Investigations into the Involvement of Tie2 in Endothelial:Mural Cell Interactions

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Abstract

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The interaction of endothelial cells and mural cells play a critical role in the stabilisation and maturation of the embryonic and postnatal vasculature. Several pathological situations exhibit poor and abnormal endothelial and mural cell association. In this study immunohistochemical analysis revealed dysmorphogenic microvessels within atherosclerotic plaques are lacking mural cells. Similar dysmorphogenic mural cell poor vessels were also observed in human myocardium following laser induced myocardial injury. In both cases elevated VEGF was detected associated with the mural cell poor vessels. To gain insight into the mechanisms controlling mural cell recruitment assays were established to examine directly smooth muscle cell migration and adhesion between smooth muscle cells and endothelial cells in culture. These assays were used to study the involvement of the angiopoietin/Tie2 system in recruitment and retention of mural cells. Ang-1 was observed to stimulate endothelial-directed migration of putative mural cell precursors. The ligand also increased adhesion between endothelial cells and smooth muscle cells. To investigate further the possible role of Tie2 in interaction between endothelial cells and mural cells a mutant form of the receptor found in inherited venous malformations (VM) was studied. The form of VM associated with this mutant receptor is characterised by dysmorphogenic, enlarged mural cell vessels. Surprisingly, mutant Tie2 did not inhibit endothelial-induced mural cell migration or adhesion between endothelial cells and mural cells. Taken together these data suggest Tie2 has a role in regulating endothelial:mural cell interaction. Furthermore, the presence of mural cell poor vessels in the atherosclerotic plaque, injured myocardium and inherited VM may reflect escape of these vessels from regression by the presence of survival factors in the case of the plaque and injured myocardium, or constitutive anti-apoptotic signalling from mutant Tie2 in the case of VM.

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Abbreviations

ABIN-2	A20 binding inhibitor of NF-κB activation
AEBSF	4-(2-aminoethyl) benzene-sulphonyl fluoride
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
Ang-3	Angiopoietin-3
Ang-4	Angiopoietin-4
ATCC	American type culture collection
BAE cells	Bovine aortic endothelial cells
BSA	Bovine serum albumin
CHO cells	Chinese hamster ovary cells
DAB	Diaminobenzidine
DABCO	1,4-diazobicyclo-(2,2,2)-octane
DAG	Diacyglycerol
DAPI	4,6-diamidino-2-phenylindole-2-HCl
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
Dok-R	Dok-related docking protein
DTT	Dithiothreitol
ECL	Enhanced chemiluminesence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FAK	Focal adhesion kinases
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGF-2	Fibroblast growth factor-2
Flk-1	Fetal liver kinase-1
Flt-1	Fms-like tyrosine kinase-1
Flt-4	Fms-like tyrosine kinase-4

GAP	GTPase activating protein
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
Grb2	Growth factor receptor bound protein 2
Grb7	Growth factor receptor bound protein 7
Grb14	Growth factor receptor bound protein 14
GST	Glutathione S-transferase
HRP	Horseradish peroxidase
HUVE cells	Human umbilical vein endothelial cells
HIF-1/2	Hypoxia-inducible transcription factors
HRE	Hypoxia-responsive element
IAP	Inhibitor of apoptosis protein
Ig	Immunoglobulin
IL	Interleukin
Kb	Kilobase
kDa	Kilodalton
KDR	Kinase domain region
LB	Luria bertani
MAPK	Mitogen activated protein kinase
MEM	Minimum essential media
MHE cells	Mouse heart endothelial cells
MMP	Matrix metalloproteinase
N-cadherin	Neural cadherin
NF-κB	Nuclear factor-κB
PAGE	Polyacrylamide gel electrophoresis
Pak	p21-activating kinase
PBS	Phosphate buffered saline
PDGF	Platelet derived endothelial cell growth factor
PDGFR-α	Platelet derived endothelial cell growth factor receptor-α
PDGFR-β	Platelet derived endothelial cell growth factor receptor-β
PDK 1/2	Phosphoinositide dependent kinases 1/2
PECAM-1	Platelet endothelial cell adhesion molecule-1
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase

PIP$_3$	Phosphatidylinositol 3,4,5-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PlGF	Placenta growth factors
PLCγ	Phospholipase Cγ
PMR	Percutaneous myocardial revascularisation
PTB	Phosphotyrosine binding domains
RGD	Arginine-glycine-aspartic acid
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
SH2	Src-homology-2
SHP2	tyrosine phosphatase SH2-containing protein
SMA	Smooth muscle actin
STAT	Signal transducer and activator of transcription
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
TIAs	Transient Ischaemic Attacks
Tie1	Tyrosine kinase with immunoglobulin like loops and EGF homology domains
Tie2/Tek	Tunica interna endothelial cell kinase
TNF-α	Tumour necrosis factor-α
Tx100	Triton X-100
VCAM-1	Vascular cell adhesion molecule-1
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor-1
VEGFR-2	Vascular endothelial growth factor receptor-2
VSM cells	Vascular smooth muscle cells

