, lane 1) and is

not phosphorylated when phosphatase activity is *not* suppressed by pervanadate treatment (Fig.3.2, lower panel, lane 2). In addition to these findings, it was also observed for CHO cell co-expressing the Tie1 endodomain and the chimeric receptor TrkA/Tie2 that had been treated with pervanadate, the Tie1 endodomain was auto-phosphorylated too. Furthermore, when cells had not been subjected to pervanadate treatment, hence when phosphatase activity had not been suppressed, auto-phosphorylation of the Tie1 endodomain still occurred (Fig 3.2. lower panel, lane 4). This result was contrary to data that had been obtained when TrkA/Tie2 was not present. These data suggest for the first time, that Tie2 activity is sufficient enough to lead to the phosphorylation of Tie1.

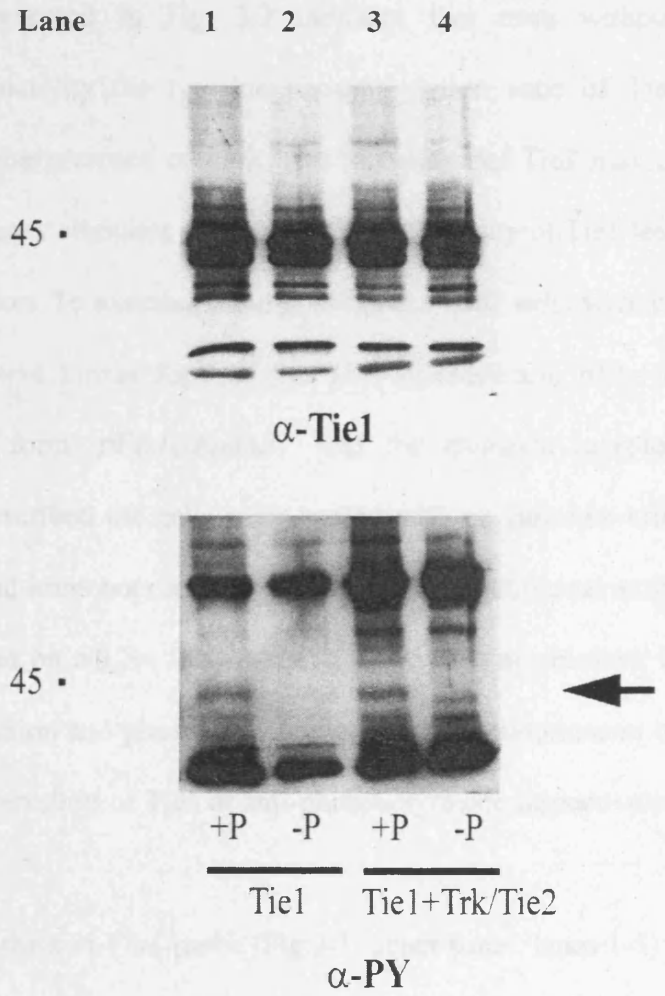


Fig. 3.2 **Involvement of Tie2 in the phosphorylation of Tie1.** CHO cells were transfected with Tie1 endodomain (*pFLAGEndoB1^{WT}*) alone *(lanes 1 and 2)* and TrkA/Tie2 *(lanes 3 and 4)* and treated with 5μg/ml pervanadate (+P) for 10 min. CHO cells were lysed and immunoprecipitated using FLAG monoclonal antibody M2. Proteins were separated by SDS-PAGE and Western blotted. Blots were probed with antibodies to the carboxyl terminus of Tie1 (*upper panel*) or anti-phosphotyrosine (PY) (*lower panel*). Blots representative of at least three independent experiments are shown. The position of the Tie1 endodomain is indicated by an arrow. Relative mobility of molecular mass markers is indicated in kDa.

3.2.3 Tie2 activates tyrosine kinase activity of Tie1

The data presented in Fig. 3.2 indicates that even without suppression of phosphatase activity the tyrosine phosphorylation state of Tie1 endodomain is increased in the presence of Tie2. This suggests that Tie2 may transphosphorylate Tie1 or Tie2 may stimulate endogenous kinase activity of Tie1 leading to Tie1 auto-phosphorylation. To examine these possibilities CHO cells were co-transfected with either the active kinase form of the Tie1 endodomain, *pFLAGEndoB1*^{WT} or the kinase dead form, *pFLAGEndoB1*$^{-}$ and the chimeric receptor TrkA/Tie2. As previously described the cells were treated with pervanadate after 24 hours. Cells were lysed and immunoprecipitated using FLAG monoclonal antibody M2. Proteins were separated on a 12% SDS-PAGE gel and Western blotted. Blots were probed for the expression and phosphorylation of the Tie1 endodomain using antibodies to the carboxyl terminus of Tie1 or anti-phosphotyrosine respectively (Fig. 3.3).

As shown by the anti-Tie1 probe (Fig.3.3, upper panel, lanes 1-5) all transfected/co-transfected cells, expressed the truncated form of Tie1, Tie1 endodomain at 45 kDa.

The anti-phosphotyrosine probe (Fig. 3.3, lower panel) revealed a repeated finding for the phosphorylation of Tie1, when CHO cells expressed the kinase active construct and were treated with pervanadate (Fig.3.3, lower panel, lane 1 and Fig 3.3b), auto-phosphorylation of Tie1 occurred. Cells expressing Tie1 and not treated

with pervanadate (Fig.3.3, lower panel, lane 2), did not exhibit Tie1 phosphorylation.

Co-expression of the Tie1 endodomain and the chimeric TrkA/Tie2 receptor with pervanadate treatment (Fig.3.3, lower panel, lane 3 and Fig 3.3b) resulted in the stimulation of Tie1, due to the presence of Tie2, a result that had been seen in earlier experiments (Fig.3.2, lower panel, lane 3). This stimulation by Tie2 appeared to be blocked when Tie1 has negative kinase activity; despite suppression of phosphatase action on the receptor (Fig.3.3, lower panel, lane 4) a result that was exacerbated if phosphatase activity was not suppressed (Fig.3.3, lower panel, lane 5 and Fig 3.3.b).

Close analysis reveals a low level of phosphorylation in lane 4; one possibility is that this reflects Tie2 transphosphorylating Tie1 but that in the absence of Tie1 kinase activity, Tie1 is unable to undergo any further autophosphorylation.

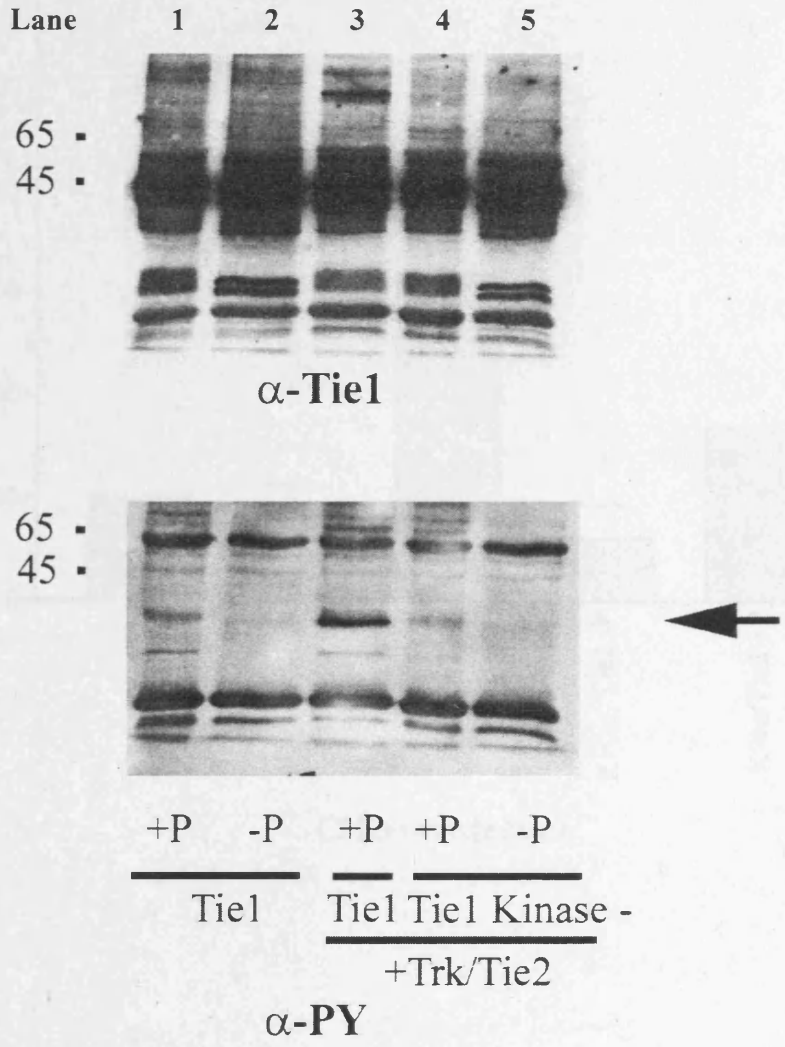


Fig. 3.3. **Tie2 activates tyrosine kinase activity of Tie1.** CHO cells were transfected with Tie1 endodomain (*pFLAGEndoB1^{WT}*) alone *(lanes 1 and 2)* and TrkA/Tie2 *(lane 3);* Tie1 kinase dead cDNA (*pFLAGEndoB1^{-}*) and TrkA/Tie2 *(lanes 4 and 5)* and treated with 5μg/ml pervanadate (+P) for 10 min. CHO cells were lysed and immunoprecipitated using FLAG monoclonal antibody M2. Proteins were separated by SDS-PAGE and Western blotted. Blots were probed with antibodies to the carboxyl terminus of Tie1 (*upper panel*) or anti-phosotyrosine (PY) (*lower panel*). Blots representative of at least three independent experiments are shown. The position of the Tie1 endodomain is indicated by an arrow. Relative mobility of molecular mass markers is indicated in kDa.

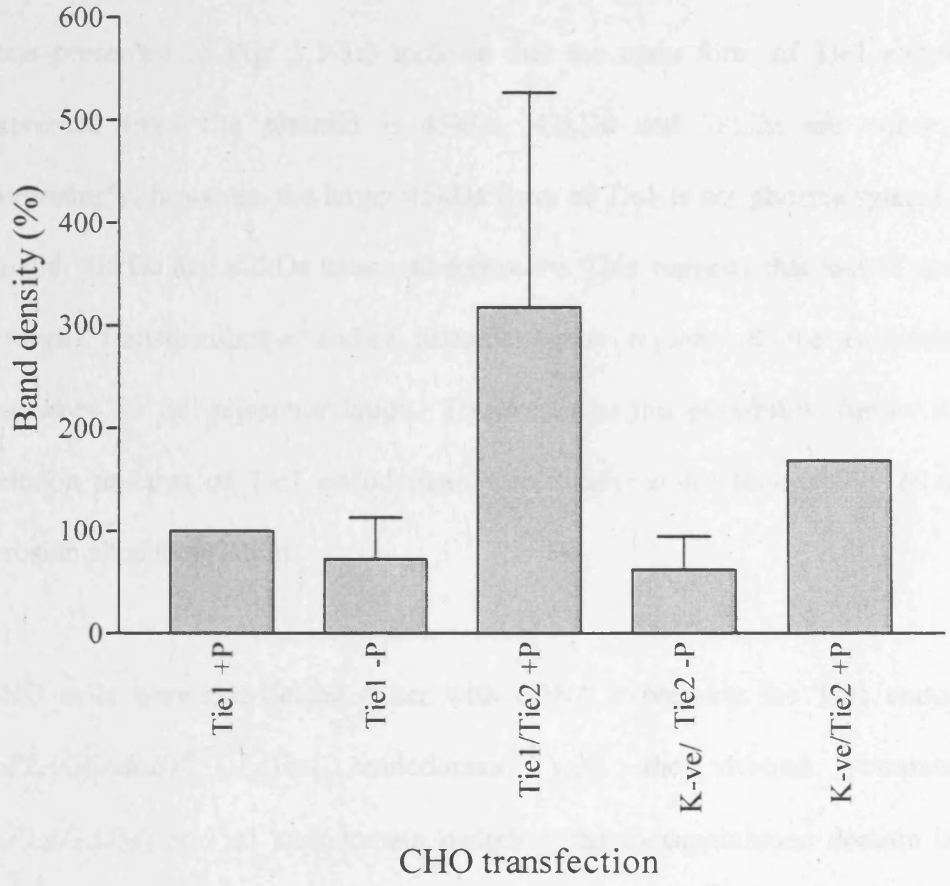


Fig 3.3b Tie-2 effect on Tie-1 kinase activity. CHO cells were transfected with Tie1 endodomain alone, Tie1 endodomain co-transfected with Tie2; Tie1 kinase dead cDNA co-transfected with Tie2 , in the presence (+P) or absence (-P) of 5μg/ml pervanadate for 10 min. CHO cells were lysed and immunoprecipitated using FLAG monoclonal antibody M2. Proteins were separated by SDS-PAGE and Western blotted. Blots were probed with antibodies to the carboxyl terminus of Tie1 or anti-phosphotyrosine. The relative level of tyrosine phosphorylation for each of the different transfected CHO cells was quantified by densitometric analysis for three independent experiments.

3.2.4 The intracellular domain is involved in Tie1 kinase activity

Data presented in Fig. 3.1-3.3 indicate that the main form of Tie1 endodomain expressed from the plasmid is 45kDa, 42kDa and 38kDa are minor forms. Surprisingly, however, the larger 45kDa form of Tie1 is not phosphorylated but the smaller 38kDa and 42kDa truncated forms are. This suggests that loss of the amino terminal transmembrane and/or juxtamembrane regions of the endodomain is necessary for full phosphorylation. To investigate this possibility, further different deletion mutants of Tie1 endodomain were analysed for their ability to undergo tyrosine phosphorylation.

CHO cells were transfected either with cDNA expressing the Tie1 endodomain (*pFLAGEndoB1*^{WT}), Tie1 endodomain with the deleted juxtamembrane (*pFLAGΔJM*) or Tie1 endodomain including the transmembrane domain in-frame with the leader sequence (*pSecTag TM+LS*). In addition to these transfections, CHO cells were co-transfected with the above cDNA, along with the chimeric receptor TrkA/Tie2. After 24 hours, all transfections/co-transfections were treated with pervanadate for 10min. Cells were lysed and immunoprecipitated using FLAG monoclonal antibody M2, for constructs expressing an amino FLAG epitope tag or a polyclonal antibody to the carboxyl terminus of Tie1. Proteins were separated on a 12% SDS-PAGE gel and Western blot analysis performed. Blots were probed for the expression and phosphorylation of the Tie1 receptor, using an anti-phosphotyrosine antibody and anti-Tie1 antibody.

Probing with the anti-Tie1 antibody (carboxyl terminus), resulted in the detection of the truncated form of Tie1 at 45 kDa and truncation products, in cells transfected with the Tie1 endodomain, both in the presence and absence of TrkA/Tie2 (Fig.3.4, upper panel, lanes 1 and 2). The *pFLAGΔJM* transfected cells exhibited a single ~30 kDa band corresponding to the construct in lane 4. In three independent experiments the *pFLAGΔJM* construct could not be detected unless co-expressed with TrkA/Tie2. In cells transfected with *pSecTag TM+LS* construct, the predominant form of Tie1 expressed was at ~55 kDa and truncation products of ~45 kDa and ~40 kDa were also seen (Fig. 3.4, lanes 5 and 6).

Examination of tyrosine phosphorylation of these expressed forms of Tie1 endodomain revealed little or no tyrosine phosphorylation of the full-length Tie1 endodomain in the absence or presence of TrkA/Tie2 (Fig 3.4, lanes 1,2,5 and 6) but detectable tyrosine phosphorylation of the ~30 kDa truncated form of Tie1. Taken together these data indicate that the presence of Tie1 transmembrane and juxtamembrane regions limit the ability of the receptor endodomain to become tyrosine phosphorylated.

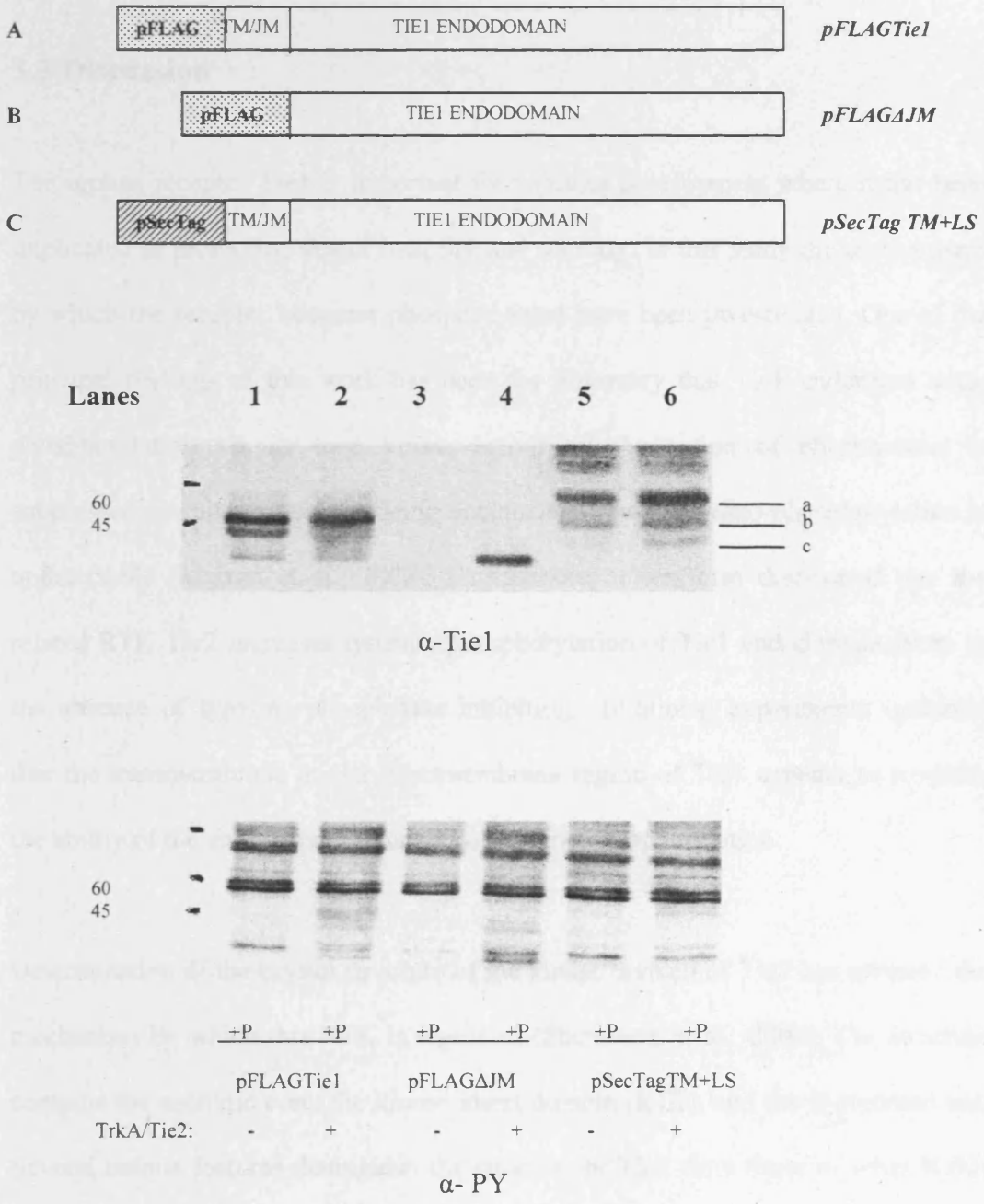


Fig.3.4 **The intracellular domain is involved in Tie1 kinase activity.** CHO cells were transfected either with cDNA expressing the Tie1 endodomain (*pFLAGTie1* construct A) *lane 1* , Tie1 endodomain with the deleted juxtamembrane (*pFLAGΔJM*; construct B), *lane 3* or Tie1 endodomain including the transmembrane domain in-frame with the leader sequence (*pSecTag TM+LS;* construct C), *lane 5*. Also co-transfected with TrkA/Tie2 *(lanes 2,4 and 6)* and treated with 5μg/ml pervanadate (+P) for 10 min. CHO cells were lysed and immunoprecipitated using FLAG monoclonal antibody M2. Proteins were separated by SDS-PAGE and Western blotted. Blots were probed with antibodies to the carboxyl terminus of Tie1 (*upper panel*) or anti-phosotyrosine (PY) (*lower panel*). Tie1 truncation products are indicated by letters *a* (45kDa), *b* (42kDa) and *c* (38kDa) Blots representative of at least three independent experiments are shown. Relative mobility of molecular mass markers is indicated in kDa.