Suppliers

Abcam – Cambridge, Cambridgeshire, UK

Amersham – Little Chalfont, Buckinghamshire, UK

ATCC – Manassas, Virginia, USA

BDH Laboratories – Poole, Dorset, UK

Becton Dickinson – Cowley, Oxford, UK

Bibby Sterillin – Stone, Staffordshire, UK

Calbiochem – supplied by Merck Biosciences, Beeston, Nottinghamshire, UK

Cambrex – Wokingham, Berkshire, UK

Cell Signaling Technology – Hitchin, Hertfordshire, UK

Dako Cytomation – Ely, Cambridgeshire, UK

Duchefa Biochemie – Amsterdam, Netherlands

Fahrenheit – Rotherham, South Yorkshire, UK

Fisher Scientific – Loughborough, Leicestershire, UK

Harlan Sera Laboratories – Bicester, Oxon, UK

Invitrogen – Paisley, Scotland, UK

Melford Laboratories – Ipswich, Suffolk, UK

Millipore – Watford, Hertfordshire, UK

Molecular Probes – Eugene, Oregon, USA

Nalge Nunc International – Rochester, New York, USA

New England Biolabs – Hitchin, Hertfordshire, UK

Novocastra – supplied by Vector Laboratories, Peterborough, UK

Oncogene (CN Biosciences Ltd) – Beeston, Nottinghamshire, UK

Perkin Elmer – Beaconsfield, Buckinghamshire, UK

Promega –Southampton, Hampshire, UK

Qiagen – Crawley, West Sussex, UK

R & Systems – Abingdon, Oxfordshire, UK

Roche – Lewes, East Sussex, UK

Santa Cruz Biotechnology – Santa Cruz, California, USA

Scanalytics – Fairfax, Virginia, USA

Sigma – Poole, Dorset, UK

Stratagene – La Jolla, California, USA

Targeting Systems – Santee, California, USA

Chapter One

Introduction

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Introduction

1.1 Introduction

The vascular network is essential for the distribution of nutrients and oxygen as well as the removal of waste products, and is therefore one of the first organ systems to be developed in the embryo (Hirschi et al. 1998). Formation of new blood vessels is essential for embryonic development as well as maintaining the adult vasculature in situations such as wound healing, ovulation and placental development. The vascular network is made up of thick walled arteries and thin, flexible walled veins which become progressively smaller giving rise to arterioles and venules, respectively. Arterioles and venules are connected by capillaries allowing the exchange of nutrients, oxygen and waste between blood and tissue.

A blood vessel is made up of several distinct layers; the innermost layer, the intima, which consists of endothelial cells (Adams and Watt 1993); the media, consisting of mural cells, smooth muscle cells or pericytes (large vessels and microvessels, respectively) (Sims 1986); and the adventitia which contains loose connective tissue and small vessels (Hirschi et al. 1998).

1.2 Vasculogenesis and angiogenesis

Nascent vessels form by two distinct processes, vasculogenesis and angiogenesis. Vasculogenesis is the process whereby endothelial cell precursors called angioblasts associate to form vessel tubules (Risau 1991 and 1995). Directed by fibroblast growth factor-2 (FGF-2), angioblasts originate in the mesoderm giving rise to endothelial cells (Poole et al. 2001). Differentiation of angioblasts into endothelial cells together with proliferation and aggregation result in the formation of primitive tubular structures defining the earliest parts of the vasculature (Noden 1989; Risau 1997).

The primitive embryonic network is further refined by sprouting and budding into a larger network, a process called angiogenesis. Several physiological processes, such as, embryonic development and endometrial proliferation require angiogenesis (Blood and Zetter 1990; Paku and Paweletz 1991; Fidler and Ellis 1994). Angiogenesis is not restricted to the developing embryonic vasculature and can occur during the female reproductive cycle, during pregnancy and in wound healing. It has been defined into two types, sprouting angiogenesis and non-sprouting

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angiogenesis (Risau 1997), and it is thought that the vasculature begins life as a plexus of primitive capillary tubes before subsequent modifications give rise to a more complex network of vessels in the adult (Beck and D'Amore 1997).

There have been many angiogenic factors identified that regulate vasculogenesis and angiogenesis. These include members of the fibroblast growth factor (FGF) family (Esch et al. 1985; Lobb et al. 1985; Shing et al. 1985), tumour necrosis factor-α (TNF-α) (Leibovich et al. 1987; Frater-Schroder et al. 1987), transforming growth factor-α and β (TGF-α and β) (Roberts et al. 1986; Schreiber et al. 1986) and platelet derived growth factor (PDGF) (Ishikawa et al. 1989).

The best characterised physiological angiogenic factor is vascular endothelial growth factor (VEGF) (Connolly et al. 1989; Ferrara and Henzel 1989; Levy et al. 1989). VEGF and its receptors regulate vasculogenesis and angiogenesis (Kim et al. 1993; Millauer et al. 1994; Shalaby et al. 1995). The growth factor is relatively specific for endothelial cells and is involved in vascular patterning and capable of stimulating endothelial cell proliferation and chemotaxis (Ferrara 1999). There are five forms of VEGF (A to E), as well as, placenta growth factor (PlGF) and they all have differential binding to three related receptors VEGFR-1/Flt-1, VEGFR-2/Flk-1 and VEGFR-3/Flt-4. Briefly, the well characterised VEGF-A seems to induce endothelial cell proliferation, migration and tubule sprouting. These effects are mediated mainly via the VEGFR-2 receptor (Gale and Yancopoulos 1999). Gene knockout studies observed that VEGF-A and VEGFR-2 are critical in early vasculogenesis as blood islands, endothelium and tubules fail to develop in the absence of ligand or receptor (Shalaby et al. 1995; Carmeliet et al. 1996; Ferrara et al. 1996). Lack of VEGFR-1 does not affect formation of vascular tubules but vessels become poorly organised (Fong et al. 1995). The role of VEGF and receptors in angiogenesis are covered in more detail in Section 1.5.

A second family of ligands involved in vessel formation and relatively specific for endothelial cells are the angiopoietins (Davis et al. 1996; Suri et al. 1996; Maisonpierre et al. 1997; Valenzuela et al. 1999). The angiopoietins interact with the mainly endothelial cell specific Tie receptor, Tie2. So far, four angiopoietins have been discovered and they all interact with Tie2. The two best characterised ligands are Ang-1 and Ang-2. Ang-1 activates Tie2 whereas at least in some situations Ang-2 has been shown to antagonise the stimulatory effects of Ang-1 (Maisonpierre et al. 1997). Tie2 is a member of the Tie family of receptor tyrosine kinases

(RTKs) (Dumont et al. 1993). The other member of this family is Tie1 and as yet no ligand has been identified for Tie1. Mice deficient in Ang-1 and Tie2 have been reported to form a normal primitive vasculature but exhibit embryonic lethality as a result of increased vessel remodelling and decreased vascular network stabilisation (Dumont et al. 1994; Sato et al. 1995; Folkman and D'Amore et al. 1996; Suri et al. 1996). Mice deficient in Tie1 also result in embryonic lethality due to endothelial cells lacking integrity, thus increasing hyper-permeability of smaller vessels (Puri et al. 1995; Sato et al. 1995). The roles of the angiopoietins and Tie receptors in vessel development are discussed in more detail in Section 1.6.

1.2.1 Overview of vessel formation

A model for vessel formation in vasculogenesis and angiogenesis has been formulated. In vasculogenesis, it appears that VEGF activates VEGFR-2 receptor to induce differentiation of angioblasts into endothelial cells and activation of endothelial cell migration and proliferation. Furthermore, VEGF binding to VEGFR-1 appears to regulate endothelial cell:cell and cell:extracellular matrix contact initiating endothelial tubule formation. Mature endothelial tubules form upon the activation of the Tie2 receptor by Ang-1, coinciding with the recruitment of mural cells thus maintaining vessel quiescence and integrity (Hanahan 1997).