3.3 Discussion

The orphan receptor Tie1 is important for vascular development where it has been implicated in promoting vessel integrity and stability. In this study the mechanisms by which the receptor becomes phosphorylated have been investigated. One of the principal findings of this work has been the discovery that Tie1 undergoes auto-phosphorylation via its own kinase activity if the action of phosphatases is suppressed; despite previously being documented that Tie1 auto-phosphorylation is undetectable (Marron et al., 2000). Furthermore, it was also discovered that the related RTK Tie2 increases tyrosine phosphorylation of Tie1 endodomain, even in the absence of tyrosine phosphatase inhibition. Additional experiments indicated that the transmembrane and/or juxtamembrane region of Tie1 appears to suppress the ability of the endodomain to undergo tyrosine phosphorylation.

Determination of the crystal structure of the kinase domain of Tie2 has revealed the mechanism by which this RTK is regulated (Shewchuk et al., 2000). The structure contains the catalytic core, the kinase insert domain (KID), and the C-terminal tail. Several unique features distinguish the structure of Tie2 from those of other RTKs whose structures are known. Namely the Tie2 nucleotide binding loop, this region of the receptor contains residues responsible for binding the triphosphate moiety of ATP in the correct position for catalysis (Johnson et al., 1996). It was discovered that the nucleotide-binding loop adopts a unique self-inhibitory conformation with residues 832-836 occupying the ATP binding site.

Secondary, the activation loop of the Tie2 structure has been shown to play an important role in its kinase activity. The activation loop, is a large flexible loop contained in protein kinases, the conformation of the activation loop is thought to regulate kinase activity. The activation loop in Tie2 adopts an 'activated like' conformation. The crystallographic structure of Tie2 revealed the end of the C-terminal tail blocks access to the substrate-binding site, in the absence of phosphorylation. And hence, the C-terminal must undergo a conformational change upon activation of the protein in order to expose both the substrate binding site and Y1101 and Y1112 for phosphorylation and signalling. The overall amino acid identity between Tie1 and Tie2 is 44% homology at the amino acid level and 76% homology in the intracellular domain therefore Tie1 probably has the same structure as Tie2 (Wilks, 1989; Partanen *et al.*, 1992).

It is possible that in Tie1 a single tyrosine residue might be responsible for relieving inhibition of Tie1 from a de-phosphorylated state, to become auto-phosphorylated, a theory that has been widely documented (Johnson et al, 1996, Shewchuk et al., 2000; Wybenga-Groot et al., 2001). For Tie2, tyr-897, located in the N-terminal domain, is thought to have a role in negatively regulating the activity of the receptor by preventing the dimerisation of the kinase domains or recruiting phosphatases when it is phosphorylated (Shewchuk et al., 2000). This scenario may have possibly been recreated when kinase-negative cDNA was used in conjunction with the TrkA/Tie1 chimeric receptor, in experiments described in this study.

Greater understanding to the mechanism by which auto-phosphorylation of Tie1 takes place is suggested by the data presented in this study. The theory for transphosphorylation of Tie1 by Tie2 is highly conceivable, for findings in this report have shown that in the presence of Tie2, the Tie1 receptor is repeatedly phosphorylated even without inhibition of tyrosine phosphatases, the extent to which Tie2 affects Tie1 phosphorylation cannot be better illustrated, than when Tie2 is not present, as this results in no/ negligible kinase activity. Subsequent experiments by Saharinen et al.(2005) have confirmed Tie2 can trans-activate Tie1. It has been shown that a chimeric form of Ang1 can stimulate Tie1 although the level of phosphorylation is less than that of Tie2, further that Tie2 enhances phosphorylation of Tie1; this finding is consistent with the data presented in this chapter (Saharinen et al., 2005).

It has previously been documented that Tie1 binding to Tie2 is mediated by the intracellular domains of the receptors i.e. juxtamembrane region, transmembrane domain and carboxyl terminus (Tsiamis et al, 2002). Data presented in this study examining the effect on Tie1 endodomain kinase activity, when the juxtamembrane region is deleted, showed that no phosphorylation of the endodomain occurs, despite tyrosine phosphatase suppression. However, when Tie2 is co-expressed, this led to phosphorylation of the endodomain.

The relevance of the Tie1: Tie2 complex in vascular development has been documented (Tsiamis et al., 2002) but moreover the role of *cross talk* in the co-ordination of regulating blood vessel formation during development and in postnatal

angiogenesis. The mechanism of cross talk, by which receptors elicit their cellular effects, is not exclusive to the Tie family of RTKs. Recently, it has been documented that the placental growth factor (PGF, also known as PIGF) regulates inter- and intramolecular cross talk between the VEGF receptor tyrosine kinases Flt-1 and Flk-1 (Autiero et al., 2003). Activation of Flt-1 by PGF resulted in intermolecular transphosphorylation of Flk-1; which causes the amplification of VEGF-driven angiogenesis through Flk-1. Furthermore, it was found that the VEGF/PGF heterodimer activated intramolecular VEGF receptor cross talk through formation of Flk1/Flk1 heterodimers.

To conclude, the findings in this study to date have in part, contributed to understanding how the orphan RTK Tie1 functions and signals. It has been revealed that the receptor does become auto-phosphorylated when tyrosine phosphatase are suppressed suggesting that Tie1 is a target for tyrosine phosphatases and that this is one of the mechanisms by which the phosphorylation state of the receptor is maintained at a low level.

It has also been shown in this study that Tie1 phosphorylation is stimulated by Tie2 and it is speculated that this occurs by transphosphorylation via hetero-oligomerisation, to form the Tie1: Tie2 complex. The intracellular domains of the Tie1 (as well as Tie2) have been documented to play a vital role in the formation of this complex but moreover, for the intracellular binding of Tie1 to Tie2. Also in this study, the influences of both the transmembrane domain and the juxtamembrane region have been investigated and indicate that the presence of these domains

suppresses phosphorylation of the Tie1 endodomain. For the receptor to have sufficient kinase activity, to bring about auto-phosphorylation, both the transmembrane domain and the juxtamembrane region are required. Subsequent to the formation of this complex, the two individual receptors, Tie1 and Tie2, communicate with each other via the concept of 'cross talk'. It is thought that this cross talk enables the two receptors to co-ordinate inter- and intermolecular signalling in endothelial cells; such signalling has yet to be elucidated. The co-ordination of cellular signalling, contributes to the control of both embryonic and postnatal vascular development, stability and maintenance during angiogenesis.

Chapter Four

Investigations into the signalling mechanisms of Tie1 following stimulation by the soluble Angiopoietin-1 protein, COMP-Ang1

4.1 Introduction

Tie1 signalling and function was initially explored in the previous chapter. It was discovered that Tie2 can stimulate autophosphorylation of Tie1 by trans-phosphorylating the endodomain of Tie1 and thus may induce receptor activation. In subsequent work by another group, it was found that full length Tie1 becomes trans-phorylated by Tie2 (Saharinen et al., 2005). Earlier attempts to activate Tie1 were unsuccessful; this was attributed to negligible kinase activity of the chimeric form of Tie1 used in the study (Marron et al., 2000). Indeed, in this thesis (refer to Chapter Three) the critical importance of Tie1 kinase activity in Tie1 signalling was investigated and was shown to be vital in order for the receptor to become activated; a similar finding, using a kinase inactive mutant form of Tie1, has also been demonstrated by Kontos et al. (2002).

Activation of Tie1 has previously only been achieved through use a of chimeric receptor system due to no ligand having been identified for Tie1 (Marron et al., 2000; Kontos et al., 2002). A limitation of the chimeric approach is that it required overexpression of the receptor in non-endothelial cells. Although providing useful insights into potential Tie1 signalling mechanisms and functions, additional work is required in endothelial cells to define physiologically relevant functions and signalling.

Recently, the possibility that the angiopoiteins may affect Tie1 function was reassessed, it was discovered that native Ang1 and Ang4 induced Tie1 tyrosine phosphorylation in

human dermal blood vascular endothelial cells (BEC) and in lymphatic endothelial cells (LEC). In addition to the ability of the native angiopoiteins to induce Tie1 tyrosine phosphorylation, it was found that a chimeric Ang1 protein, COMP-Ang1, also induced Tie1 tyrosine phosphorylation.

Cartilage oligomeric matrix protein (COMP) Ang1 was originally produced as an alternate to Ang1, as it was recognised that Ang1 was a suitable candidate for use in therapeutic angiogenesis by enhancing endothelial cell survival and preventing vascular leakage. Large-scale production of recombinant Ang1 has proven to be difficult due to the structure of Ang1, which results in aggregation and insolubility of the protein. The generation of COMP-Ang1 (Cho et al.,2004a; Cho et al., 2004b) based on the original structure of Ang1, provided a solution to the problem of aggregation and insolubility experienced with Ang1; the modified structure containing a minimal coiled-coil domain allows for oligomerisation of the protein but is short enough to overcome protein aggregation and insolubility. COMP-Ang1 has also been found to be more potent than native Ang1 in tyrosine phosphorylating both Tie2 (Cho et al., 2004) and Tie1 (Saharinen et al., 2005).

The aim of the studies described in this chapter was to investigate the effect of COMP-Ang1 on Tie1 signalling mechanisms.

4.2 Results

4.2.1 The effect of COMP Ang1 on Tie1 tyrosine phosphorylation

To confirm that COMP-Ang1 was biologically active, the ability of the ligand to stimulate Tie1 tyrosine phosphorylation was investigated. The effect of COMP-Ang1 on Tie1 tyrosine phosphorylation was investigated in two types of vascular endothelial cells, HUVEC and HMEC-1.

A 90% confluent monolayer of HUVE cells was serum-starved for 2 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2 as previously described by Saharinen et al., (2005). COMP-Ang1 used in experiments described in this thesis, was a gift from Gou Young Koh, Korea Advanced Institute of Science and Technology, Republic of Korea. Cells were then lysed as described in section 2.3.2. Phosphorylated tyrosine proteins were immunoprecipitated according to the immunoprecipitation protocol, as described in section 2.3.3.3, using a mouse monoclonal phospho-tyrosine specific antibody. For whole cell lysate (WCL) samples, an equal volume of 2x SB-DTT was added to each cell sample and boiled for 5 minutes. Proteins were then loaded onto a 12% SDS-PAGE gel, electrophoresed and the proteins were transferred to a nitrocellulose membrane and probed with a phospho-tyrosine antibody, for the detection of tyrosine-phosphorylated proteins. The nitrocellulose was then stripped and re-probed with an anti-Tie1 antibody for the detection of Tie1.

In experiments, using HUVEC cells, phosphorylated tyrosine proteins were detected at 130kDa, in both COMP-Ang1 treated and untreated samples (Figures 4.1 A and 4.2 A). The amount of tyrosine phosphorylation was identical for both treated and untreated cells. To determine whether the 130kDa protein was Tie1, the nitrocellulose membrane was stripped and re-probed with an anti-Tie1 antibody. Immunoblotting with an anti-Tie1 antibody suggested that the tyrosine phosphorylated protein of 130kDa, present in WCL and phospho-tyrosine immunoprecipitation samples (refer to Figures 4.1 B and 4.2 B respectively) was Tie1. To establish if the 130kDa protein was Tie1, immunoprecipitation of Tie1 was performed.

A 90% confluent monolayer of HMEC-1 cells was serum starved for 18 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. Cells were then lysed as described in section 2.3.2. Tie1 was immunoprecipitated following the protocol as given in section 2.3.3.1 of Materials and Methods chapter, using a mouse monoclonal anti-Tie1 antibody. Phosphorylated tyrosine proteins were detected by immunoblotting with an anti phospho-tyrosine antibody (Figures 4.3 A). Untreated and treated cells displayed a tyrosine phosphorylated protein of 130kDa; the level of tyrosine phosphorylation of this protein, for untreated and treated cells was identical. Following Tie1 immunoprecipitation and immunoblotting with an anti-Tie1 antibody, it was determined that the tyrosine phosphorylated protein was Tie1 (Figure 4.3 B).

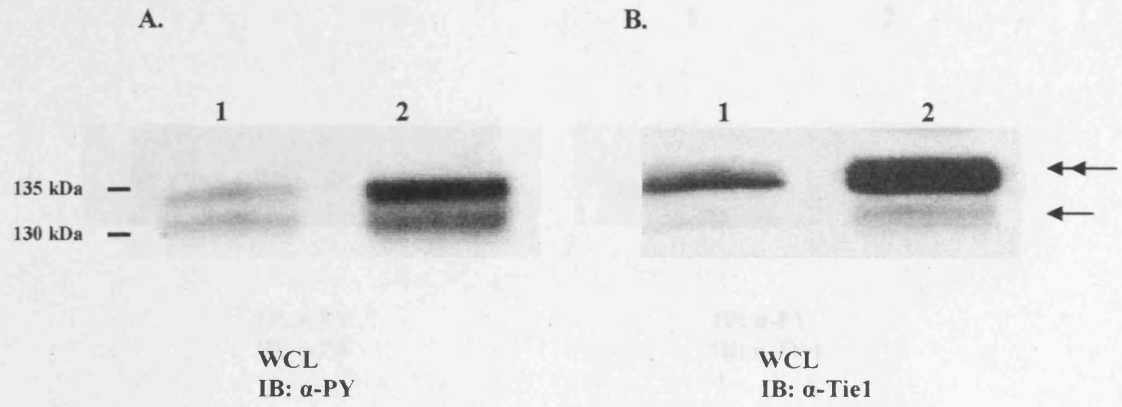


Figure 4.1 The effect of COMP-Ang1 stimulation on Tie1 phosphorylation. A 90% confluent monolayer of HUVE cells were serum-starved for 2 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. Whole cell lysate (WCL) samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected tyrosine phosphorylated protein at 130kDa (**A**). Tie1 was detected as a doublet at 130kDa and 135kDa, which represents the partial (single arrowhead) and fully glycosylated (double arrowhead) receptor, by immunoblotting with a Tie1 specific antibody (**B**). Figure shows results obtained for control HUVE cells (**1**) and COMP-Ang1 stimulated HUVE cells (**2**). Data represents three independent experiments.

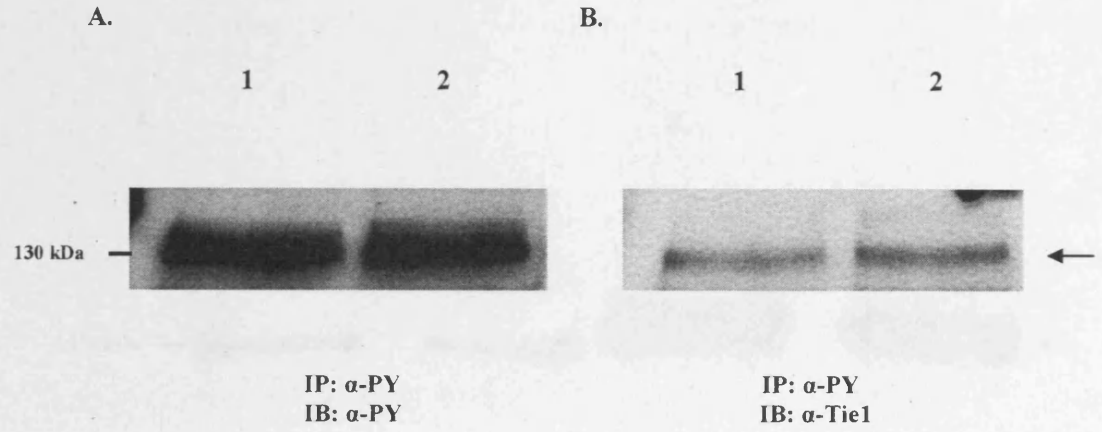


Figure 4.2 The effect of COMP-Ang1 stimulation on Tie1 phosphorylation. A 90% confluent monolayer of HUVE cells were serum-starved for 2 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. Immunoprecipitation (IP) with an anti phospho-tyrosine antibody (α-PY) was carried out and samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected tyrosine phosphorylated protein at 130kDa (**A**). Tie1 was detected at 130kDa by immunoblotting with a Tie1 specific antibody (**B**). Figure shows results obtained for control HUVE cells (**1**) and COMP-Ang1 stimulated HUVE cells (**2**). Data represents three independent experiments.

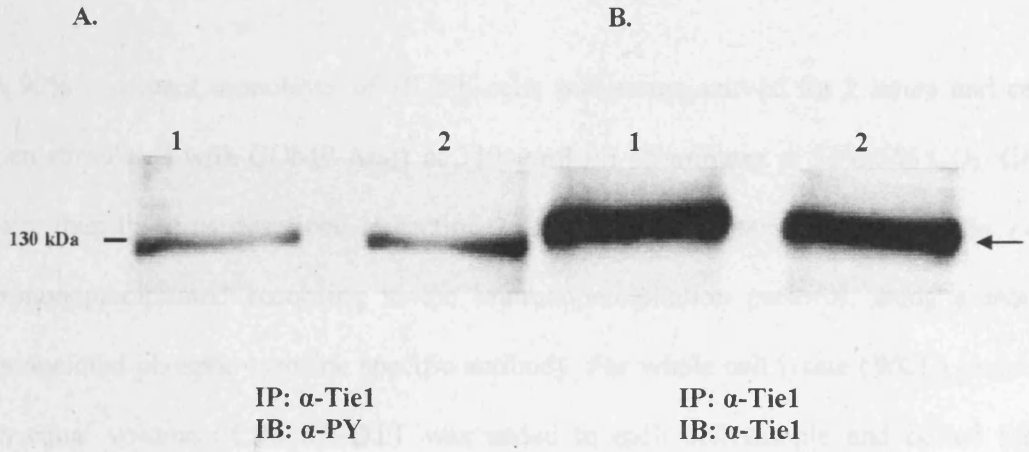


Figure 4.3 The effect of COMP-Ang1 stimulation on Tie1 phosphorylation. A 90% confluent monolayer of HMEC-1 cells were serum-starved for 18 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. Immunoprecipitation (IP) with a Tie1 specific antibody (α-Tie1) was carried out and samples were loaded on to a 7.5% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected tyrosine phosphorylated protein at 130kDa (**A**). Tie1 was detected at 130kDa by immunoblotting with a Tie1 specific antibody (**B**). Control HMEC-1 cells (**1**), COMP-Ang1 stimulated HMEC-1 cells (**2**). Data represents three independent experiments.

4.2.2 The effect of COMP Ang1 on Tie2 tyrosine phosphorylation

To confirm that COMP-Ang1 was biologically active, the ability of the ligand to stimulate Tie2 tyrosine phosphorylation was examined. The effect of COMP-Ang1 on Tie2 tyrosine phosphorylation was investigated in two types of vascular endothelial cells, HUVEC and HMEC-1.