In angiogenesis, the formation of new blood vessels from the existing vasculature can be divided into four phases. 1) Angiogenic growth factors signal through their respective receptor to initiate the secretion of proteolytic enzymes by endothelial cells which breakdown the basement membrane of the existing capillary network; 2) Endothelial cells migrate and proliferate forming capillary tubules (Ausprunk and Folkman 1977); 3) Growth factor expression by endothelial cells is altered resulting in the recruitment of mural cells towards the newly formed endothelial tubule and mural cell proliferation; 4) Association of mural cells marks the stabilisation of the vessel. Figure 1.1 represents the simplified model for the roles of VEGF and receptors, and Tie2 plus Ang-1 and Ang-2 in vasculogenesis and angiogenesis (Hanahan 1997). These receptors and ligands are reviewed in more detail in Sections 1.5 and 1.6.
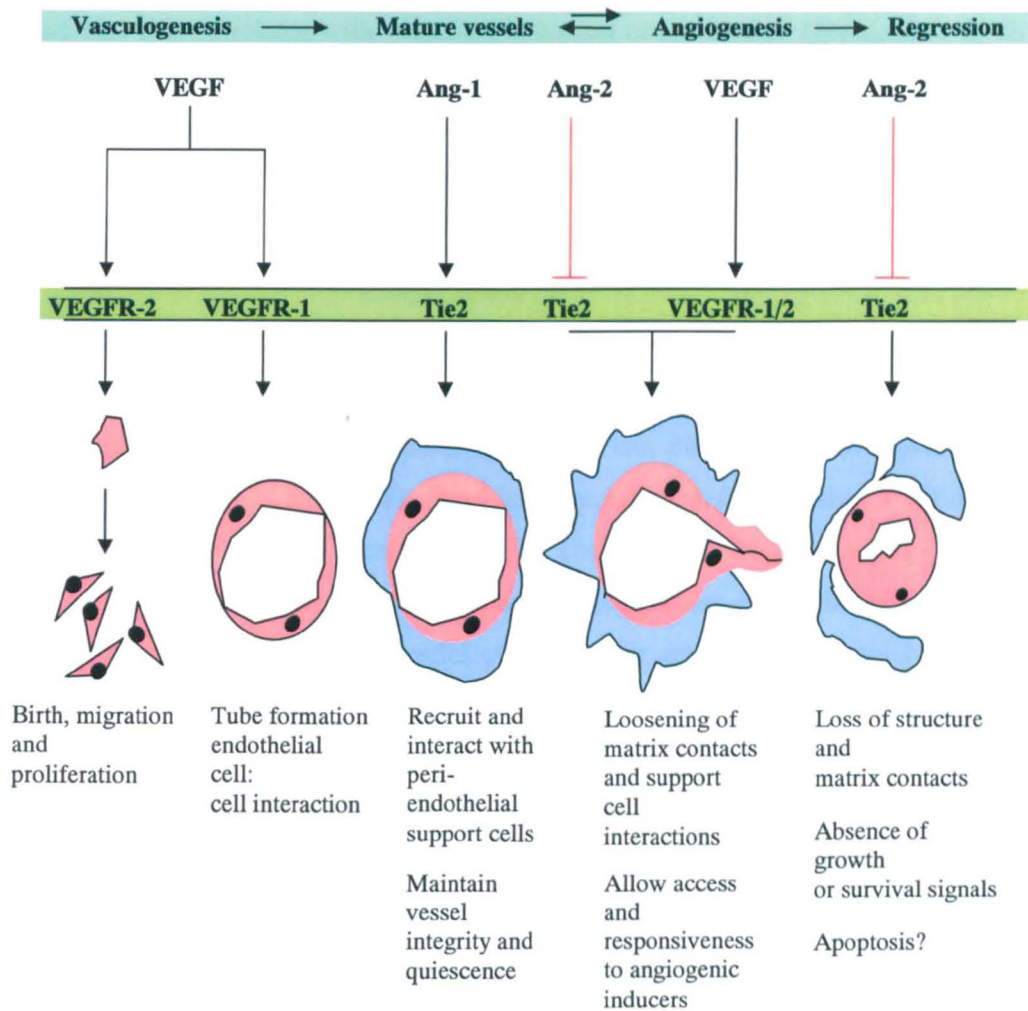
Figure 1.1 **Schematic representations of vessel morphogenesis, maintenance and remodelling by the principal endothelial receptor tyrosine kinases, VEGF receptors and Tie2 plus their ligands.** VEGF, Ang-1 and Ang-2 have similar cytoplasmic signalling domains yet elicit distinctive signalling responses. In vasculogenesis, VEGF elicits endothelial proliferation via VEGFR-2, inducing formation, migration and proliferation of endothelial cells. Binding of VEGF by VEGFR-1 appears to modulate the endothelial cell interaction and the formation of tubules resulting in the primitive vessel network. Evidence suggests that Ang-1 binding to Tie2 recruits and maintains the association of mural cells thus stabilising the newly formed blood vessels. The role of Ang-2 remains unclear although it was initially established as an antagonist of Ang-1 activation of Tie2. Ang-2 expression appears to result in loosening of endothelial cell contacts with extracellular matrix and mural cells. It has been suggested that loosening of endothelial cell contacts increases vessel susceptibility to VEGF and other angiogenic growth factors. The lack of VEGF co-expression results in endothelial cell apoptosis, possibly due to the absence of a survival signal. Adapted from Hanahan 1997.

1.3 Vessel maturation

During embryonic and adult vessel development, mural cells associate with the newly formed vasculature and this correlates with vessel stabilisation or maturation (Hungerford et al. 1996). Microvessels lacking mural cell support are prone to endothelial death and vessel regression (Benjamin et al. 1998 and 1999).

1.3.1 Mural cells

Mural cells are cells of smooth muscle cell lineage and there are two types, pericytes or vascular smooth muscle cells (Sims 1986). It has been postulated that the smooth muscle cell precursor is fibroblastic, and under specific conditions these cells can differentiate into several cell types. The 10T1/2 cell line is an example of a fibroblast population with the ability to differentiate into smooth muscle cells (Pinney and Emerson 1989). The origin of the vascular smooth muscle cell appears to be the undifferentiated mesoderm surrounding primitive endothelial tubules (Drake et al. 1998). However, there is controversial evidence that smooth muscle cells could originate from embryonic endothelial cells (Williams et al. 1994; Williams 1995; De Ruiter et al. 1997).

In the developmental process mural cells are derived from the surrounding mesenchyme of the endothelium or by migration and de-differentiation of arterial smooth muscle cells (Nehls et al. 1992; Nicosia and Villaschi 1995; Beck and D'Amore 1997; Benjamin et al. 1998; Liu et al. 2000). In general, pericytes are associated with small and medium sized vessels and smooth muscle cells are associated with large vessels (Carmeliet 2003). Pericytes, also known as Rouget cells, abluminally associate with vascular capillaries and post-capillary venules (Hirschi and D'Amore 1996). They are surrounded by basement membrane and closely associate with endothelial cells of these microvessels (Diaz Flores et al. 1991). The expression of smooth muscle actin (αSMA) suggests a contractile function for the pericyte and vascular smooth muscle cell. In blood vessels vascular smooth muscle cells are found in several phenotypic states showing a high degree of plasticity in response to vascular injury (Moiseeva 2001). Pericytes are also a heterogenous population and their morphology is very much dependent upon their specific microenvironment (Hirschi and D'Amore 1996; Thomas 1999; Liu et al. 2000). In development as the levels of VEGF expression return to normal following angiogenesis, mural cells proliferate and migrate to the site of branching or sprouting (Nicosia and Villaschi 1995; Beck and D'Amore 1997; Benjamin et al. 1998).