A 90% confluent monolayer of HUVE cells was serum-starved for 2 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. Cells were then lysed as described in section 2.3.2. Tyrosine-phosphorylated proteins were immunoprecipitated according to the immunoprecipitation protocol, using a mouse monoclonal phospho-tyrosine specific antibody. For whole cell lysate (WCL) samples, an equal volume of 2x SB-DTT was added to each cell sample and boiled for 5 minutes. Proteins were then loaded onto a 12% SDS-PAGE gel, electrophoresed followed by protein transfer to a nitrocellulose membrane and probed with a phospho-tyrosine antibody, for the detection of tyrosine phosphorylated proteins. The nitrocellulose was then stripped and re-probed with an anti-Tie2 antibody for the detection of Tie2.

As previously described for experiments examining the effects of COMP-Ang1 on tyrosine phosphorylation of Tie1; in experiments using HUVEC cells, phosphorylated tyrosine proteins were detected at 145kDa, in both COMP-Ang1 treated and untreated samples (Figures 4.4 A and 4.5 A). The amount of tyrosine phosphorylation was

identical for both treated and untreated cells. To determine whether the 145kDa protein was Tie2, the nitrocellulose membrane was stripped and re-probed with an anti-Tie2 antibody. Immunoblotting with an anti-Tie2 antibody suggested that the tyrosine phosphorylated protein of 145kDa, present in WCL and phospho-tyrosine immunoprecipitation samples (refer to Figures 4.4 B and 4.5 B respectively) was Tie2. Tyrosine phosphorylation detected in WCL experiments represents the overall level of tyrosine phosphorylation in the cell, hence further confirmation as to whether the tyrosine phosphorylation detected corresponded to Tie2 was needed, as this would suggest overall Tie2 phosphorylation. For this reason immunoprecipitation of Tie2 was performed.

A 90% confluent monolayer of HUVE cells was serum-starved for 2 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. Cells were then lysed as described in section 2.3.2. Immunoprecipitation of Tie2 was carried out according to the immunoprecipitation protocol, using a rabbit anti-Tie2 specific antibody, (refer to section 2.3.3.2). For whole cell lysate (WCL) samples, an equal volume of 2x SB-DTT was added to each cell sample and boiled for 5 minutes. Proteins were then loaded onto a 7.5% SDS-PAGE gel, electrophoresed followed by protein transfer to a nitrocellulose membrane and probed with a phospho-tyrosine antibody, for the detection of tyrosine phosphorylated proteins. The nitrocellulose was then stripped and re-probed with an anti-Tie2 antibody for the detection of Tie2.

Untreated and treated cells displayed a tyrosine phosphorylated protein of 145kDa; the level of tyrosine phosphorylation of this 145kDa protein was greater for HUVEC cells treated with COMP-Ang1 compared to that of untreated HUVEC cells (Figure 4.6 A). Following Tie2 immunoprecipitation and immunoblotting with an anti-Tie2 antibody, it was determined that the tyrosine phosphorylated protein corresponded to Tie2 (Figure 4.6 B).

In summary, the results obtained from experiments in both HUVEC and HMEC-1 vascular endothelial cells to examine the effects of COMP-Ang1 on Tie1 and Tie2, suggest that COMP-Ang1 stimulates tyrosine phosphorylation of Tie1 and Tie2.

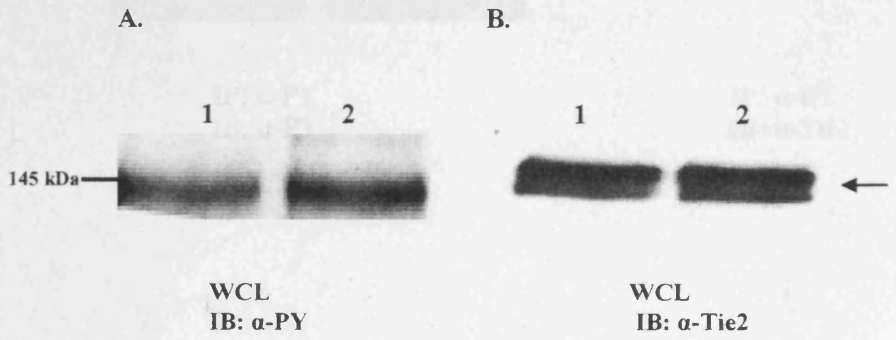


Figure 4.4 The effect of COMP-Ang1 stimulation on Tie2 phosphorylation. A 90% confluent monolayer of HUVE cells were serum-starved for 2 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. Whole cell lysate (WCL) samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected tyrosine phosphorylated protein at 145kDa (**A**). Tie2 was detected by immunoblotting with a Tie2 specific antibody (**B**). Control HUVE cells (**1**) and COMP-Ang1 stimulated HUVE cells (**2**). Data represents three independent experiments.

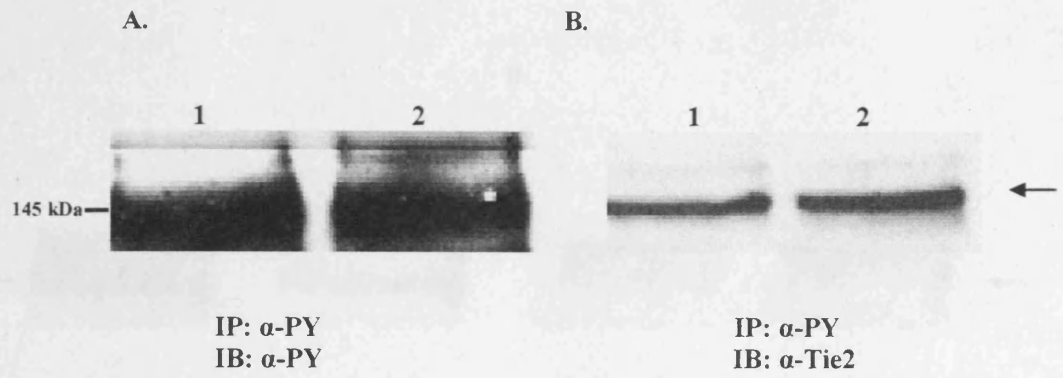


Figure 4.5 The effect of COMP-Ang1 stimulation on Tie2 phosphorylation. A 90% confluent monolayer of HUVE cells were serum-starved for 2 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. Immunoprecipitation (IP) with a phospho-tyrosine antibody (α-PY) was carried out and samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected tyrosine phosphorylated protein at 145kDa (**A**). Tie2 was detected by immunoblotting with a Tie2 specific antibody (**B**). Control HUVE cells (**1**) and COMP-Ang1 stimulated HUVE cells (**2**). Data represents three independent experiments.

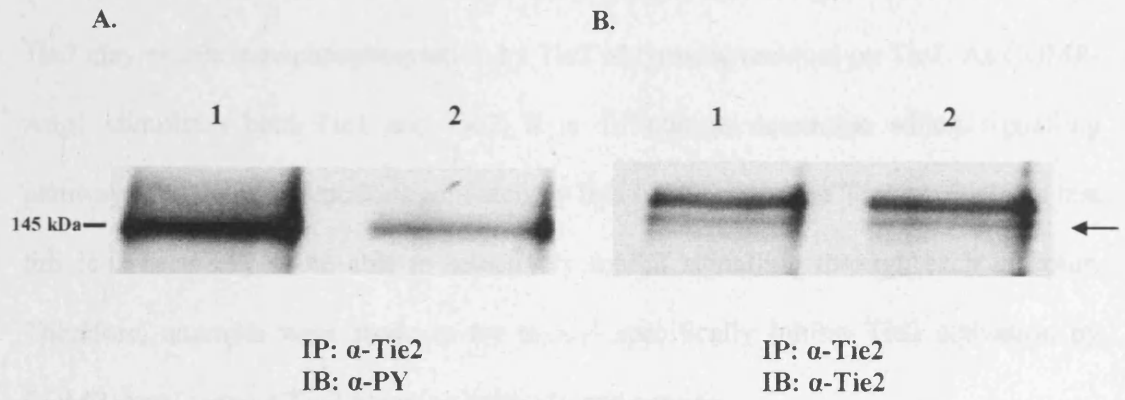


Figure 4.6 The effect of COMP-Ang1 stimulation on Tie2 phosphorylation. A 90% confluent monolayer of HUVE cells were serum-starved for 18 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. Immunoprecipitation (IP) with a Tie2 specific antibody (α-Tie2) was carried out and samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected tyrosine phosphorylated protein at 145kDa (**A**). Tie2 was detected by immunoblotting with a Tie2 specific antibody (**B**). COMP-Ang1 stimulated HUVE cells (**1**) and control HUVE cells (**2**). Data represents three independent experiments.

4.2.3 Blockade of Tie2 to investigate Tie1 activation

The Tie receptors are present in endothelial cells as pre-formed heteromeric complexes (Marron et al., 2000; Saharinen et al., 2005) this physical interaction between Tie1 and Tie2 may enable transphosphorylation by Tie2 of tyrosine residues on Tie1. As COMP-Ang1 stimulates both Tie1 and Tie2, it is difficult to determine which signalling pathways and cellular functions activated by this ligand occur via Tie1 or Tie2. To test this it is necessary to be able to selectively inhibit signalling through each receptor. Therefore, attempts were made to try to and specifically inhibit Tie2 activation by COMP-Ang1 using a Tie2 blocking antibody and peptide.

4.2.3.1 Tie2 blocking antibody

To investigate Tie1 signalling in endothelial cells, a polyclonal goat antibody specific for Tie2 was used to try to inhibit Tie2 activation by preventing COMP-Ang1 binding to Tie2. The antibody (AF313, goat polyclonal antibody) was chosen as it was claimed by the manufacturer (R&D) to inhibit angiopoietin binding to Tie2.

HUVE cells were serum starved overnight in serum free MEM 199 media. Following overnight serum-starvation, the cell media was aspirated off the cells and washed gently in sterile PBS. Serum-free MEM 199 media was then replaced and the cells incubated with a polyclonal goat antibody specific for Tie2 10μg/ml, for 2 hours at 37°C/5% CO_2

The cells were then stimulated with COMP-Ang1 at 340ng/ml for 15 minutes at 37°C/5% CO_2. For whole cell lysates (WCL), cells were lysed with an equal volume of 2x SB DTT and boiled for 5 minutes. Tie2 immunoprecipitation was carried out as detailed in section 2.3.3.2 using the same polyclonal goat anti-Tie2 antibody. Equal amounts of protein were loaded onto a 12% SDS-PAGE gel and electrolysed. The proteins were then transferred onto nitrocellulose membrane by Western blotting. Tyrosine phosphorylated proteins were detected by immunoblotting of the nitrocellulose membrane with an anti phospho-tyrosine antibody. The nitrocellulose membrane was stripped and re-probed with an anti-Tie2 antibody for the detection of Tie2 protein.

Analysis of the results obtained from whole cell lysates revealed phosphorylated tyrosine proteins detected at 145kDa in all three of the treatment conditions (Figure 4.7 A). The amount of tyrosine phosphorylation was the same in all three of the treatment conditions. To determine if the 145kDa tyrosine phosphorylated protein was Tie2, the nitrocellulose membrane was stripped and re-probed with an anti-Tie2 antibody. Immunoblotting with an anti-Tie2 antibody confirmed the tyrosine phosphorylated protein of a molecular mass of 145kDa was Tie2 (Figure 4.7 B). Further confirmation was obtained from immunoprecipitation of Tie2 (Figure 4.8). HMEC-1 cells stimulated with COMP-Ang1 were tyrosine phosphorylated, close inspection of the level of tyrosine phosphorylation, indicated that the level of tyrosine phosphorylation was

identical in both COMP-Ang1 treated samples, despite one of the samples having been treated with the Tie2 blocking antibody.

In summary, for cells treated with Tie2 blocking antibody, the ability of COMP-Ang1 to tyrosine phosphorylate the receptor was maintained and subsequently Tie2 was activated. This finding confirms that the attempt to block Tie2 activation by use of a Tie2 specific polyclonal goat antibody was unsuccessful.

Attempts to block the activation of Tie2 using the anti-Tie2 antibody were carried out more than three times in HUVE and HMEC-1 cells, despite this, Tie2 blockade was never achieved. For this reason, another method to intervene in the activation of Tie2 was employed.

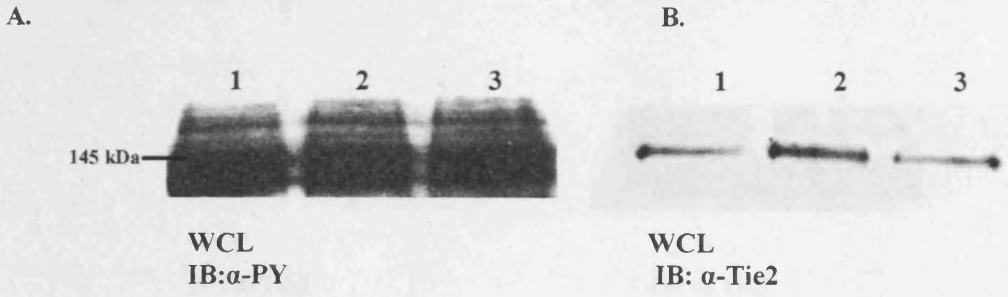


Figure 4.7 Attempt to block Tie2 to investigate Tie1 activation. HUVE cells were serum-starved overnight, serum-free MEM 199 medium was replaced and the cells incubated with a polyclonal goat antibody specific for Tie2 10μg/ml, for 2 hours at 37°C/5% CO_2. The cells were then stimulated with COMP-Ang1 at 340ng/ml for 15 minutes at 37°C/5% CO_2. Whole cell lysate (WCL) samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected tyrosine phosphorylated protein at 145kDa (**A**). Tie2 was detected by immunoblotting with a Tie2 specific antibody (**B**). Control HUVE cells (**1**); COMP-Ang1 stimulated HUVE cells alone (**2**); COMP-Ang1 + Tie2 blocking antibody stimulated HUVE cells (**3**). Data represents three independent experiments.

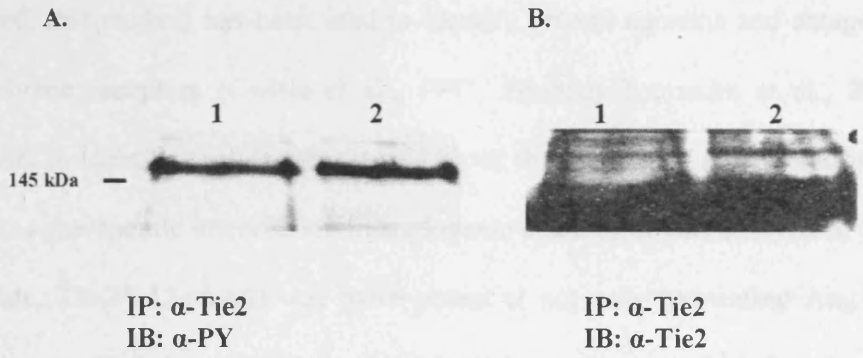


Figure 4.8 Attempt to block Tie2 transphosphorylation to investigate Tie1 signalling. HUVE cells were serum starved overnight, serum-free MEM 199 medium was replaced and the cells incubated with a polyclonal goat antibody specific for Tie2 10μg/ml, for 2 hours at 37°C/5% CO_2. The cells were then stimulated with COMP-Ang1 at 340ng/ml for 15 minutes at 37°C/5% CO_2. Immunoprecipitation (IP) with a Tie2 specific antibody (α-Tie2) was carried out and samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected tyrosine phosphorylated protein at 145kDa (**A**). Tie2 was detected by immunoblotting with a Tie2 specific antibody (**B**). COMP-Ang1 stimulated HUVE cells alone (**1**); COMP-Ang1 + Tie2 blocking antibody stimulated HUVE cells (**2**). Data represents three independent experiments.

4.2.3.2 Tie2 blocking peptide

The screening of phage-displayed libraries is a powerful method for identifying peptides that mimic protein surfaces and display target selectivity (Smith, 1985). Indeed, this method has been used to identify several agonists and antagonists of cell membrane receptors (Cwirla et al., 1997; Binétruy-Tournaire et al., 2000). In the attempt to identify peptides that would block the binding of angiopoietins to Tie2, for use as a therapeutic intervention in angiogenic diseases; it was discovered that one such peptide, T4-(NLLMAAS) was more potent at not only preventing Ang1 and Ang-2 binding to Tie2 but specifically inhibited the activation of Tie2 induced by Ang1 (Tournaire et al., 2004). Based on this evidence, the T4-(NLLMAAS) peptide was used in the attempt to block the activation of Tie2 in order to explore Tie1 signalling, independent of Tie2.

HUVE cells were grown to 90% confluence in 80cm^2 flasks and were serum starved for 2 hours at 37°C/5% CO_2 in serum free MEM 199 media. The peptide T4-(NLLMAAS), 0.5mM was added for the second hour before the addition of COMP-Ang1 (340ng/ml) for a further 45 minutes at 37°C/5% CO_2. Cells that were not treated with the peptide T4-(NLLMAAS) but only treated with COMP-Ang1, were serum starved for 2 hours and then treated with COMP-Ang1 for 45 minutes at 37°C/5% CO_2. Cells were lysed as described in Section 2.3.2. Immunoprecipitation of Tie2 was carried using a goat polyclonal anti-Tie2 antibody following the protocol detailed in Section 2.3.3. Proteins

were loaded on to a 12% SDS-PAGE gel and electrophoresed. The proteins were then transferred onto a nitrocellulose membrane by Western blotting. Tyrosine phosphorylated proteins were detected by immunoblotting with a phospho-tyrosine antibody. The nitrocellulose membrane was stripped and re-probed with an anti-Tie2 antibody, for the detection of Tie2.

In whole cell lysate samples, tyrosine phosphorylation of a protein with a molecular mass of 145kDa was detected; noticeably cells stimulated with COMP-Ang1 had greater tyrosine phosphorylation at 145kDa than control HUVE cells. Tyrosine phosphorylation for cells treated with the Tie2 blocking peptide, exhibited the same level of tyrosine phosphorylation as cells without Tie2 blocking peptide treatment (Figure 4.9 A). To confirm whether the tyrosine phosphorylation of the 145kDa was Tie2, the nitrocellulose membrane was stripped and re-probed with an anti-Tie2 antibody. Tie2 was detected at 145kDa for all samples; this observation therefore showed that Tie2 tyrosine phosphorylation was greater for cells stimulated with COMP-Ang1. Furthermore, that treatment with the Tie2 blocking peptide had not suppressed Tie2 activity.

In further experiments in HUVE cells, immunoprecipitation of Tie2 was carried out and tyrosine phosphorylation examined, to confirm further if Tie2 activity, following treatment with the Tie2 blocking peptide had been suppressed. Tyrosine phosphorylation was detected with an anti phospho-tyrosine antibody. As previously seen in experiments for WCL, tyrosine phosphorylation of a protein with a molecular

mass of 145kDa was detected and that this protein showed greatly more tyrosine phosphorylation in cells that had been stimulated with COMP-Ang1. These data gave further confirmation that Tie2 activity had not been suppressed in cells treated with the Tie2 blocking peptide, as tyrosine phosphorylation was identical to that of cells which had only been stimulated with COMP-Ang1 (Figure 4.10). Results shown are representative of four experiments.

To summarise, the attempt to block Tie2 activity using the peptide T4-(NLLMAAS) was made in HUVE cells, the data obtained from these experiments confirmed that Tie2 activity was upheld and that the peptide did not block Tie2 activity. Secondary to this observation, it was further shown that COMP-Ang1 induced tyrosine phosphorylation of Tie2 is greater than endogenous tyrosine phosphorylation of Tie2. These data are consistent with the work in human dermal blood vascular endothelial cells (BEC) and lymphatic endothelial cells (LEC) (Saharinen et al., 2005). It was therefore not possible to inhibit Tie2, using this approach. This prevented determination of the role of Tie2 in stimulating tie1 phosphorylation in endothelial cells. Furthermore, it was therefore not possible to examine downstream signalling for Tie1 in the absence of Tie2 activation.