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1.3.2 Mural cells regulate vessel regression

The contact of endothelial cells and mural cells has been postulated to inhibit endothelial cell proliferation, stabilise vessels and promote endothelial cell survival (Lindahl et al. 1997; Benjamin et al. 1998; Hirschi et al. 1998). Both endothelial and mural cells undergo phenotypic changes on interaction (Orlidge and D'Amore 1987; Sato and Rifkin 1989; Hirschi et al. 1999). A key regulator of these processes is TGF-β.

Endothelial cell contact with either mesenchymal cells or smooth muscle cells results in TGF-β activation by the proteolytic cleavage of the precursor form of the ligand (Antonelli-Orlidge et al. 1989; Rohovsky et al. 1996; Hirschi et al. 1998). TGF-β inhibits endothelial cell proliferation and migration, as well as enhancing differentiation of the mesenchyme into pericytes (Orlidge and D'Amore 1987; Sato and Rifkin 1989; Rohovsky et al. 1996; Hirschi et al. 1998). Targeted mutations of the TGF-β gene significantly affected the signalling pathways regulating mural cell differentiation (Li et al. 1999; Oh et al. 2000).

Observations in retinal vascularisation revealed that vessels lacking pericyte support were prone to regression (Benjamin et al. 1998). The requirement for endothelium and pericyte association was also demonstrated in the selective ablation of pericyte free vessels of the xenografted glioma tissue (Benjamin et al. 1999). Upon removal of VEGF microvessels negative for pericyte association regressed, whereas pericyte positive vessels were maintained (Benjamin et al. 1999). It appears that the mural cell provides a key survival signal for the vasculature. A potential survival signal in vessel stabilisation is the angiogenic ligand, Ang-1. This ligand is expressed by the mural cell and is a potent anti-apoptotic signal for endothelial cells (Kwak et al. 1999). Implications for Ang-1 in endothelial cell survival are discussed in more detail later in this study.

Another angiogenic-associated ligand, platelet-derived growth factor (PDGF), has a key role in the acquisition of mural cells by newly formed vessels. The PDGF family consists of homo (PDGF-A or B) or heterodimers (PDGF-AB) assembled from the two PDGF chains, A and B. The receptors are dimers arranged in a similar manner to the ligands. PDGFR-$\alpha\alpha$ and PDGFR-$\alpha\beta$ bind both PDGF-A and AB, whereas PDGFR-β can only bind to B. PDGF is known to regulate a diverse array of cellular processes including cell migration, proliferation, transformation and apoptosis (Deuel 1987; Kim et al. 1995; Unlu et al. 1998; Rosenkranz and

Kazlauskas 1999). For example, both PDGF-A and B are strong mitogens and inducers of cell motility. PDGF-A appears to specifically mediate fibroblast migration but, PDGF-B mediates fibroblast, endothelial cell and smooth muscle cell migration (Yu et al. 2001). Furthermore, PDGF-B induces proliferation of endothelial cells via the PDGFR-β receptor specifically in tubule formation (Battegay et al. 1994).

There is vast evidence for PDGF in the recruitment of pericytes to newly formed vessels in angiogenesis. Mice deficient for PDGF-B or PDGFR-β result in identical phenotypes causing widespread microvascular leakage and haemorrhage (Leveen et al. 1994; Soriano 1994). The microvascular dysfunction is due to a severe deficiency of pericytes (Lindahl et al. 1997). Although, in the absence of PDGF-B or PDGFR-β pericytes appear to be induced expansion and spreading does not occur (Hellstrom et al. 1999). The failure of pericyte expansion and spreading is partly due to a reduction in pericyte proliferation. Therefore, disruption of PDGF-B/PDGFR-β signalling could also contribute to impaired pericyte migration (Gerhardt and Betsholtz 2003).

Expression of PDGF-B is observed from the sprouting endothelium and PDGFR-β is expressed by pericytes/smooth muscle cells (Lindahl et al. 1997; Hellstrom et al. 1999). This suggests a paracrine mode of interaction between endothelial cells and mural cells (Gerhardt and Betsholtz 2003). Research by Crosby et al. (1998) using chimeras composed of PDGFR-β positive and negative cells, found only positive cells populated pericyte/smooth muscle cell compartments. Therefore, pericytes require PDGFR-β expression for development. Further evidence, is observed in endothelial cell specific ablation of PDGF-B resulting in pericyte/smooth muscle cell deficiency (Enge et al. 2002). However, ablation of PDGF-B in haematopoietic cells or neurons has no effect on the vascular network (Buetow et al. 2001; Kaminski et al. 2001; Enge et al. 2003). This research suggests the requirement of the endothelial cell PDGF-B signal in pericyte recruitment.

Regulation in the amount of PDGF-B released by endothelial cells is also crucial. PDGF-B is only produced by a subset of endothelial cells in angiogenesis (Hellstrom et al. 1999). In developing arteries PDGF-B expression is observed at the site of pericyte recruitment and smooth muscle cell proliferation (Lindahl et al. 1997; Hellstrom et al. 1999). Furthermore, in tumour vessels expression of PDGF-B in endothelial cells is heterogenous (Abramsson et al. 2002).