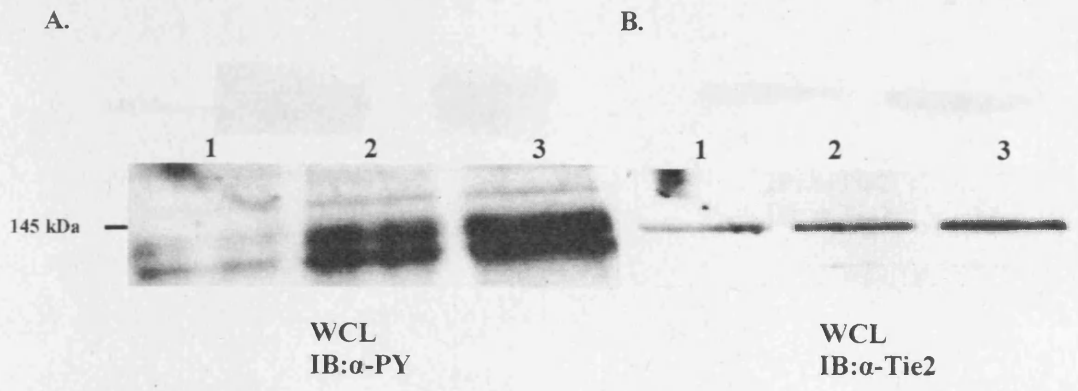


Figure 4.9 Attempt to block Tie2 to investigate Tie1 activation. HUVE cells were serum-starved overnight, serum-free MEM 199 medium was replaced and the cells incubated with a polyclonal goat antibody specific for Tie2 10µg/ml, for 2 hours at 37°C/5% CO_2. The cells were then stimulated with COMP-Ang1 at 340ng/ml for 15 minutes at 37°C/5%CO_2. Whole cell lysate (WCL) samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected phosphorylated tyrosine protein at 145 kDa (**A**). Tie2 was detected by immunoblotting with a Tie2 specific antibody (**B**). Control HUVE cells (**1**); COMP-Ang1 stimulated HUVE cells alone (**2**); COMP-Ang1+ Tie2 blocking peptide stimulated HUVE cells (**3**). Data represents three independent experiments.

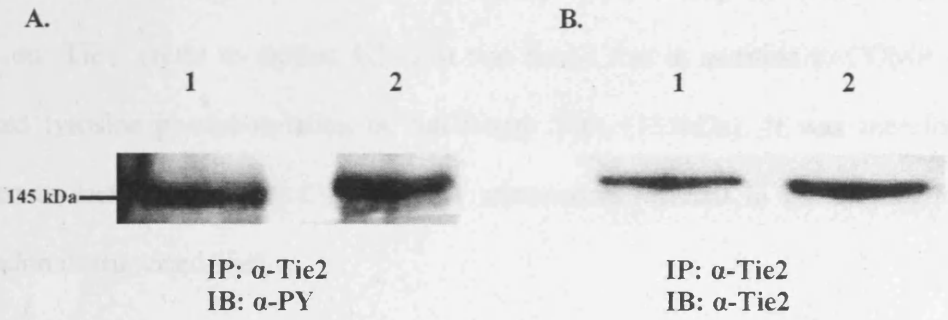


Figure 4.10 Attempt to block Tie2 to investigate Tie1 activation. HUVE cells were serum-starved overnight, serum-free MEM 199 medium was replaced and the cells incubated with a polyclonal goat antibody specific for Tie2 10μg/ml, for 2 hours at 37°C/5% CO_2. The cells were then stimulated with COMP-Ang1 at 340ng/ml for 15 minutes at 37°C/5% CO_2. Immunoprecipitation (IP) with a Tie2 specific antibody (α-Tie2) was carried out and samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected tyrosine phosphorylated protein at 145kDa (**A**). Tie2 was detected by immunoblotting with a Tie2 specific antibody (**B**). COMP-Ang1 stimulated HUVE cells (**1**); COMP-Ang1 + Tie2 blocking peptide stimulated HUVE cells (**2**). Data represents three independent experiments.

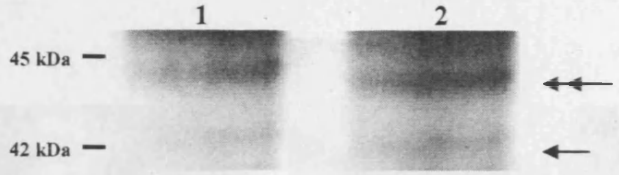
4.2.4 Effect of COMP-Ang1 on Tie1 truncation & tyrosine phosphorylation of Tie1 intracellular domain (TICD)

In initial experiments, described within this chapter examining the effect of COMP-Ang1 on Tie1 (refer to section 4.2.1). it was found that in addition to COMP-Ang1 induced tyrosine phosphorylation of full-length Tie1 (135kDa). It was therefore of interest to examine whether COMP-Ang1 stimulation resulted in the stimulation or inhibition of truncated Tie1.

To do this a 90% confluent monolayer of HUVE cells were serum starved for 2 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. For whole cell lysate (WCL) samples, an equal volume of 2x SB-DTT was added to each cell sample and boiled for 5 minutes. Proteins were then loaded onto a 12% SDS-PAGE gel, electrophoresed followed by protein transfer to a nitrocellulose membrane and probed with a mouse monoclonal anti-Tie1 specific antibody. The data obtained shows that COMP-Ang1 stimulation does not affect Tie1 truncation in endothelial cells (Figure 4.11).

For protein immunoprecipitation, a 90% confluent monolayer of HUVE cells was serum starved for 2 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. Cells were then lysed as described in section 2.3.2. Phosphorylated tyrosine proteins were immunoprecipitated according to the immunoprecipitation protocol, as described in section 2.3.3.3, using a mouse

monoclonal specific antibody. Proteins were then loaded onto a 12% SDS-PAGE gel, electrophoresed and the proteins were transferred to a nitrocellulose membrane and probed with a phospho-tyrosine antibody, for the detection of tyrosine-phosphorylated proteins. The nitrocellulose was then stripped and re-probed with an anti-Tie1 antibody for the detection of Tie1. It was observed that for cells stimulated with COMP-Ang1, tyrosine phosphorylation of truncated Tie1 was greater than cells that had not been stimulated with COMP-Ang1 (Figure 4.12). Subsequent to this observation, further experiments were carried out.



WCL
IB: α-Tie1

Figure 4.11 Effect of COMP-Ang1 on Tie1 truncation of Tie1 intracellular domain (T1CD). A 90% confluent monolayer of HUVE cells were serum-starved for 2 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. Whole cell lysate (WCL) samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Tie1 was detected at 45kDa (double arrowhead) and at 42kDa (single arrowhead) by immunoblotting (**IB**) with a Tie1 specific antibody. Figure shows results obtained for control HUVE cells (**1**) and COMP-Ang1 stimulated HUVE cells (**2**). Data represents three independent experiments.

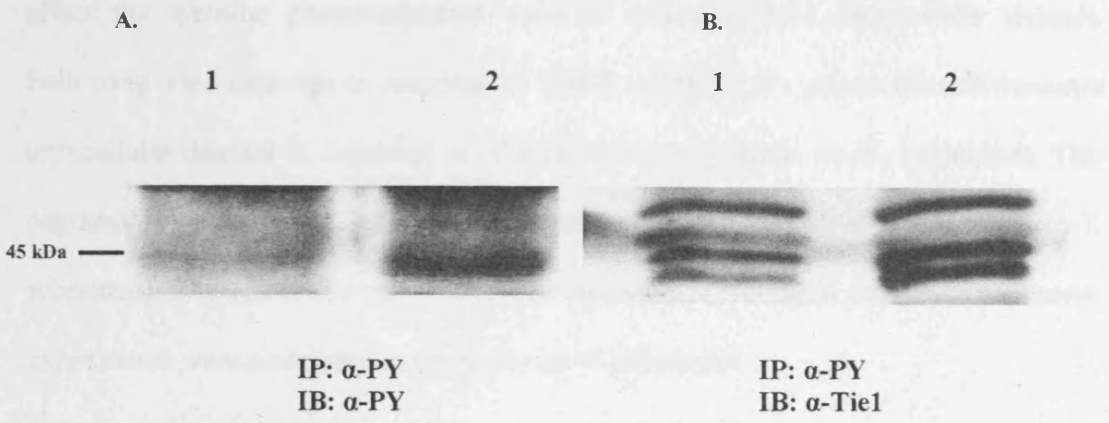


Figure 4.12 Effect of COMP-Ang1 on Tie1 truncation: tyrosine phosphorylation of Tie1 intracellular domain (TICD). A 90% confluent monolayer of HUVE cells were serum-starved for 2 hours and cells then stimulated with COMP-Ang1 at 340ng/ml for 45 minutes at 37°C/5% CO_2. Immunoprecipitation with an anti phospho-tyrosine antibody was carried out and samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected tyrosine phosphorylated proteins at 42kDa and 45kDa (**A**). Tie1 was detected by immunoblotting with a Tie1 specific antibody (**B**). Figure shows results obtained for control HUVE cells (**1**) and COMP-Ang1 stimulated HUVE cells (**2**). Data represents three independent experiments.

To date, the effect of COMP-Ang1 on Tie1 intracellular domain has not been documented. It was of interest therefore, to determine whether COMP-Ang1 could affect the tyrosine phosphorylation state of truncated Tie1 intracellular domain. Following Tie1 cleavage in response to VEGF or PMA, the newly formed truncated intracellular domain is degraded via the proteosome (Marron et al., submitted). This degradation is inhibited by the proteosomal inhibitor lactacystin (Marron et al., submitted). Therefore, in order to enhance detection of truncated intracellular domain, experiments were performed in the presence of lactacystin.

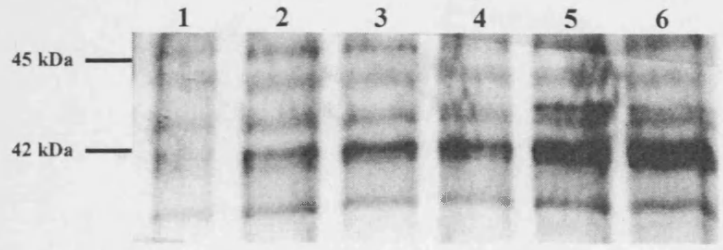
HUVE cells were grown to 90% confluence in six-well cell culture plates. Complete medium was replaced with serum-free MEM 199 media and cells were serum starved overnight whilst in the presence of 0.1mM lactacystin at 37°C/5% CO_2 .The cells were then washed in sterile PBS and serum-starved for 2 hours at 37°C/5% CO_2. After 2 hours, the cells were then stimulated with COMP-Ang1 at 340ng/ml over a 60-minute time-course at 37°C/5% CO_2. Cells were then lysed as described in Section 2.3.2. Proteins were loaded on to a 12% SDS-PAGE gel and electrolysed. The proteins were then transferred onto a nitrocellulose membrane by Western blotting. Tyrosine phosphorylated proteins were detected by immunoblotting with a phospho-tyrosine antibody. The nitrocellulose membrane was stripped and re-probed with an anti-Tie1 antibody, for the detection of Tie1.

Immunoblotting with an anti phospho-tyrosine antibody detected tyrosine phosphorylated proteins of a molecular mass of 45-38kDa, in all samples that had been stimulated with COMP-Ang1. Negligible tyrosine phosphorylation was detected in control HUVE cells. Whilst tyrosine phosphorylation of protein at 45kDa was consistent throughout the time course, tyrosine phosphorylation of a protein at 42kDa was markedly greater and as the time course progressed, the level of tyrosine phosphorylation of the 42kDa protein increased (Figure 4.13 A).

The nitrocellulose membrane was stripped and re-probed with an anti-Tie1 antibody (Figure 4.13 B). Proteins were detected by the Tie1 antibody at 45-38kDa which corresponded to the tyrosine phosphorylated proteins previously mentioned, as shown in Figure 4.13 A. The level of the 42kDa protein remained constant throughout the time course, at 10 and 30 minutes of COMP-Ang1 stimulation, the level 42kDa protein did not increase. This observation was interesting as it indicated that the increase (occurring with 10 and 30 minutes after COMP-Ang1 stimulation) in tyrosine phosphorylation of the 42kDa protein was not due to an increase in protein, but exclusively due to an increase in tyrosine phosphorylation. To further confirm the increase in tyrosine phosphorylation, the density of tyrosine phosphorylation relative to Tie1 protein over the 60-minute time course was quantified (Figure 4.13 C). As shown in Figure 4.13 C, tyrosine phosphorylation at the start of the time course increases gradually with a marked increase between 5 and 10 minutes of COMP-Ang1 stimulation. Following this 3-fold increase in tyrosine phosphorylation, a plateau between 10 and 30 minutes occurs. Tyrosine phosphorylation gradually decreases between 30 and 60 minutes to a

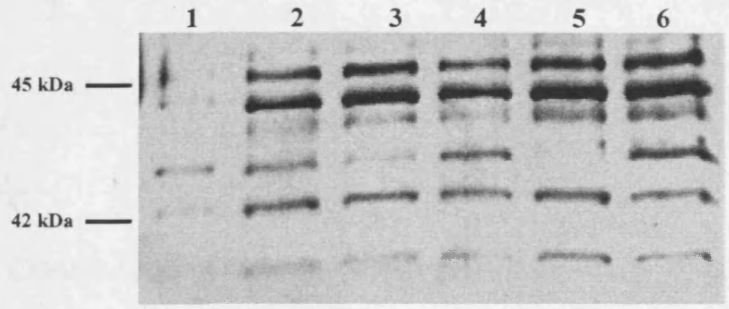
level of tyrosine phosphorylation comparable to levels seen at the start of the time course hence 5 minutes after COMP-Ang1 stimulation.

A.



WCL
IB: α-PY

B.



WCL
IB: α- Tie1

Figure 4.13 Time course of COMP-Ang1 stimulation of truncated Tie1 in endothelial cells. HUVE cells were grown to 90% confluence in six-well cell culture plates. Complete medium was replaced with serum-free MEM 199 medium and cells were serum-starved overnight with 0.1mM lactacystin at 37°C/5% CO_2 After 2 hours, cells were stimulated with COMP-Ang1 at 340ng/ml over a 60-minute time course at 37°C/5% CO_2. Whole cell lysate (WCL) samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected tyrosine phosphorylated protein at 45kDa and 42kDa (**A**). Truncated Tie1 and protein was detected by immunoblotting with a Tie1 specific antibody (**B**). Control HUVE cells, 0 minutes (**1**); 1 minute (**2**); 5 minutes (**3**); 10 minutes (**4**); 30 minutes (**5**) and 60 minutes (**6**). Data represents three independent experiments.

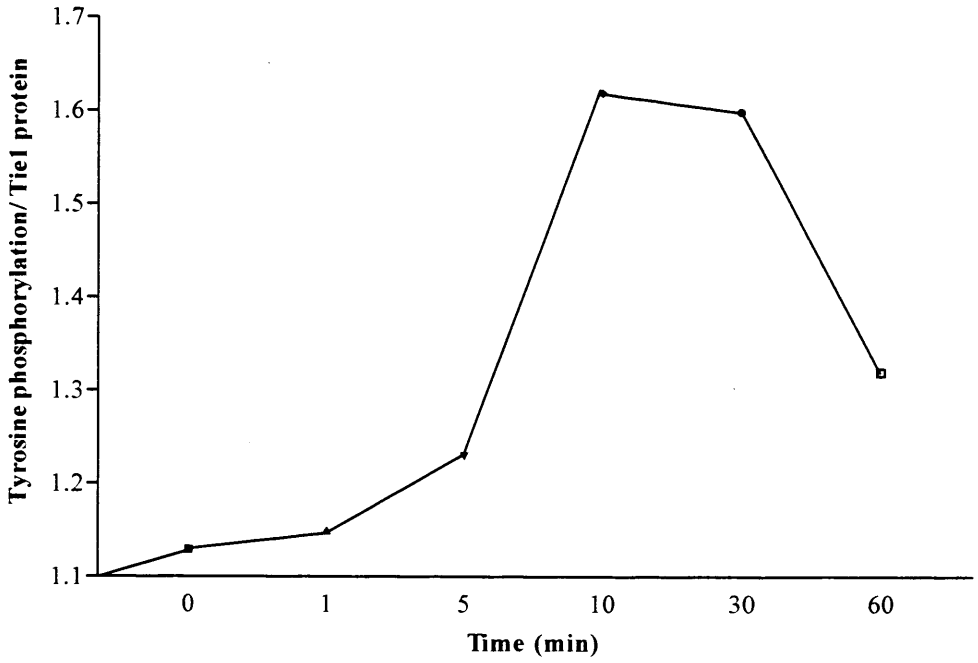


Figure 4.13 C

Quantified COMP-Ang1 induced tyrosine phosphorylation of 42kDa protein in HUVE cells. Tyrosine phosphorylation detected at 42kDa by immunoblotting with an anti phospho-tyrosine antibody was densitometrically analysed. Tie1 protein detected at 42kDa by immunoblotting with an anti-Tie1 specific antibody was also densitometrically analysed. Tyrosine phosphorylation was calculated relative to Tie1 protein (tyrosine phosphorylation/Tie1 protein). Maximal tyrosine phosphorylation of the 42kDa protein occurs within 10 minutes of COMP-Ang1 stimulation. Figure is representative of a single experiment for data presented in Figure 4.13.

4.2.5 Effects of γ-secretase inhibitor on tyrosine phosphorylation of the 42kDa protein

Following initial cleavage of Tie1, the transmembrane and intracellular domain undergo a secondary cleavage which is mediated by γ-secretase. This secondary cleavage event results in a further truncated form of Tie1 intracellular domain (ICD); this newly truncated protein is approximated to have a molecular mass of 42kDa. The phosphorylated protein observed after COMP-Ang1 stimulation may correspond to the 42kDa protein produced by Tie1 cleavage. Therefore, as a further test to determine whether the 42kDa phosphorylated protein is Tie1, the effects of inhibiting γ-secretase were tested.

HUVE cells were grown to 90% confluence in six-well cell culture plates. Complete media was replaced with serum-free MEM 199 media and cells were serum starved overnight in the presence of 0.1mM lactacystin at 37°C/5% CO_2. Prior to the addition of 0.1mM lactacystin, the cells were pre-incubated with 20nM γ-secretase inhibitor (variant 458) for 5 hours in serum-free media at 37°C/5% CO_2. Following overnight treatment, serum-free media was replaced and cell treatments were replenished, before treating cells with 340ng/ml COMP-Ang1 for 45 minutes at 37°C/5% CO_2. Cells were then lysed as described in Section 2.3.2. Proteins were loaded onto a 12% SDS-PAGE gel and electrolysed. The proteins were then transferred onto a nitrocellulose membrane by Western blotting. Tyrosine phosphorylated proteins were detected by

immunoblotting with a phospho-tyrosine antibody. The nitrocellulose membrane was stripped and re-probed with an anti-Tie1 antibody, for the detection of Tie1.

Immunoblotting with an anti phospho-tyrosine antibody revealed several bands of molecular mass of 42-45kDa in all samples, regardless of γ-secretase inhibitor treatment. Tyrosine phosphorylation was the same in all samples (Figure 4.14 A). To confirm whether this 42kDa phospho-protein could be related to Tie1, the nitrocellulose membrane was then stripped and re-probed with an anti-Tie1 antibody. For cells not treated with the γ-secretase inhibitor, proteins with a molecular mass of 45kDa and 42kDa were detected. For cells treated with the γ-secretase inhibitor, proteins were also detected at 45kDa and 42kDa (Figure 4.14 B). These data suggest that the 42kDa protein is not truncated Tie1, since the secondary cleavage event that results in the truncated Tie1 product of 42kDa is mediated by γ-secretase and thus would be blocked following treatment with the γ-secretase inhibitor. Furthermore, the phospho-protein observed at 42 kDa does not signify tyrosine-phosphorylated Tie1.

In summary, COMP-Ang1 induced tyrosine phosphorylation of a 42kDa protein has been shown to occur in endothelial cells. Furthermore the tyrosine phosphorylation status of this protein is not affected following treatment with a γ-secretase inhibitor, thus the identity of the 42kDa phospho-protein remains to be elucidated. These findings are representative of three experiments carried out in HUVE cells.

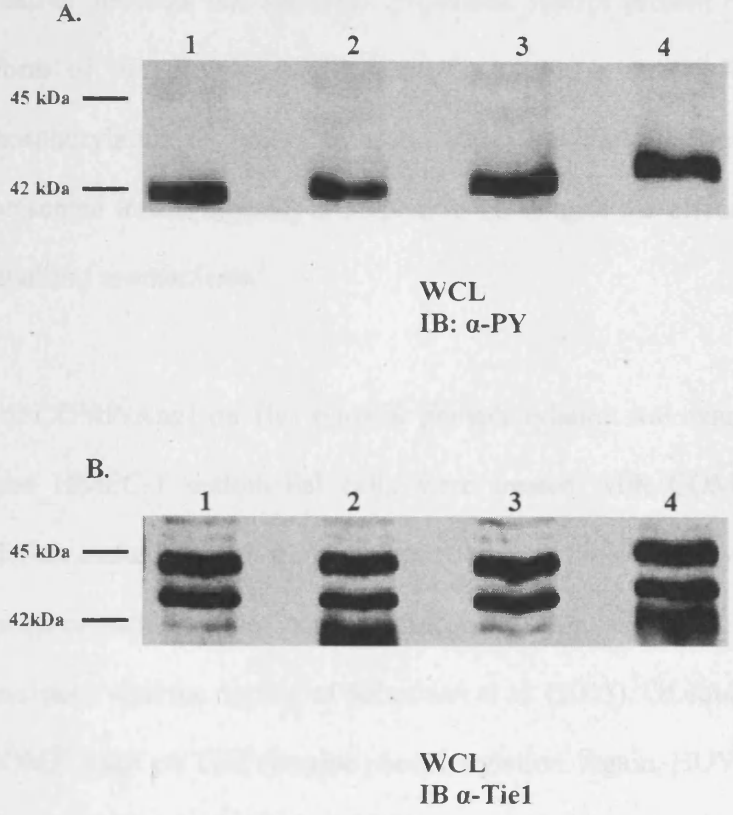


Figure 4.14 Effects of γ-secretase inhibitor on tyrosine phosphorylation of the 42kDa protein HUVE cells were grown to 90% confluence in six-well cell culture plates. Complete media was replaced with serum-free MEM 199 media and cells were serum starved overnight with 0.1mM lactacystin at 37°C/5% CO_2. Prior to the addition of 0.1mM lactacystin, the cells were pre-incubated with 20nM γ- secretase inhibitor (variant 458) for 5 hours in serum-free media at 37°C/5% CO_2. Following overnight treatment, serum-free media was replaced and cell treatments were replenished, before treating cells with 340ng/ml COMP-Ang1 for 45 minutes at 37°C/5% CO_2. Prior to the addition of 0.1mM lactacystin, the cells were pre-incubated with 20nM γ- secretase inhibitor (variant 458) for 5 hours in serum-free media at 37°C/5% CO_2. Following overnight treatment, serum-free media was replaced and cell treatments were replenished, before treating cells with 340ng/ml COMP-Ang1 for 45 minutes at 37°C/5% CO_2. Whole cell lysate (WCL) samples were loaded on to a 12% SDS-PAGE gel and detected by Western Blotting. Immunoblotting (IB) with a phospho-tyrosine antibody detected tyrosine phosphorylated protein at 45kDa and 42kDa (**A**). Truncated Tie1 and protein was detected by immunoblotting with a Tie1 specific antibody (**B**). Figure shows results obtained for control HUVE cells + Lacta. (1); γ-secretase inhibitor + Lacta. (2); COMP-Ang1 stimulated HUVE cells + Lacta. (3) COMP-Ang1 stimulated HUVE cells + γ-secretase inhibitor + Lacta. (4). Lactacystin treatment has been abbreviated to 'Lacta'.

4.3 Discussion

Based on earlier findings that cartilage oligomeric matrix protein (COMP) Ang1, a chimeric form of the protein Angiopoietin-1 (Ang1), is successful in stimulating tyrosine phosphorylation of Tie2 (Cho et al., 2004) and Tie1 (Saharinen et al., 2005); the work presented in this chapter, attempted to investigate the effect of COMP-Ang1 on Tie1 signalling mechanisms.

The effect of COMP-Ang1 on Tie1 tyrosine phosphorylation was examined. To do this, HUVEC and HMEC-1 endothelial cells were treated with COMP-Ang1 and the phosphorylation status of Tie1 studied. From the data obtained, it would appear that tyrosine phosphorylation of Tie1 occurred following stimulation by COMP-Ang1. This result is consistent with the finding of Saharinen et al. (2005). Of equal interest was the effect of COMP-Ang1 on Tie2 tyrosine phosphorylation. Again, HUVEC and HMEC-1 endothelial cells were treated with COMP-Ang1 and the phosphorylation status of Tie2 examined. The data obtained suggests that Tie2 tyrosine phosphorylation is induced following COMP-Ang1 stimulation; this finding is consistent with earlier work by Cho et al.(2004). Having found that COMP-Ang1 stimulation of Tie1 and Tie2 induces tyrosine phosphorylation of both receptors, the next stage of investigation was to dissect by what mechanism COMP-Ang1 stimulates Tie1 tyrosine phosphorylation. Data presented in Chapter Three of this study, demonstrates the transactivation of Tie1 by Tie2 in non-endothelial cells overepressing Tie1 and Tie2. In line with these results, Saharinen et al. (2005) have reported Tie2 directly phosphorylates Tie1 in a COMP-

Ang1 dependent manner. It was of interest therefore to investigate whether COMP-Ang1 stimulates Tie1 phosphorylation through the receptor, autonomous of Tie2. In order to do this, two strategies to block Tie2 activity were adopted. The first strategy was the use of a Tie2 blocking antibody. The manufacturer of the antibody states its ability to neutralise receptor (Tie2)-ligand (Ang1) interaction and has been used with effectiveness to investigate Ang2/VEGF interaction independent of Tie2 (Lobov et al., 2002). However, despite numerous attempts to block Tie2 activity, the data obtained suggests that blockade of Tie2 was not achieved. For this reason, an alternate strategy to block Tie2 activity was used.

The peptide T4-(NLLMAAS) has been reported as more potent at not only preventing Ang1 and Ang2 binding to Tie2 but specifically inhibits the activation of Tie2 induced by Ang1 (Tournaire et al., 2004). For this reason, the peptide was deemed a suitable candidate to block Tie2 concomitant with COMP-Ang1 stimulation. The data from the series of experiments carried out indicate unsuccessful blockade of Tie2 with the peptide. In addition to the use of Tie2 blocking antibody and peptide, the use of stable transfected cells was also considered, in the attempt to study Tie1 activity independent of Tie2. However, despite rigorous attempts to produce stable transfected cells that expressed only Tie1 or Tie2, unsatisfactory expression in CHO cells continuously occurred (data not shown). Thus, this prevented determination of the role of Tie2 in COMP-Ang1 induced Tie1 phosphorylation in endothelial cells. Furthermore, it was not possible therefore, to examine the downstream signalling for Tie1 in the absence of Tie2 activation.

Studies have revealed the extracellular domain of Tie1 can be proteolytically cleaved following treatment of cultured endothelial cells with phorbol myristate acetate (PMA), vascular endothelial growth factor (VEGF), protein kinase C, and tumour necrosis factor alpha (TNF-α), which results in the release of soluble Tie1 into the tissue culture supernatant. Additionally, exposure to shear stress within blood vessels also contributes to Tie1 cleavage (Yabkowitz et al., 1997a, 1999b; McCarthy et al., 1999; Tsiamis et al., 2002; Chen-Konak et al., 2003). Subsequent to the cleavage of the extracellular domain, a membrane-bound Tie1 endodomain is generated which contains the intracellular kinase and transmembrane domains. Based on these findings, experiments to investigate a potential mechanism by which Tie1 endodomain is activated were carried out. The data presented in this study (Chapter Three) has contributed to a greater understanding of how Tie1 endodomain becomes activated (kinase-dependent/relief of the inhibitory action by phosphatases) and the significant role Tie2 has in the transphosphorylation of Tie1 endodomain. Data in this study have also shown that upon Tie1 endodomain activation, truncation of the endodomain occurs to give rise to smaller sized proteins that are thought to be Tie1-related (Chapter Three). It was of further interest therefore, to examine the effects of COMP-Ang1 stimulation on Tie1 endodomain and subsequent truncation products in endothelial cells.

To do this, HUVEC and HMEC-1 endothelial cells were treated with COMP-Ang1 and the phosphorylation status of Tie1 truncation products studied. Results from these experiments demonstrated that following COMP-Ang1 stimulation, tyrosine phosphorylation of Tie1 endodomain and truncated products occurred. Furthermore, it

was also found that the truncation pattern, previously seen in earlier experiments with Tie1 endodomain (refer to data presented in Chapter Three) was not affected following stimulation by COMP-Ang1; specifically, the production of both 42kDa and 45kDa truncation products. It was observed however, that the 42kDa protein was significantly more tyrosine phosphorylated than the protein at 45kDa (Tie1 endodomain). It was therefore of great importance to establish the identity of the 42kDa protein. It is conceivable that this protein could represent the truncated form of Tie1; conversely, it is also possible that the tyrosine phosphorylation seen does not represent phosphorylation of Tie1 but phosphorylation of a protein that has associated to the intracellular domain of Tie1.

Following initial cleavage of Tie1, the transmembrane and intracellular domains undergo a secondary cleavage which is mediated by γ-secretase. Based on this knowledge, the strategy employed to determine whether the 42kDa phosphorylated protein is Tie1 examined the effects of inhibiting γ-secretase action in endothelial cells. From the experiments carried out, it was observed that tyrosine phosphorylation of the 42kDa protein was upheld despite inhibition of γ-secretase and the presence of the 42kDa protein was detected in all samples, including that of γ-secretase inhibitor treated cells, by immunoblotting with a Tie-1 specific antibody. However, taken together, these data suggest that the 42kDa protein is not truncated Tie1, since the secondary cleavage event that results in the truncated Tie1 product of 42kDa is mediated by γ-secretase and thus would be blocked following treatment with the γ-

secretase inhibitor. The studies in this chapter have therefore revealed for the first time the presence of a phospho-protein associated to the intracellular domain of Tie1 that has yet to be identified.

Chapter Five

Tie1 protects against apoptosis and promotes the release of an anti-apoptotic factor from endothelial cells

5.1 Introduction

The importance of Tie1 during embryonic vascular development and both physiological and pathological angiogenesis is well established. Genetic studies in mice have demonstrated the importance of Tie1 in regulating vascular integrity; mice lacking Tie1 die during late gestation or in immediate postnatal period with oedema and haemorrhage (Puri et al. 1995; Sato 1993). In addition to the observed oedema and haemorrhage, the numbers of endothelial cells in mice lacking Tie1 is reduced (Puri et al. 1995) and in chimeric mice, endothelial cells lacking Tie1 were involved in angiogenesis in early gestation but in later stages of embryonic development were progressively selected against (Partanen et al., 1996). Both pieces of evidence therefore suggest a role for Tie1 in the maintenance of endothelial cell survival.

The function and mechanism by which the generated Tie1 endodomain signals was investigated earlier in Chapter Three. Tie1 endodomain signalling, albeit as part of a chimeric receptor, has been implicated in the activation of PI 3 kinase/Akt pathway to inhibit apoptosis (Kontos et al., 2002). In breast and colon tumours, overexpression of Tie1 endodomain has been hypothesised to be a direct result of the endodomain's involvement in activating the PI 3 kinase/Akt pathway, thereby allowing invading cells to suppress apoptotic signals (Craven et al. 2003; Yang et al. 2003).

In this Chapter the potential involvement of Tie1 endodomain in suppressing endothelial cell apoptosis was examined.

5.2 Results

5.2.1 VEGF induced endothelial cell survival

Endothelial apoptosis is accompanied by a loss of adherent cells into tissue culture medium and the rate of appearance of such cells has previously been used as a marker of apoptosis (Levkau et al., 1998a; 1998b). This method was therefore used in the initial experiments to examine the effects of Tie1 endodomain. Preliminary studies were performed to confirm the utility of this assay by examining the affects of a known anti-apoptotic ligand, VEGF, on serum-deprived HUVE cells.

HUVE cells were serum-starved for 18 hours with or without 100ng/ml VEGF at 37°C/5% CO_2 and the number of non-adherent cells determined. Treatment with VEGF resulted in a 68.33± 19.65% (mean ± SD, n=3) reduction in the number of non-adherent cells in the medium at 18 hours (Figure 5.1). This reduction was statistically significant ($p \leq 0.05$).

These data are representative of three independent experiments and demonstrate well that in preliminary studies carried out with VEGF, an established anti-apoptotic ligand, suppressed the appearance of non adherent cells. This observation is consistent with an effect on apoptosis as described by Levkau et al., (1998).

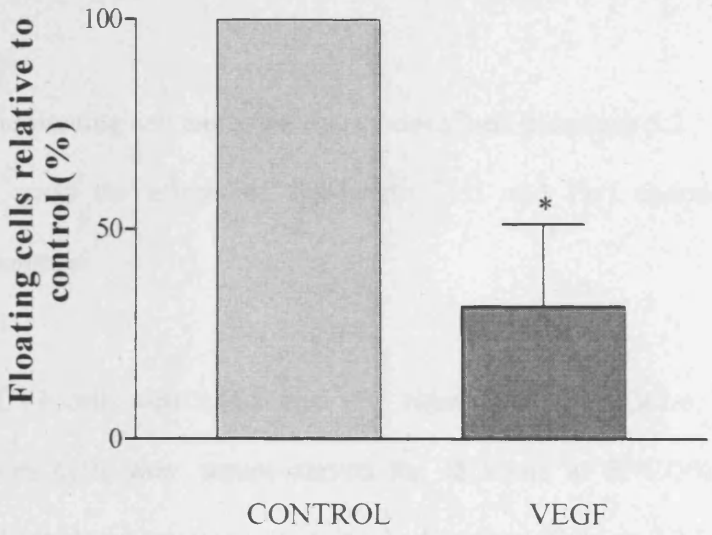


Figure 5.1 **Vascular Endothelial Growth Factor protects endothelial cells against apoptosis.**
HUVE cells were serum-starved for 18 hours with or without 100ng/ml VEGF at 37°C/5% CO_2. Non-adherent cells were collected from the medium by centrifugation and counted as previously described (Section 2.2.6.1). HUVE cells treated with 100ng/ml VEGF had increased endothelial cell survival. VEGF treatment resulted in a 68.33 ± 19.65% (mean ± SD, n=3) reduction in the number of non-adherent cells. This reduction was statistically significant ($p \leq 0.05$, Students't' test). Data presented as mean ± SD, n=3.

5.2.2 Overexpression of Tie1 in HUVE cells to examine the effects on endothelial cell apoptosis

The floating cell assay previously described in section 5.2.1 of this chapter was utilised to assay the effects of full-length Tie1 and Tie1 endodomain on endothelial cell apoptosis.

HUVE cells were transfected with either full-length Tie1 or Tie1 endodomain. After 48 hours cells were serum-starved for 18 hours at 37°C/5% CO_2. Floating cells were collected and counted as previously described (Section 2.2.6.1).

HUVE cells overexpressing full-length Tie1 exhibited a reduction in endothelial cell apoptosis, indicated by an approximate 50% decline in the number of apoptotic cells measured (49.63 ± 13.15% for full-length Tie1 overexpressing HUVE cells, mean ± SD, n=3; $p \leq 0.05$, Figure 5.2). A similar effect was observed for HUVE cells overexpressing the endodomain of Tie1; the percentage of apoptotic cells measured was less than 50% (46.10 ± 8.55%, mean ± SD, n=3; $p \leq 0.01$ of non-transfected cells).

These data suggest a potential anti-apoptotic effect of Tie1 overexpression in HUVE cells.

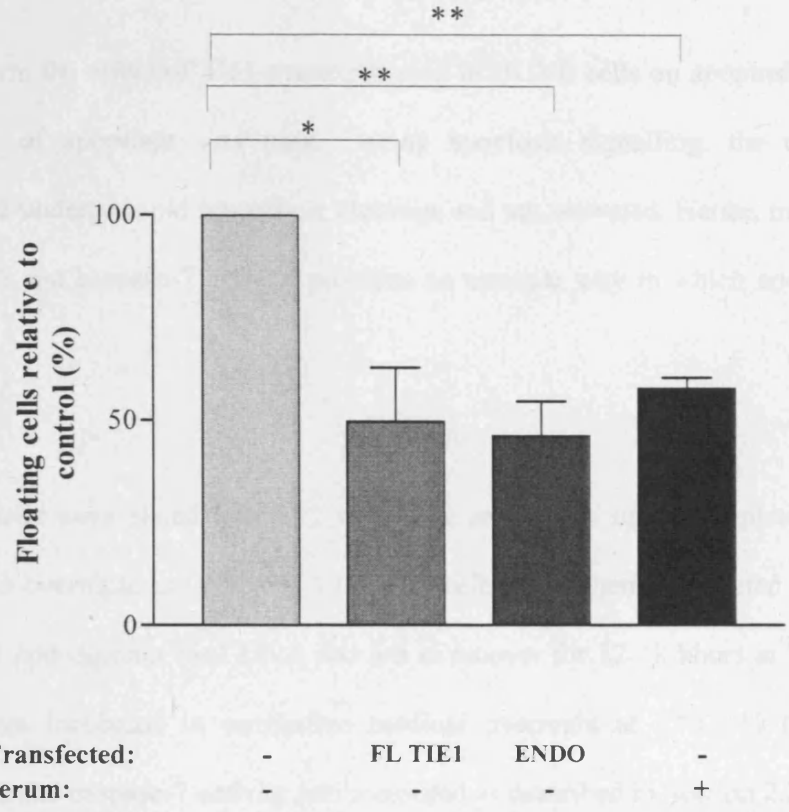


Figure 5.2 Overexpression of full-length Tie1 and Tie1 endodomain has an anti-apoptotic effect in endothelial cells.
HUVE cells were transfected with either full-length or endodomain Tie1. After 48 hours cells were serum-starved for 18 hours at 37°C/5% CO_2. Cells were counted as previously described (Section 2.2.6.1). HUVE cells overexpressing full-length Tie1 or the endodomain of Tie1 exhibited an increase in cell survival compared to HUVE cells alone (49.63 ± 13.15% full-length Tie1 and 46.10 ± 8.55% Tie1 endodomain). Data presented as mean ± SD (n=3). * $p \leq 0.05$; ** $p \leq 0.01$ Students't' test. Overexpression of full-length Tie1 and Tie1 endodomain in HUVE cells resulted in a significant reduction of non adherent cells.

5.2.3 Effect of Tie1 overexpression in HUVE cells on apoptosis: cleaved caspase-3 and caspase-7 activity

To confirm the effect of Tie1 overexpression in HUVE cells on apoptosis, an alternate indicator of apoptosis was used. During apoptosis signalling, the caspase-3 and caspase-7 undergo rapid proteolytic cleavage and are activated. Hence, measurement of caspase-3 and caspase-7 activity provides an accurate way in which apoptosis can be assessed.