PDGF-B heterozygote mice causing a reduction in PDGF expression by half resulted in decreased pericyte density (Hammes et al. 2002). Unpublished data by Lindholm and Betsholtz from transgenic over-expression of PDGF-B in mice produces embryonic lethality due to pericyte abundance (Gerhardt and Betsholtz 2003). Therefore, PDGF-B and PDGFR-β are critically required for regulated mural cell recruitment and coverage.

1.3.3 Endothelial:mural cell adhesion

The mechanisms whereby mural cells interact with endothelial cells in newly formed microvessels are poorly understood. One molecule implicated in mediating adhesion between endothelial cells and mural cells is neural cadherin (N-cadherin). Originally identified in the nervous tissue it is expressed in the developing and mature central nervous system (Utton et al. 2001), and functions in several neuronal processes including axon growth and guidance (Bixby and Zhang 1990; Doherty et al. 1991; Riehl et al. 1996; Arndt et al. 1998) and synaptic adhesion (Fannon and Colman 1996; Uchida et al. 1996).

In the developing mouse embryo N-cadherin is expressed initially in the mesoderm and primitive streak at embryonic day (E) 7.5. At E8.5 expression is evident in the neural plate and tube, pre-cardiac mesoderm and somites. Expression is then observed in the notochord, otic and optic vesicles and in the heart tube on E9.5. Mutating the N-cadherin gene caused several neuronal and somitic defects, as well as, vascular defects. Homozygous mutants died at embryonic day 10, due to the failure of the primitive heart to develop (Radice et al. 1997).

Gilbertson-Beadling and Fisher (1993) observed expression of N-cadherin in both endothelial cells and vascular smooth muscle cells. Within the rat aorta N-cadherin is localised to the endothelium and the smooth muscle cell layer beneath the internal elastic membrane. Adhesion of rat endothelial cells and smooth muscle cells in culture could be inhibited by approximately 25% in the presence of N-cadherin antibodies (Gilbertson-Beadling and Fisher 1993).

Further studies, revealed that there was expression and regulation of N-cadherin in endothelial cells and pericytes, in early angiogenesis of the brain and eye of chick embryos (Gerhardt et al. 1999). N-cadherin expression was observed at the contact zones of the two cell types. Using blocking antibodies to N-cadherin injected into the chick brain at embryonic days 4 and 5 resulted in defective pericyte adhesion, increased pericyte recruitment and disturbed vascular

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morphogenesis. These defects produced massive haemorrhaging of the brain (Gerhardt et al. 2000).

1.4 Endothelial RTKs regulating blood vessel formation

Receptor tyrosine kinases have central roles in early and later stages of blood vessel formation. Two families of RTKs expressed predominantly in endothelial cells have been clearly implicated in vascular formation, the VEGF receptor family and the Tie family.

RTKs possess the ability to transfer γ phosphate of ATP to hydroxyl groups of tyrosine upon target proteins (Hunter 1998). These receptors are composed of an extracellular domain that binds ligands/growth factors, a transmembrane domain and an intracellular domain containing a catalytic core (kinase domain), as well as non-catalytic regulatory sequences (Hunter 1998; Hubbard et al. 1998). Apart from the insulin receptor family, all known RTKs including the VEGF receptors and Tie family are expressed as monomers on the cell surface. The general consensus for activation of these receptors is via ligand binding to the extracellular domain causing receptor dimerisation (Schlessinger 1988; Lemmon and Schlessinger 1994; Jiang and Hunter 1999). Dimerisation mediates receptor kinase activation resulting in autophosphorylation of catalytic and non-catalytic domains. Catalytic domain phosphorylation causes direct activation of receptor kinases and phosphorylation of the non-catalytic domains revealing docking sites for downstream signalling molecules in the cytoplasm (Pawson 2002). Potential docking sites are available for adaptor proteins possessing SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains (Pawson and Scott 1997).