HUVE cells were plated into a 12 well plate and grown up in complete medium and incubated overnight at 37°C/5% CO_2. The cells were then transfected with the full-length or endodomain Tie1 DNA and left to recover for 12-18 hours at 37°C/5% CO_2. Cells were incubated in serum-free medium overnight at 37°C/5% CO_2. Cleaved caspase-3 and caspase-7 activity was measured as described in Section 2.2.6.2.

Cleaved caspase activity was found to be reduced for non-transfected HUVE cells that had not been serum-starved (65.03 ± 1.67%, data as mean ± SD, n=3, $p \leq 0.001$ Students't' test; Figure 5.3). This result was consistent with earlier experimental findings presented in Figure 5.2.

Measurement of cleaved caspase activity in HUVE cells overexpressing full-length Tie1 indicated a reduction in apoptosis; concluded from an approximate 25% decline in cleaved caspase activity for full-length Tie1 overexpression in HUVE cells compared to control HUVE cells (75.8 ± 0.62%, mean ± SD, n=3, $p \leq 0.001$ Students 't' test; Figure 5.3).

Measurement of cleaved caspase activity in HUVE cells overexpressing Tie1 endodomain also indicated a reduction in endothelial cell apoptosis. Cleaved caspase activity was reduced by approximately 45% in relation to control HUVE cells (55.43 ± 5.33%, mean ± SD, n=3; $p \leq 0.01$ Students't' test).

Comparison of cleaved caspase activity for full-length Tie1 overexpressing HUVE cells with that of the Tie1 endodomain overexpressing HUVE cells would indicate that full-length Tie1 overexpressing HUVE cells exhibited greater cell apoptosis than endodomain expressing cells (75.8% ± 0.62% comparison to 55.43% ± 5.33%, mean ± SD, n=3, $p < 0.05$ Students 't' test) This comparison would therefore suggest that the endodomain of Tie1, when overexpressed in HUVE cells, significantly protects HUVE cells from apoptosis more effectively than full-length Tie1 overexpression.

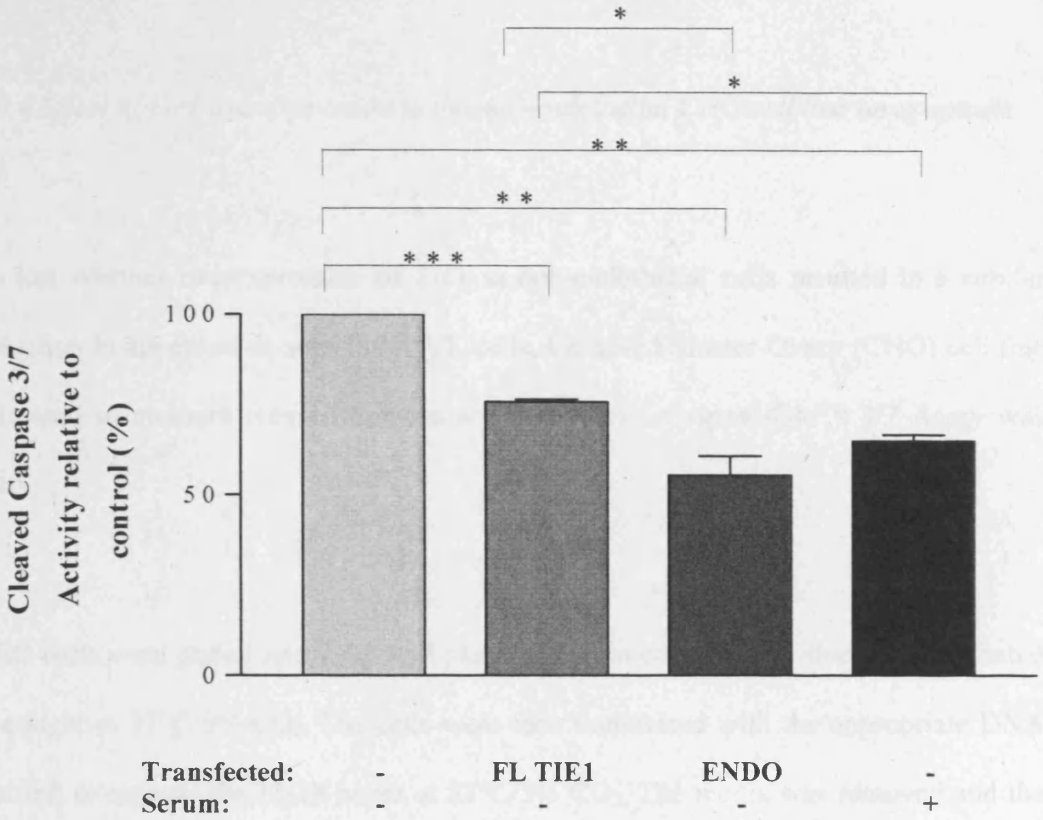


Figure 5.3 Effect of Tie1 overexpression in HUVE cells on apoptosis: cleaved caspase-3 and caspase-7 activity. HUVE cells were plated into a 12 well plate and grown up in complete medium and incubated overnight at 37°C/5% CO_2. The cells were then transfected with the full-length or endodomain Tie1 DNA and left to recover for 12-18 hours at 37°C/5% CO_2. Cells were incubated in serum-free medium overnight at 37°C/5% CO_2. Cleaved caspase-3 and caspase-7 activity was measured as described in Section 2.2.6.2. HUVE cells overexpressing full-length Tie1 exhibited an approximate 25% decrease in cell survival compared to HUVE cells alone (75.8 ± 0.62%). HUVE cells overexpressing Tie1 endodomain showed cleaved caspase activity was reduced by approximately 45% in relation to control HUVE cells (55.43 ± 5.33%). Comparison of data obtained for HUVE cells overexpressing full-length or endodomain Tie1, indicates greater reduction in cell apoptosis for Tie1 endodomain overexpressing HUVE cells (75.8% ± 0.62% comparison to 55.43% ± 5.33%). Data presented as mean ± SD (n=3). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, Students 't' test.

5.2.4 Effect of Tie1 overexpression in the non-endothelial CHO cell line on apoptosis

To test whether overexpression of Tie1 in non-endothelial cells resulted in a similar reduction in apoptosis as seen in HUVE cells, Chinese Hamster Ovary (CHO) cell line was used to measure cleaved caspase activity. Again, Caspase-Glo™ 3/7 Assay was used.

CHO cells were plated into a 12 well plate, grown in complete medium and incubated overnight at 37°C/5% CO_2. The cells were then transfected with the appropriate DNA and left to recover for 12-18 hours at 37°C/5% CO_2. The media was removed and the cells gently washed twice in PBS. Complete medium was replaced with serum-free medium. Cells were incubated overnight (18 hours) at 37°C/5% CO_2. On the day of performing the Caspase-Glo™ 3/7 Assay, the serum-free medium was removed from the cells and cleaved caspase activity was measured as described (Section 2.2.6.2).

For CHO cells overexpressing full-length Tie1, caspase activity was found to be similar to control non-transfected CHO cells (control CHO cell caspase activity was normalised to be 100% compared to full-length Tie1 overexpressing CHO cells, 99.10 ± 13.94%, mean ± SD, n=3; refer to Figure 5.4).

Measurement of caspase activity in CHO cells overexpressing Tie1 endodomain indicates a small statistically significant increase in apoptosis occurring in these cells in comparison to control CHO cells (113.2 ± 3.90%; mean ± SD, n=3 , $p \leq 0.05$ Students't' test).

Taken together these data show that Tie1 overexpression in CHO cells does not protect against cell apoptosis and therefore suggests the anti-apoptotic effect of Tie1 overexpression seen in HUVE cells might be exclusive to endothelial cells.

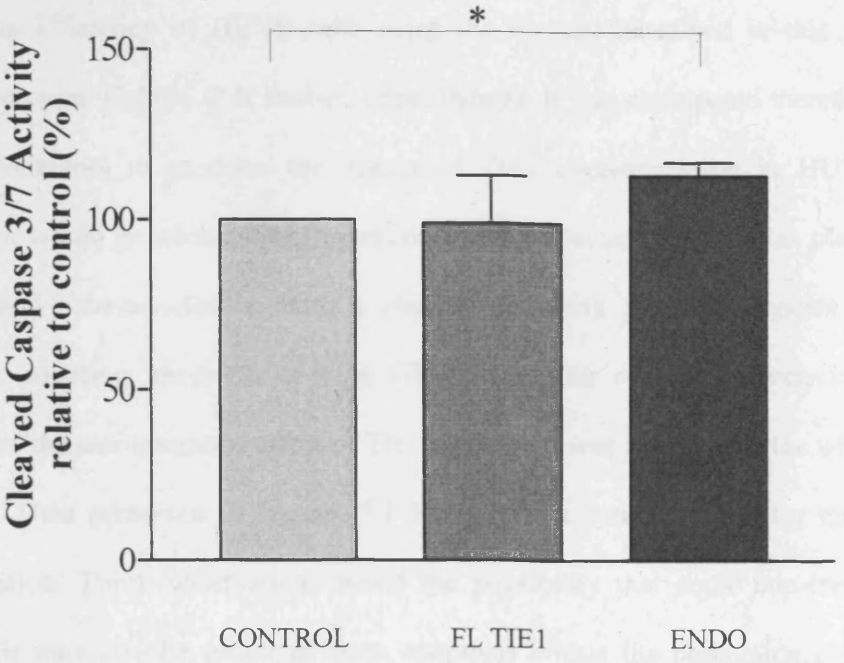


Figure 5.4 Effect of Tie1 overexpression in the non-endothelial CHO cell line on apoptosis. CHO cells were plated into a 12 well plate, grown in complete medium and incubated overnight at 37°C/5% CO_2. The cells were then transfected with the appropriate DNA and left to recover for 12-18 hours at 37°C/5% CO_2. The medium was removed and the cells gently washed twice in PBS. Complete medium was replaced with serum-free medium. Cells were incubated for18 hours at 37°C/5% CO_2. Apoptosis was measured as an index of cleaved caspase 3/7 activity and expressed as a percentage relative to control CHO cells. Cleaved caspase 3/7 activity for CHO cells overexpressing full-length Tie1 was almost equivalent to control CHO cells, a marginal 1% decrease in apoptosis occurred. Cleaved caspase 3/7 activity for CHO cells overexpressing Tie1 endodomain, exhibited approximately 13% increase in cell apoptosis. Data presented as mean ± SD (n=3). * $p \leq 0.05$ Students 't' test.

5.2.5 Effect of conditioned medium from HUVE cells overexpressing Tie1 on HUVE cell apoptosis

Transfection efficiency of HUVE cells using the method described in this study is routinely between 40-70% (J R Barber, unpublished). It was anticipated therefore, that in the experiments to examine the effects of Tie1 overexpression in HUVE cell apoptosis, it would be necessary to examine only transfected cells. It was planned to achieve this by co-transfection with a plasmid encoding green fluorescent protein (GFP) and counting apoptosis only in GFP expressing cells. However, in initial experiments, the anti-apoptotic effect of Tie1 expression was observed in the whole cell population. Data presented in Figures 5.1-5.4 represent measurements for the whole cell population. These observations raised the possibility that some non-transfected HUVE cells may also be protected from apoptosis within the population containing transfected cells. One mechanism for this would be if the transfected cells secreted an anti-apoptotic factor.

To test this theory, HUVE cells were transfected with either full-length Tie1 or endodomain Tie1. After 48 hours cells were serum starved for 18 hours at 37°C/5% CO_2. Serum free conditioned media from control, full-length Tie1 and Tie1 endodomain expressing HUVE cells was aspirated, cleared of particulate material by centrifugation and added to fresh HUVE cells. These HUVE cells were incubated in serum free

conditioned media for 18 hours at 37°C/5% CO_2. Apoptotic cells were measured for cleaved caspase activity, as previously described (Section 2.2.6.2).

As shown in Figure 5.5, measurement of cleaved caspase activity in HUVE cells grown in conditioned medium from HUVE cells overexpressing full-length Tie1, was approximately 45% less than HUVE cells grown in conditioned medium from untransfected HUVE cells (54.2 ± 0.6%, mean ± SD, n=3, $p \leq 0.001$).

HUVE cells grown in conditioned medium from HUVE cells overexpressing Tie1 endodomain, exhibited an approximate 50% decrease in cleaved caspase 3/7 activity compared to HUVE cells grown in conditioned medium from non-transfected HUVE cells (44.6 ± 5.3% mean ± SD, n=3, $p \leq 0.01$ Students't' test) (Figure 5.5).

A comparison between the data obtained for HUVE cells grown in conditioned medium from overexpressing full-length Tie1 HUVE cells and HUVE cells grown in conditioned medium from HUVE cells overexpressing Tie1 endodomain, demonstrates that the latter conditioned medium i.e. Tie1 endodomain overexpression, protects HUVE cells against apoptosis 5% more, than conditioned medium from full-length Tie1 expressing HUVE cells. Together these data suggest HUVE cells overexpressing full-length or truncated Tie1 secrete an anti-apoptotic factor or factors.

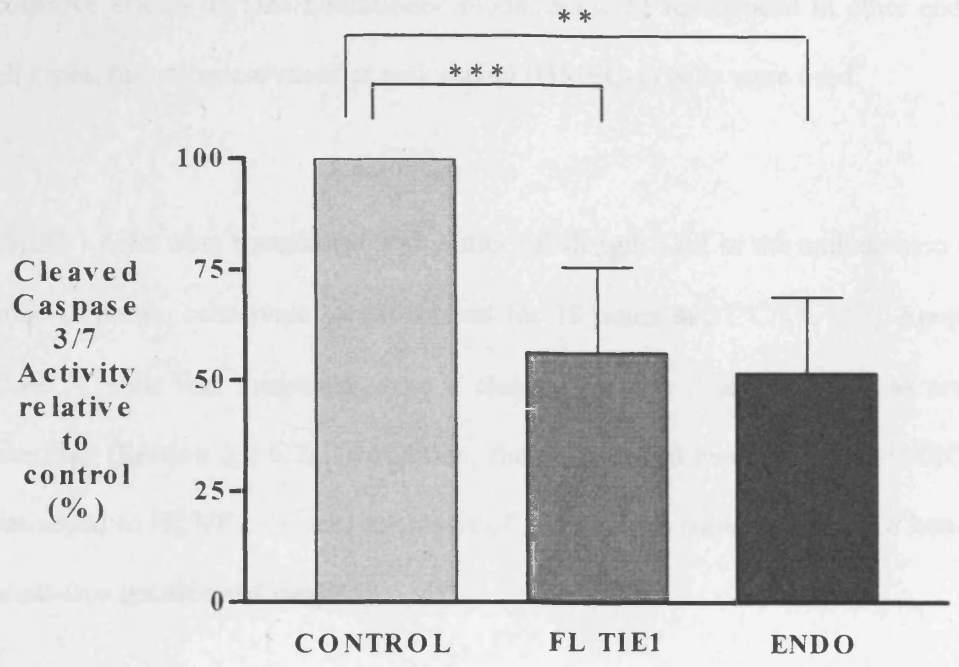


Figure 5.5 Effect of conditioned medium from HUVE cells overexpressing Tie1 on HUVE cell apoptosis. HUVE cells were transfected with either full-length or endodomain Tie1. Conditioned medium from Tie1 overexpressing HUVE cells was added to HUVE cells and apoptosis of HUVE cells was measured after 18 hours in serum-free conditioned medium. Apoptosis was measured as an index of cleaved caspase 3/7 activity and expressed as a percentage relative to control HUVE cells. Cleaved caspase 3/7 activity for HUVE cells grown in conditioned medium from full-length Tie1 transfected HUVE cells was decreased by almost 45%. Cleaved caspase 3/7 activity for HUVE cells grown in conditioned medium from HUVE cells transfected with Tie1 endodomain was decreased by approximately 50%. Data presented as mean ± SD (n=3). ** $p \leq 0.01$; *** $p \leq 0.001$, Students 't' test.

5.2.6 Effect of conditioned medium from HMEC-1 cells overexpressing Tie1 on HUVE cell apoptosis

To explore whether this phenomenon was exclusive to HUVE cells or if the cyto-protective effects of Tie1 conditioned media could be reproduced in other endothelial cell types, human microvascular endothelial (HMEC-1) cells were used.

HMEC-1 cells were transfected with either full-length Tie1 or the endodomain of Tie1. After 48 hours, cells were serum-starved for 18 hours at 37°C/5% CO_2. Apoptosis of HMEC-1 cells was measured using a cleaved caspase 3 and 7 assay, as previously described (Section 2.2.6.2). In addition, the conditioned medium from HMEC-1 cells was added to HUVE cells and apoptosis of HUVE cells measured after 18 hours in the serum-free conditioned medium.

Measurement of cleaved caspase activity in HUVE cells grown in conditioned medium from HMEC-1 overexpressing full-length Tie1 cells, exhibited approximately 50% less apoptosis than HUVE cells grown in conditioned medium from HMEC-1 cells alone ($47.16 \pm 6.08\%$, mean ± SD, $n=3$, $p \leq 0.01$ Students't' test) (Figure 5.6).

For HUVE cells grown in conditioned medium from HMEC-1 cells overexpressing Tie1 endodomain, caspase activity was measured to be approximately 50% when

compared to HUVE cells grown in conditioned medium from HMEC-1 cells alone (46.57 ± 16.44%, mean ± SD, n=3, $p \leq 0.05$ Students't' test).

A comparison between the data obtained for cleaved caspase 3/7 activity for HMEC-1 overexpressing full-length Tie1 or Tie1 endodomain, shows that conditioned medium from HMEC-1 cells, protects HUVE cells against apoptosis equally. Furthermore, the cyto-protective affect demonstrated in HUVE cells grown in medium from Tie1 expressing HUVE cells (refer to Figure 5.5), is also seen in HMEC-1 cells. This would suggest that conditioned medium from endothelial cells may contain soluble growth factors which protect endothelial cells against apoptosis.

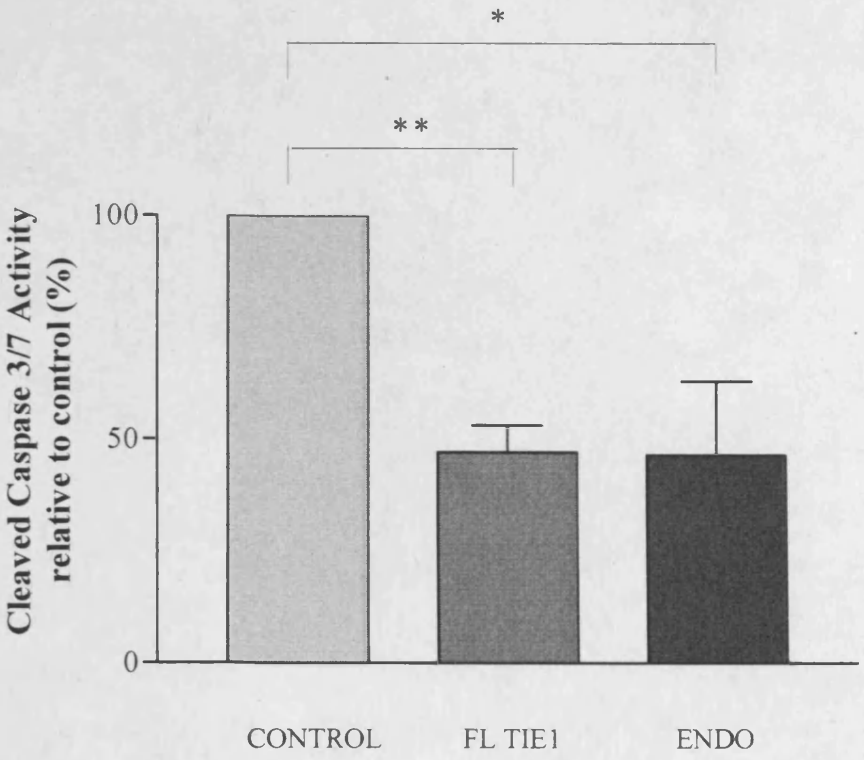


Figure 5.6 Effect of conditioned medium from HMEC-1 cells overexpressing Tie1 on HUVE cell apoptosis. HMEC-1 cells were transfected with either full-length or endodomain Tie1. Conditioned medium from Tie1 overexpressing HMEC-1 cells was added to HUVE cells and apoptosis of HUVE cells was measured after 18 hours in serum-free conditioned medium. Apoptosis was measured as an index of cleaved caspase 3/7 activity and expressed as a percentage relative to control HUVE cells. Cleaved caspase 3/7 activity for HUVE cells grown in conditioned medium from full-length Tie1 transfected HMEC-1 cells was decreased by almost 50%. Cleaved caspase 3/7 activity for HUVE cells grown in conditioned medium from HMEC-1 cells transfected with Tie1 endodomain was decreased by approximately 50%. Data presented as mean ± SD (n=3). * $p \leq 0.05$; ** $p \leq 0.01$, Students 't' test.

5.2.7 Effect of conditioned medium from CHO cells overexpressing Tie1 on HUVE cell apoptosis

Previous experiments investigating the effect of Tie1 overexpression in non-endothelial cells on apoptosis, suggested that the anti-apoptotic effect was exclusive for HUVE endothelial cells. It was of interest, therefore, to determine whether non-endothelial cells overexpressing full-length or Tie1 endodomain also produced a soluble anti-apoptotic factor for endothelial cells. To answer this question, CHO cells were transfected with either full-length Tie1 or the endodomain of Tie1. After 48 hours, cells were serum-starved for 18 hours at 37°C/5% CO_2. Cells were pelleted and measured for cleaved caspase 3 and 7, as previously described (Section 2.2.6.2).

Measurement of cleaved caspase activity in HUVE cells grown in conditioned medium from CHO overexpressing full-length Tie1 cells, exhibited approximately 69% more apoptosis than HUVE cells grown in conditioned medium from CHO cells alone (180.3 ± 23.76% compared to 100 ± 0.00%, mean ± SD, n=3) (Figure 5.7).

For HUVE cells grown in conditioned medium from CHO cells overexpressing Tie1 endodomain, caspase activity was measured to be almost 41% more than control CHO conditioned medium (140.5 ± 49.82 % compared to 100 ± 0.00 %, mean ± SD, n=3) (Figure 5.7).

Evaluation of the anti-apoptotic effect on HUVE cells grown in conditioned medium from Tie1 overexpressing CHO cells, suggests cell specificity required by Tie1 to function as an anti-apoptotic receptor or at least mediate an anti-apoptotic response. The ability of Tie1 to protect cells from apoptosis was observed exclusively in endothelial cells, HUVE and HMEC-1 cells. Overexpression in non- endothelial cells failed to result in reduced apoptosis; conversely, an increase in cell apoptosis was seen.

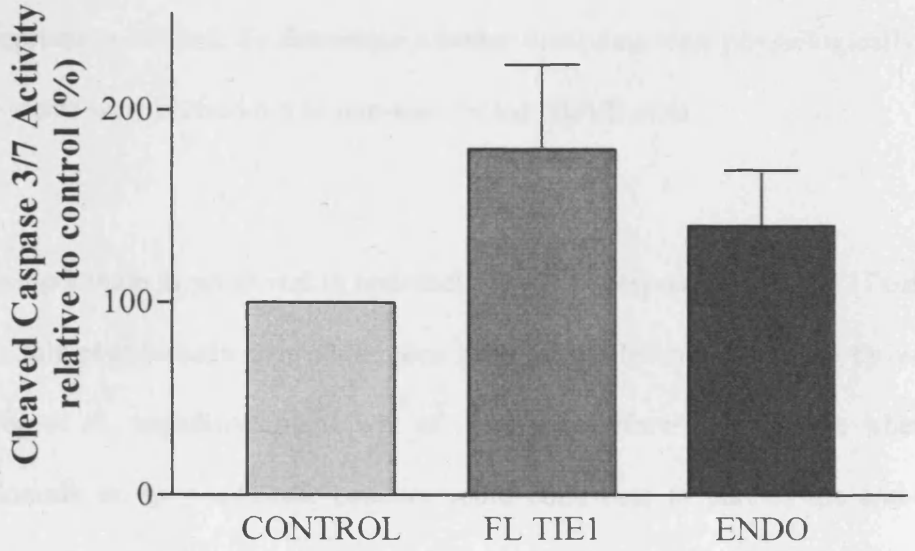


Figure 5.7 Effect of conditioned medium from CHO cells overexpressing Tie1 on HUVE cell apoptosis. CHO cells were transfected with either full-length or endodomain Tie1. Conditioned media from Tie1 overexpressing CHO cells was added to HUVE cells and apoptosis of HUVE cells was measured after 18 hours in serum-free conditioned medium. Apoptosis was measured as an index of cleaved caspase 3/7 activity and expressed as a percentage relative to control HUVE cells. Cleaved caspase 3/7 activity for HUVE cells grown in conditioned medium from full-length Tie1 transfected CHO cells was increased by 80.3%. Cleaved caspase 3/7 activity for HUVE cells grown in conditioned medium from CHO cells transfected with Tie1 endodomain, exhibited a 41% increase. Data is representative of repeated experiments, n=3.

5.2.8 Effects of TAPI and γ-secretase inhibitor treatment on endothelial cell apoptosis

Data presented herein, implicates the endodomain of Tie1 to possess a greater ability to prevent apoptosis than full-length Tie1. However, experiments thus far have used overexpression of Tie1. To determine whether these data were physiologically relevant, experiments were carried out in non-transfected HUVE cells.

Tie1 endodomain is produced in endothelial cells in response to VEGF (Tsiamis et al. 2002), this endodomain then undergoes subsequent cleavage mediated by γ-secretase (Marron et al., unpublished). It was of interest therefore to determine whether Tie1 endodomain or its γ-secretase product could contribute to part of the anti-apoptotic activity of VEGF. To test this endothelial cells were stimulated with VEGF in the absence of serum and the effects of inhibiting endodomain production by preventing cleavage of full-length Tie1 , using a metalloprotease inhibitor, TAPI, on apoptosis was examined. In addition, to test the effect of suppressing processing of endodomain by γ-secretase, the effect of a γ-secretase inhibitor was tested.

HUVE cells were plated into a 12 well plate and grown up in Complete Media and incubated overnight at 37°C/5% CO_2. The media was removed and the cells gently washed twice in PBS. Complete Media was replaced with serum-free media to enable cell starvation. To the serum-free media 0.1mM TAPI-2 or 20nM γ-secretase inhibitor

were added 30 minutes prior to the addition of 100ng/ml VEGF. Cells were incubated overnight at 37°C/5% CO_2. On the day of performing the Caspase-Glo™ 3/7 Assay, the serum-free media was removed from the cells and cleaved caspase activity was measured as described (Section 2.2.6.2).

For HUVE cells treated with VEGF, cleaved caspase 3/7 activity was observed to be reduced in comparison to HUVE cells that had been serum-starved without VEGF treatment (84.83 ± 5.829% for VEGF treated HUVE cells compared to 100 ± 0.00% HUVE cells with no VEGF treatment, data presented as mean ± SD, n=3; $p \leq 0.05$ Students't' test). It must be noted that the reduction in cleaved caspase 3/7 activity for HUVE cells treated with VEGF was much lower than expected.

For HUVE cells treated with VEGF but also with the metalloprotease inhibitor, TAPI-2, cleaved caspase 3/7 activity declined significantly (47.07 ± 2.386%, data presented as mean ± SD, n=3; $p \leq 0.01$, $p \leq 0.001$ Students't' test).

γ-secretase inhibitor treatment of HUVE cells in addition to VEGF treatment, resulted in marginal increase in measured cleaved caspase 3/7 activity (101.8 ± 13.38%, data presented as mean ± SD, n=3).

In summary, endothelial cell apoptosis was reduced most significantly upon treatment with a metalloprotease inhibitor, TAPI-2. This would suggest that Tie1 is most effective at protecting against cell death when the action of metalloprotease, i.e. cleavage of full-length Tie1, is inhibited. This is an interesting observation as it is contradictory to the observations seen for endothelial cells overexpressing full-length and endodomain Tie1.

γ-secretase treatment resulted in an marginal increase in endothelial cell apoptosis and thus the anti-apoptotic property of Tie1 endodomain was maintained.

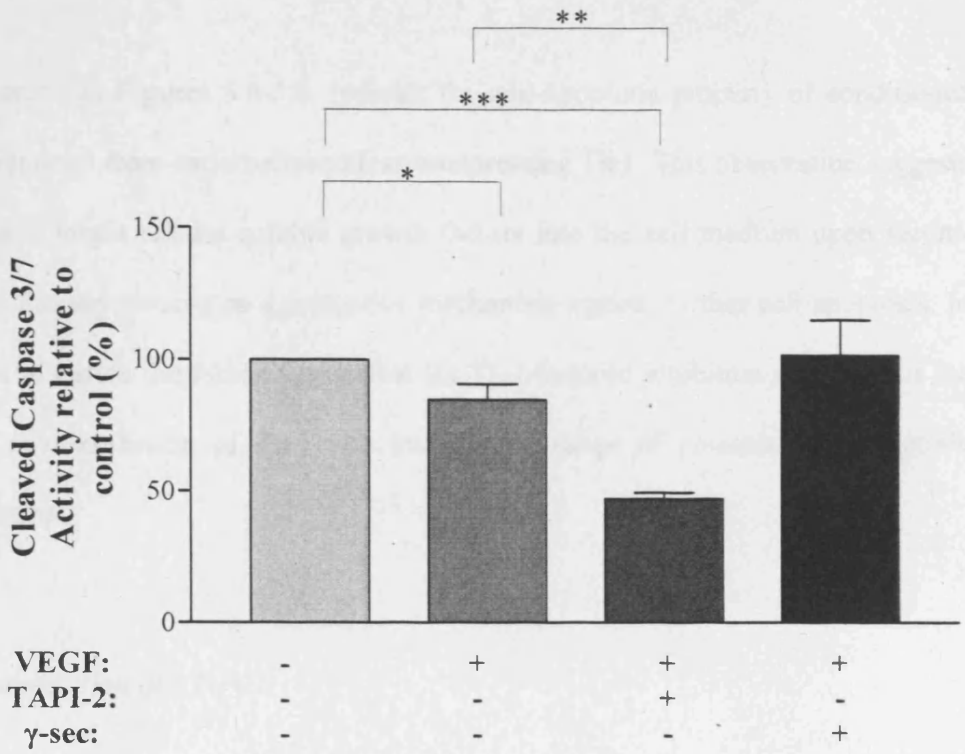


Figure 5.8 Effects of TAPI and γ-secretase inhibitor treatment on endothelial cell apoptosis. HUVE cells were plated into a 12 well plate and grown up in complete medium and incubated overnight at 37°C/5% CO_2. The medium was removed and the cells gently washed twice in PBS. Complete Media was replaced with serum-free medium and 0.1mM TAPI-2 or 20nM γ-secretase inhibitor were added 30 minutes prior to the addition of 100ng/ml VEGF. Cells were incubated overnight at 37°C/5% CO_2. On the day of performing the Caspase-Glo™ 3/7 Assay, the serum-free medium was removed from the cells and cleaved caspase activity was measured as described (Section 2.2.6.2). Cleaved caspase 3/7 activity for HUVE cells treated with VEGF was decreased approximately by 16% (84.83 ± 5.829%); in cells treated with VEGF and TAPI-2, resulted in a significant decrease in apoptosis (47.07 ± 2.386%); in cells treated with γ-secretase inhibitor and VEGF, a marginal increase was observed (101.8 ± 13.38%). Data presented as mean ± SD (n=3). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, Students 't' test.

5.2.9 Human Microvascular Endothelial cell cDNA expression of Angiogenic Growth Factors in endothelial cells

Data presented in Figures 5.6-5.8, indicate the anti-apoptotic property of conditioned medium obtained from endothelial cells overexpressing Tie1. This observation suggests that the cells might release soluble growth factors into the cell medium upon serum-starvation, thereby serving as a protective mechanism against further cell apoptosis. In an attempt to define the factor responsible for Tie1-induced inhibition of apoptosis the effect of overexpression of Tie1 was tested on a range of potential anti-apoptotic growth factors.

5.2.9.1 Optimisation of RT-PCR

mRNA from HMEC-1 cells was isolated as previously described (Section 2.4.1). Briefly, HMEC-1 cells were grown to confluency and mRNA from the cells isolated using Qiagen RNA Mini-Prep Kit. The isolated RNA was quantified spectrophotometrically as described (Section 2.4.1.2). The isolated RNA was then converted to cDNA using Reverse Transcriptase as described (Section 2.4.1.3). Oligonucleotides specific to angiogenic growth factors were used to amplify HMEC-1 cell cDNA by polymerase chain reaction (PCR) (Figure 5.9 A). GAPDH primers were used to optimise PCR reactions and as a positive control (Figure 5.9 B).

A.

HGF			
Forward primer (5')	5'-ATCAAATGTCAGCCCTGGAG-3'	Tm=64.0	222bp
Reverse primer (3')	5'-TCGATAACTCTCCCCATTGC-3'	Tm=63.8	
bFGF			
Forward primer (5')	5'-AGAGCGACCCTCACATCAAG-3'	Tm=64.4	234bp
Reverse primer (3')	5'-ACTGCCCAGTTCGTTTCAGT-3'	Tm=63.6	
aFGF			
Forward primer (5')	5'-AAGGGGAAATCACCACCTTC-3'	Tm=63.9	155bp
Reverse primer (3')	5'-GTCGCTCCTGTCCCTTGTC-3'	Tm=64.9	
Ang-1			
Forward primer (5')	5'-GGGGGAGGTTGGACTGTAAT-3'	Tm=63.8	288bp
Reverse primer (3')	5'-GCTCTGTTTTCCTGCTGTCC-3'	Tm=63.9	
VEGF			
Forward primer (5')	5'-TATGCGGATCAAACCTCACC-3'	Tm=64.7	265bp
Reverse primer (3')	5'-CCTCGGCTTGTCACAGCAT-3'	Tm=66.5	

B.

GAPDH			
Forward primer (5'):	5'-GAGTCAACGGATTTGGTCGT-3'	Tm = 63.9	238bp
Reverse primer (3'):	5'-TTGATTTTGGAGGGATCTCG-3'	Tm = 63.8	

Figure 5.9 Optimisation of RT-PCR. Oligonucleotides specific for growth factors involved in the process of angiogenesis, were used to amplify HMEC-1 cell cDNA by polymerase chain reaction (PCR) (Figure 5.10 A). GAPDH primers were used to optimise PCR reactions and as a positive control (Figure 5.10 B). Forward primer (5') and reverse primer (3') oligonucleotide sequences are given along with individual melting temperature (Tm; °C) and expected product size (bp, base pairs).

PCR products were electrophoresed onto a 2% agarose gel.

PCR amplification of GAPDH (238 bp) was successfully achieved as shown in Figure 5.10.

Once successful PCR amplification of the control primer had been established, the oligonucleotides specific to angiogenic growth factors were then evaluated by PCR, following the parameters as described in Section 2.4.1.4. The sequence (5'-3') for each of the oligonucleotides tested is shown in Figure 5.9. Variable expression of each of the growth factors was observed following 2% agarose gel electrophoresis. For this reason, the initial parameters set out in Section 2.4.1.4 were altered to optimise the polymerase chain reaction. Better resolution of amplified oligonucleotides was achieved by decreasing the percentage of agarose gel used; changing from the initial 2% agarose to 1% agarose gel. Additional to this, the temperature used during the annealing phase of the polymerase chain reaction was also investigated and altered accordingly (Figure 5.11 (A.) and (B). The optimised conditions resulted in better expression of the oligonucleotides as shown in Figure 5.12.

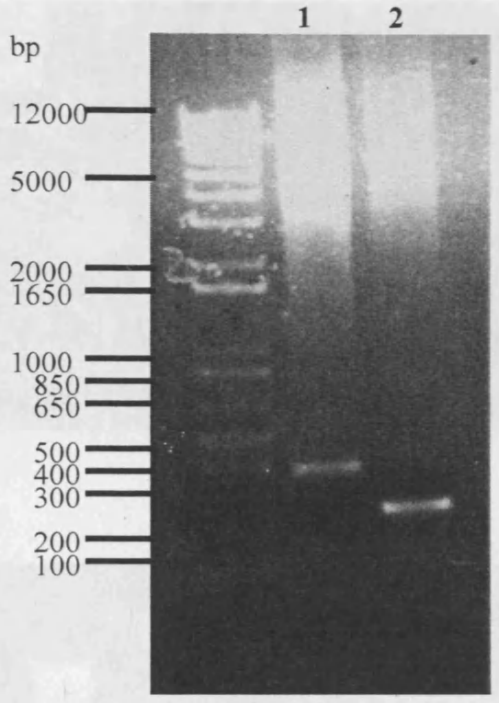


Figure 5.10 Initial PCR parameters tested with GAPDH and positive control. To test the suitability of the parameters chosen for PCR a positive control (1) with an expected product size of 400 bp and GAPDH (2) with an expected end product of 238 bp were used preliminarily. PCR products were electrophoresed onto a 2% agarose gel.

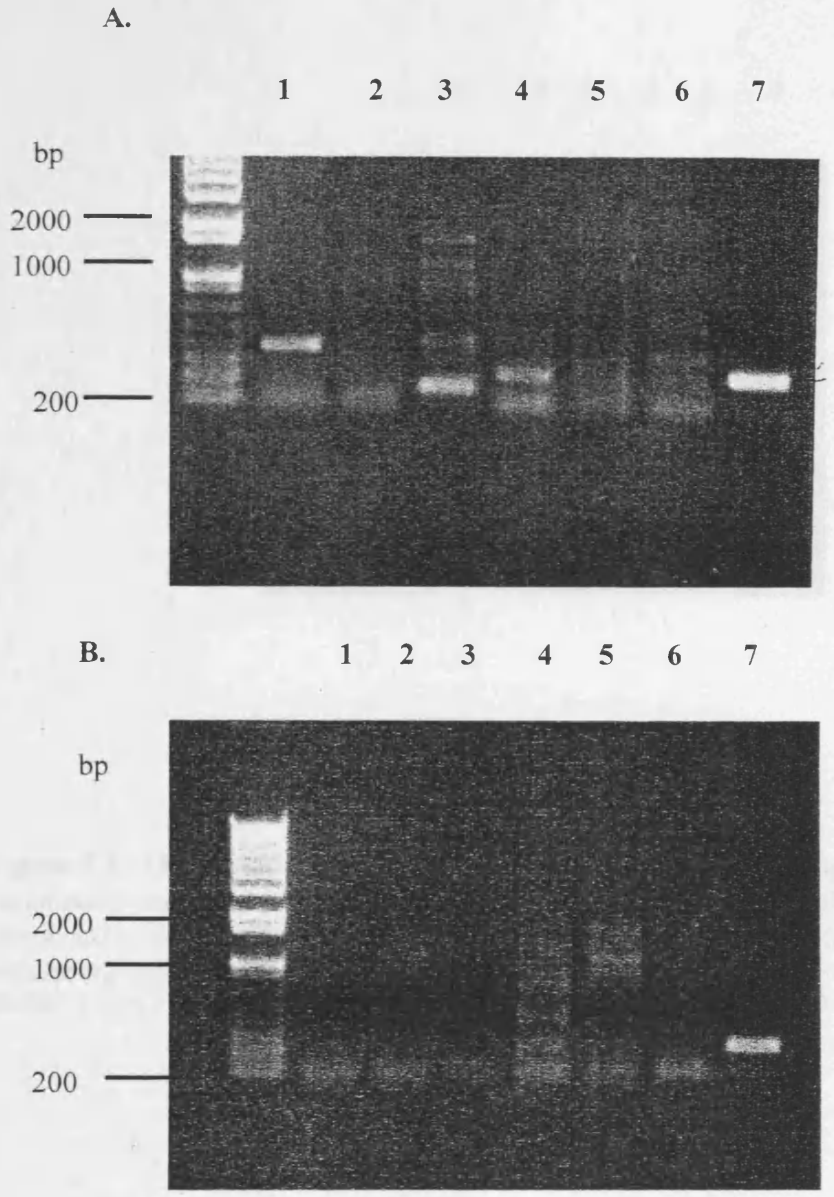


Figure 5.11 Optimisation of PCR parameters for the amplification of growth factor oligonucleotides. The initial parameters set out in Section 2.4.1.4 were altered to optimise the polymerase chain reaction. Better resolution of amplified oligonucleotides was achieved by decreasing the thickness of agarose gel used; changing from the initial 2% agarose to 1% agarose gel, as show in (A) (with original annealing temperature of 55°C). Additional to this, the temperature used during the annealing phase of the polymerase chain reaction was also investigated: the annealing temperature was increased to 60°C (B). Control, HMEC-1 only (1); HGF (2); aFGF (3); bFGF (4); Ang-1 (5); VEGF (6); GAPDH (7).