1.5 The VEGF receptor family and its ligands

There are three members of the VEGF receptor (VEGFR) family, VEGFR-1/Flt-1 (Matushime et al. 1987; Shibuya et al. 1990; de Vries et al. 1992), VEGFR-2/KDR (Terman et al. 1991; Terman et al. 1992; Millauer et al. 1993) or the murine homologue Flk-1 which has 85% homology with the human form (Matthews et al. 1991), as well as VEGFR-3/Flt-4 which is expressed on lymphatic endothelial cells and not vascular endothelial cells (Pajusola et al. 1992; Kaipainen et al. 1993 and 1995). These receptors all possess an extracellular ligand binding domain containing seven immunoglobulin-like repeats and an intracellular domain (split kinase domain) (Figure 1.2).
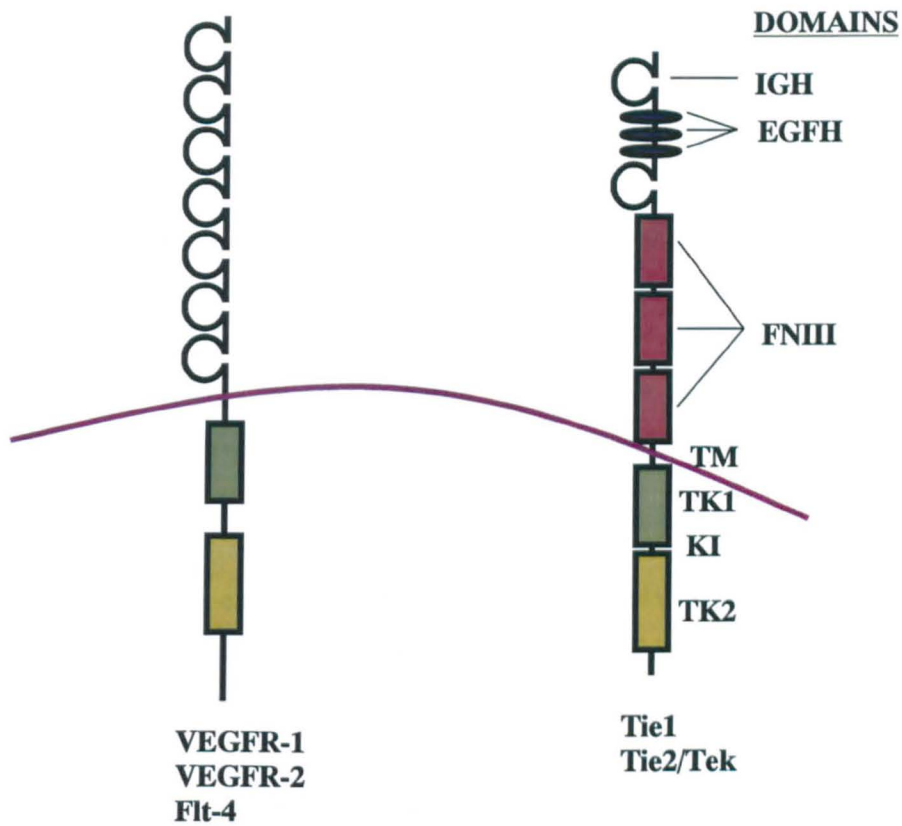
Figure 1.2 **Schematic representations of the endothelial specific receptor tyrosine kinases, Tie and VEGF receptors.** VEGF receptors comprise of a conserved extracellular domain consisting of seven immunoglobulin-like repeats and a split kinase intracellular domain. Tie receptors comprise of extracellular domains consisting of two immunoglobulin-like loops, three EGF-like repeats and three-fibronectin type III-like repeats. **EGFH** - EGF-like repeats; **FNIII** - fibronectin type III-like repeats; **IGH** - immunoglobulin-like loops; **KI** - kinase insert; **TK** - tyrosine kinase domain; **TM** - transmembrane.

The VEGFs are ligands for the VEGF receptor family of RTKs. There are six designated forms of VEGF, A through to E, as well as PlGF. VEGF-A is considered the most widely distributed form of the protein and genetic studies have underscored its requirement in vascular development. In this thesis the acronym VEGF will refer to VEGF-A, unless otherwise stated. Originally described as vascular permeability factor and then as vascular endothelial growth factor (Leung et al. 1989; Senger et al. 1993) VEGF is a 34 to 50 kDa homodimeric protein produced by a wide range of cells including macrophages and tumours (Berse et al. 1992). The VEGF-A ligand is normally observed in six isoforms generated by alternative splicing from a single gene containing eight exons forming ligands of different amino acid length – $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$ (Neufeld et al. 1999). $VEGF_{165}$ is the most abundant form in tissues (Ferrara et al. 1991). Gene expression is tightly regulated mostly by hypoxia at a transcriptional level. The hypoxia-responsive element (HRE) located in the VEGF promoter binds hypoxia-inducible transcription factors (HIF-1/2) regulating VEGF expression (Brusselmans et al. 2001; Pugh and Ratcliffe 2003). Other regulators of VEGF transcription include EGF, PDGF, TNF and TGF as well as several inflammatory cytokines (interleukins) (Neufeld et al. 1999).

VEGF regulates multiple endothelial cell functions in vascular development. VEGF modulates endothelial cell proliferation (Ferrara and Henzel 1989), induces microvascular permeability (Senger et al. 1983; Senger et al. 1990; Asahara et al. 1998), promotes endothelial cell survival (Alon et al. 1995; Pierce et al. 1996; Gerber et al 1998), and stimulates endothelial cell adhesion and migration (Byzova et al. 2000) as well as prompting endothelial cell gene expression (Senger 2001). Furthermore, VEGF modulates the expression of proteases in endothelial cells (Pepper et al. 1991; Unemori et al. 1992). In fact, VEGF actively increases proteolytic cleavage of another angiogenic growth factor receptor, Tie1 (Tsiamis et al. 2002).