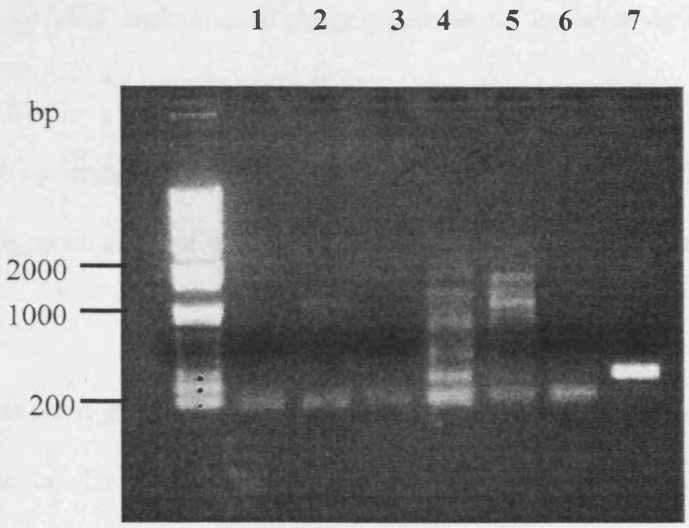


Figure 5.12 Optimised parameters for PCR of growth factor oligonucleotides. The parameters used for the polymerase chain reaction for one cycle were; annealing temperature 60°C for 1 minute, extension temperature 72°C for 2 minutes and denaturing temperature 94°C for 1 min. A total of 35 cycles were completed. Control, HMEC-1 only (1); HGF (2); aFGF (3); bFGF (4); Ang-1 (5); VEGF (6); GAPDH (7).

5.2.10 Effect of Tie1 endodomain overexpression on an array of angiogenic growth factors.

Subsequent to optimisation of RT-PCR, the effect of overexpressed Tie1 endodomain in HMEC-1 cells on an array of angiogenic growth factors was examined.

HMEC-1 cells were grown to 80-90% confluence in $80cm^2$ flasks and transfected with empty vector or Tie1 endodomain. mRNA from HMEC-1 cells was isolated as previously described (Section 2.4.1).The isolated RNA was quantified spectrophotometrically as described (Section 2.4.1.2). The isolated RNA was then converted to cDNA using Reverse Transcriptase as described (Section 2.4.1.3). Oligonucleotides specific to angiogenic growth factors were used to amplify HMEC-1 cell cDNA by polymerase chain reaction (PCR) using optimised conditions. PCR products were electrophoresed onto a 1% agarose gel.

As depicted in Figure 5.13, there was negligible difference in the level of expression of each of the angiogenic growth factors in control HMEC-1 cells. The effect of Tie1 endodomain overexpression on angiogenic growth factor expression resulted in marginal reduction; it was also observed, for each growth factor this marginal reduction was equivalent. The level of expression for each of the oligonucleotides was quantified using densitometric analysis (refer to Section 2.7). Statistical analysis by the Students't'

test, revealed no statistical significance in the level of growth factor expression for HMEC-1 cells transfected with empty vector cDNA and HMEC-1 cells transfected with Tie1 intracellular domain.

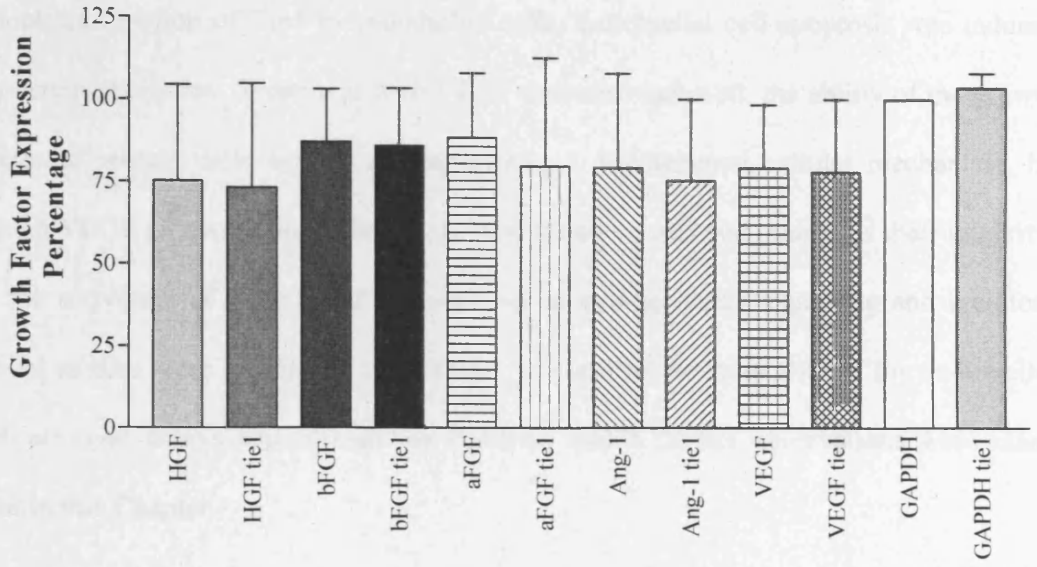


Figure 5.13 Effect of Tie1 endodomain overexpression on an array of angiogenic growth factors. HMEC-1 cells were grown 80-90% confluenece and transfected with empty vector or Tie1 endodomain. mRNA from HMEC-1 cells was isolated and converted to cDNA using Reverse Transcriptase and oligonucleotides specific to angiogenic growth factors were used to amplify HMEC-1 cell cDNA by PCR using the established optimised conditions. PCR products were electrophoresed onto a 1% agarose gel. The level of expression for each of the oligonucleotides was carried out quantifying the density of each band of the expected size product. Growth factor expression was normalised to GAPDH expression and calculated to give percentage growth factor expression. Statistical analysis of the data was done by performing the Students't' test.

5.3 Discussion

The aim of the studies described in this Chapter was to determine the potential anti-apoptotic function of Tie1 in endothelial cells. Endothelial cell apoptosis was induced by serum-starvation. Treatment with VEGF demonstrated well, the ability of the growth factor to protect cells against cell apoptosis. A fundamental cellular mechanism, by which VEGF promotes the formation of new blood vessels and maintains their integrity, is the activation of endothelial cell survival or anti-apoptotic signalling and therefore initial studies were performed with VEGF to confirm the suitability of the endothelial cell survival assays and formed the basis on which further observations were made within this Chapter.

To gain insight into a potential anti-apoptotic function of Tie1, two forms of the receptor were expressed in endothelial cells; full-length and Tie1 endodomain. It was found that expression of either full-length or Tie1 endodomain reduced endothelial cell apoptosis, furthermore this reduction in apoptosis was found to be similar to that seen in cells following addition of serum. To test if it was endothelial cell specific, experiments using non-endothelial CHO cells were performed. The anti-apoptotic effect seen in endothelial cells was not seen in CHO cells, but additional experiments would be needed with other cell types to confirm if the apparent Tie1-induced cytoprotection is endothelial cell-specific. In this regard it is interesting to note that recently there has

been a growing body of evidence linking Tie1 to epithelial cell regulation, namely in breast cancer (Tseng 2001; Craven 2003; Yang 2003).

Data produced in this study, suggests Tie1 has no cyto-protective effect when overexpressed in non-endothelial CHO cells. Over-expression in CHO cells resulted in no reduction of apoptosis, rather apoptosis was calculated to be either equivalent (CHO cells overexpressing full-length Tie1) to non-transfected CHO cells or greater (CHO cells over expressing endodomain Tie1). This phenomenon was further exemplified, in studies presented in this Chapter regarding the possible cyto-protective property of conditioned medium by the release soluble growth factors into the surrounding medium, to prevent further apoptosis.

Incubation of endothelial cells in conditioned medium from CHO cells overexpressing full-length or endodomain Tie1 resulted in a substantial increase in endothelial cell apoptosis. Notably, the amount of apoptosis measured for conditioned medium from non-transfected CHO cells, was much less than when conditioned medium was used from full-length or endodomain Tie1 overexpressing CHO cells; strongly suggesting that the overexpression of Tie1 in non-endothelial cells does not protect cells from apoptosis. It is intriguing, the profound effect non-endothelial conditioned medium had on endothelial cell survival. It is tempting to speculate that conditioned medium from non-endothelial cells abolishes cell survival completely such that cell apoptosis occurs to a greater extent.

Conversely, conditioned medium from endothelial cells used to incubate endothelial cells overexpressing Tie1 resulted in decreased endothelial cell apoptosis. Conditioned medium from either HUVE cells or HMEC-1 cells (which over-expressed full-length or endodomain Tie1), used to incubate endothelial cells resulted in a significant reduction of apoptosis, clearly indicating a cyto-protective property of conditioned medium from Tie1 overexpressing endothelial cells. The mechanism by which conditioned media protects endothelial cells against apoptosis remains to be elucidated. However, it is hypothesised that endothelial cells upon induction of apoptosis, release growth factors that promote cell survival. To examine the effects of Tie1 overexpression and growth factor expression, RT-PCR was performed to screen for the expression of angiogenic growth factors in human microvascular endothelial cells. The data obtained from experiments performed in this study, failed to identify a growth factor in endothelial cells that was modulated by Tie1 overexpression. Thus, more studies are needed to screen further for a candidate growth factor(s) which is modulated by Tie1 overexpression.

Chapter Six

Discussion

6.0 Discussion

The endothelial cell receptor tyrosine kinase, Tie1, plays an essential role in the latter stages of vascular development. It is critical in blood vessel stabilisation and ensures the differentiation of periendothelial cells. Studies have shown mice lacking Tie1 die before embryonic day 15 (E15) due to loss of microvascular integrity, which results in haemorrhaging, however, the major vessels and extra-embryonic vasculature are phenotypically normal (Puri et al., 1995; Suri et al., 1995). Tie1 deficiency also results in defects in increased vascular density in the later stages of angiogenesis (Partanen et al., 1996). Tie1 is expressed throughout the adult endothelium and plays a role in maintaining vessel stabilisation in the long term, it is therefore of interest, although has yet to be identified whether Tie1 needs to signal continuously in order to maintain this stable state in the adult endothelium.

At the time of writing this thesis, there has been no ligand identified for Tie1 and hence knowledge of how Tie1 functions and its downstream signalling intermediates had yet to be elucidated. However, *in vivo* studies have indicated an essential role for Tie1 in promoting endothelial cell survival and integrity in vascular development (Sato et al., 1995; Puri et al., 1995; Patan. 1998).

Previous studies have failed to define the cellular effects of Tie1 or a signalling mechanism(s) for this receptor. In this study, the mechanisms by which the receptor becomes phosphorylated have been investigated. One of the principal findings of this work has been the discovery that Tie1 undergoes auto-phosphorylation via its own kinase

activity if the action of phosphatases is suppressed, despite previously being documented that Tie1 auto-phosphorylation is undetectable (Marron et al., 2000). Furthermore, it was also discovered that the related RTK Tie2 increases tyrosine phosphorylation of Tie1 endodomain, even in the absence of tyrosine phosphatase inhibition. At the time of discovery, this was a novel finding and marked a significant contribution to understanding the importance of Tie2 on Tie1 activity. Subsequent experiments have since been made by Saharinen et al. (2005), to confirm the ability of Tie2 to transphosphorylate Tie1.

From studies examining the crystal structure of Tie2, it has been shown that a conformational change occurs upon activation of the protein in order to expose both the substrate-binding site and tyrosine residues, Y1101 and Y1112, for Tie2 phosphorylation and signalling. Since the overall amino acid identity between Tie1 and Tie2 is 44% homology at the amino acid level and 76% homology in the intracellular domain it is conceivable that Tie1 has the same structure as Tie2 (Wilks, 1989; Partanen *et al.*, 1992;). Moreover, it is possible that in Tie1 a single tyrosine residue might be responsible for relieving inhibition of Tie1 from a de-phosphorylated state, to become auto-phosphorylated, a theory that has been widely documented (Johnson et al, 1996, Shewchuk et al., 2000; Wybenga-Groot et al., 2001). For Tie2, tyr-897, located in the N-terminal domain, is thought to have a role in negatively regulating the activity of the receptor by preventing the dimerisation of the kinase domains or recruiting phosphatases when it is phosphorylated (Shewchuk et al., 2000). To test the hypothesis of a single tyrosine phosphorylated on Tie-2, which results in a conformational change to stimulate

Tie-1, further experiments need to be carried out. Principally, the Tie2 phosphorylation site on Tie1 should be defined, possibly by phospho-peptide mapping; mutation of tyrosine to phenylalanine in order to test the inhibition of Tie2 in Tie1; mutation of tyrosine to glutamate to test if Tie1 is auto- activated.

Tie1 binding to Tie2 is mediated by the intracellular domains of the receptors i.e. juxtamembrane region, transmembrane domain and carboxyl terminus (Tsiamis et al, 2002). Data presented in this study, indicate that the transmembrane and/or juxtamembrane region of Tie1 appears to suppress the ability of the endodomain to undergo tyrosine phosphorylation. To further confirm and define the apparent inhibitory influence of the transmembrane and/or juxtamembrane region, it would be of interest to examine the effect of swapping the Tie1 transmembrane and juxtamembrane regions with Tie-2. Additionally, based on the evidence obtained from the crystal structure of Tie2, experiments would need to examine a possible inhibitory mechanism, namely the activation loop of the carboxyl-terminus to the juxtamembrane region.

Since there has been no ligand identified for Tie1, studies to investigate Tie1 signalling and function have predominantly been dependent on the use of chimeric receptors and overexpression of Tie1 in non-endothelial cells. Cartilage oligomeric matrix protein (COMP) Ang1 was originally produced as an alternate to Ang1, as it was recognised that Ang1 was a suitable candidate for use in therapeutic angiogenesis by enhancing endothelial cell survival and preventing vascular leakage. Subsequently, COMP-Ang1 has also been found to be more potent than native Ang1 in tyrosine phosphorylating both

Tie2 (Cho et al., 2004) and Tie1 (Saharinen et al., 2005). Based on this evidence, COMP-Ang1 was used in experiments in endothelial cells to investigate the effect of COMP-Ang1 on Tie1 signalling mechanisms. The data obtained indicate tyrosine phosphorylation of Tie1 and Tie2 occurred following stimulation by COMP-Ang1. Having found that COMP-Ang1 stimulation of Tie1 and Tie2 induces tyrosine phosphorylation of both receptors, the next stage of investigation was to dissect by what mechanism COMP-Ang1 stimulates Tie1 tyrosine phosphorylation. Experiments presented in Chapter Four, attempted to reveal if COMP-Ang1 stimulation of Tie1 occurs through the receptor alone by blocking Tie2. Attempts to block Tie2 using a Tie2 blocking antibody, peptide and stable transfected cell, were unsuccessful in the blockade of Tie2. Thus, this prevented determination of the role of Tie2 in COMP-Ang1 induced Tie1 phosphorylation in endothelial cells. Furthermore, it was not possible therefore, to examine the downstream signalling for Tie1 in the absence of Tie2 activation. Future work to attempt to block Tie2 would make use of short interfering RNA (siRNA). siRNA has been used to successfully study signal transduction in endothelial cells; Tie2 expression was reduced by 80% following siRNA treatment (Tai et al, 2005).

The effects of COMP-Ang1 stimulation on Tie1 endodomain and subsequent truncation products in endothelial cells was examined. It was found that COMP-Ang1 stimulation also resulted in tyrosine phosphorylation of truncated Tie1, specifically at 42kDa and 45kDa. There was increased tyrosine phosphorylation of a protein at 42kDa following COMP-Ang1 stimulation. It is conceivable that this protein could represent the truncated form of Tie1; conversely, it is also possible that the tyrosine phosphorylation seen does

not represent phosphorylation of Tie1 but phosphorylation of a protein that has associated to the intracellular domain of Tie1.

Following initial cleavage of Tie1, the transmembrane and intracellular domains undergo a secondary cleavage which is mediated by γ-secretase. Based on this knowledge, the strategy employed to determine whether the 42kDa phosphorylated protein is Tie1 examined the effects of inhibiting γ-secretase action in endothelial cells. The data obtained confirms the 42kDa protein is not truncated Tie1, since the secondary cleavage event that results in the truncated Tie1 product of 42kDa is mediated by γ-secretase and thus would be blocked following treatment with the γ-secretase inhibitor. This finding has demonstrated for the first time the presence of a phospho-protein associated to the intracellular domain of Tie1 that has yet to be identified.

Tie1 endodomain signalling, albeit as part of a chimeric receptor, has been implicated in the activation of PI 3 kinase/Akt pathway to inhibit apoptosis (Kontos et al., 2002). In breast and colon tumours, overexpression of Tie1 endodomain has been hypothesised to be a direct result of the endodomain's involvement in activating the PI 3 kinase/Akt pathway, thereby allowing invading cells to suppress apoptotic signals (Craven et al. 2003; Yang et al. 2003). Studies in this thesis attempted to examine the potential involvement of Tie1 in suppressing apoptosis in endothelial cells. Overexpression of Tie1 in endothelial cells resulted in reduced apoptosis. Experiments were carried out to test whether this phenomenon was endothelial cell specific. Overexpression of Tie1 in the non-endothelial CHO cell line revealed that apoptosis was not suppressed. The

cytoprotective effect seen in endothelial cells overexpressing Tie1 could be due the release of soluble growth factors. For this reason, the effect of conditioned medium from endothelial cells on apoptosis was examined. The phenomenon of suppressed apoptosis in endothelial cells overexpressing Tie1 was maintained, in experiments with conditioned media. Apoptosis seen in non-endothelial cells overexpressing Tie1 treated with conditioned medium from endothelial cells was not suppressed. Further experiments in other non-endothelial cells are needed to conclude whether the anti-apoptotic effect of overexpressed Tie1 is endothelial cell specific. To examine the effect of Tie1 overexpression on growth factors in endothelial cells, an array of angiogenic growth was screened, however, data from these experiments was inconclusive and thus further work is needed.

Work presented in this thesis outlines for the first time the possible mechanisms by which Tie1 is activated and how Tie1 activity is regulated. Work presented herein has further implicated Tie1 to have a role in endothelial cell survival. To conclude, the work presented in this thesis to date has in part, contributed to understanding how the orphan RTK Tie-1 functions and signals. The identification of a ligand for Tie1 will facilitate future studies into the function of Tie1 and its downstream signalling pathways. Current research underway is based on evidence that exists relating to binding partners of Tie-2; signalling intermediates such as p85 of PI3Kinase, Shp2 and Grb proteins. This information will enhance the knowledge of the molecular mechanisms controlling angiogenesis.

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