1.5.1 VEGF-A and the VEGF receptors

In most cases VEGF receptors 1 and 2 are expressed on vascular endothelial cells (Jakeman et al. 1992; Eichmann et al. 1993; Kaipainen et al. 1993; Quinn et al. 1993; Yamane et al. 1994). However, VEGFR-1 has been observed upon monocytes and macrophages (Barleon et al. 1996; Clauss et al. 1996). VEGFR-1 has the highest affinity for recombinant human $VEGF_{165}$, K_d approximately 10-20pM, whereas VEGFR-2 has a much lower affinity K_d approximately 75-125pM (Kendall and Thomas 1993; Kendall et al. 1996). Binding analyses using ^{125}I-VEGF also

revealed K_d values of 16pM for VEGFR-1 and 760pM for VEGFR-2 confirming that VEGF binds to VEGFR-2 at a much lower affinity than to VEGFR-1 (Waltenberger et al. 1994). The binding site for VEGF on both VEGFR-1 and 2 appears to be the second Ig-like domain (Davis-Smyth et al. 1996; Barleon et al. 1997). VEGF binding to VEGFR-1 was completely abolished upon the removal of this domain (Davis-Smyth et al. 1996).

VEGFR-1 and VEGFR-2 appear to mediate distinct signalling and functional responses. VEGFR-2 expressing cells responded to VEGF stimulation by undergoing changes in cell morphology, actin reorganisation and mitogenicity, whereas cells expressing VEGFR-1 remain largely unresponsive (Waltenberger et al. 1994). Kinase activity for VEGFR-1 was reported as approximately 10-fold lower than VEGFR-2 (Mustonen and Alitalo 1995; Shibuya 1995; Sawano et al. 1996; Ferrara and Davis-Smyth 1997; Sawano et al. 1997). Sawano et al. (1996) and Seetharam et al. (1995) over-expressed VEGFR-1 in NIH3T3 and BAE cells, respectively but no proliferation was observed in the presence of VEGF. Over-expression of VEGFR-2 in the above two cell types elicited cell growth after VEGF stimulation (Takahashi and Shibuya 1997).

Gene targeting studies show substantial involvement of VEGFR-1 and VEGFR-2 in vessel formation. The VEGFR-2 mouse knockout resulted in excessive endothelial cell development causing abnormal vessel formation. Excessive endothelial cells were found in the lumens of the abnormal vessels. Embryos die between embryonic day 8.5 and 9.5 (Shalaby et al. 1995 and 1997). Embryonic mice deficient in the VEGFR-1 receptor died around the same time as the VEGFR-2 gene knockout (E8.5-9.5). However, observations revealed that unlike the VEGFR-2 knockout mouse vessels were formed but exhibited abnormal organisation (Fong et al. 1995). It has been postulated that VEGFR-1 may have an inhibitory role in embryonic vessel development having a high affinity for VEGF but low signalling activity. Indeed, transgenic mice in which the intracellular domain of VEGFR-1 is ablated exhibit normal vessel development consistent with the receptor binding of VEGF but non-mediation of signalling events critical for vessel formation (Hiratsuka et al. 1998). However, these mice exhibit defects in pathological angiogenesis suggesting the receptor does possess signalling activity in some situations (Hiratsuka et al. 1998). The receptor could down regulate VEGF activities ensuring the correct numbers of endothelial cells are generated via the VEGFR-2 receptor (Gale and Yancopoulos 1999). Recent evidence, suggests that VEGFR-1 may contribute to VEGFR-2 activity mediated by VEGF (Autiero et al. 2003). Stimulation of VEGFR-1 by PlGF resulted in enhancement of VEGFR-2 signalling following VEGF binding. Cross talk between the two receptors was found to enhance VEGFR-2

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transphosphorylation whereby VEGFR-1 appears to be activated by PlGF enhancing VEGF-A activation of VEGFR-2 (Autiero et al. 2003).

Little is known about VEGFR-1 signalling. Over-expression of VEGFR-1 was shown to tyrosine phosphorylate phospholipase Cγ (PLCγ), tyrosine phosphatase SHP2, Crk and extracellular regulated kinases (Erk1/2) upon VEGF-A stimulation (Ito et al. 2001). Therefore, VEGFR-1 could be involved in Ca^{2+}/DAG and Ras/Raf signalling. Distinct activities could also be attributed to the differential phosphorylation of the receptor when stimulated by VEGF-A or PlGF. VEGF-A activates phosphorylation of tyrosine 1213, whereas PlGF activates tyrosine 1309 phosphorylation (Autiero et al. 2003).

VEGF-A activation of VEGFR-2 elicits a number of effects both embryonically and postnatally. VEGF induces endothelial cell migration, proliferation and differentiation as well as pro-inflammation and permeabilisation. VEGFR-2 is phosphorylated on a number tyrosine residues following ligand activation (Y951, Y996, Y1054, Y1059, Y1175 and Y1214) (Dougher and Terman 1999; Takahashi et al. 2001). Phosphorylation of Y951 was shown to be essential for endothelial cell migration (Zeng et al. 2001). Y1175 and Y1205 located in the C-terminal tail are major sites of phosphorylation in endothelial cells and have been implicated in several major signalling pathways (Takahashi et al. 2001). Endothelial proliferation via VEGFR-2 is mediated by the phosphorylation of Y1175 which in turn binds PLCγ (Takahashi et al. 2001). A well characterised signalling pathway mediated by VEGFR-2 is the anti-apoptotic signal provided via phosphatidylinositol 3-kinase (PI3K)/Akt (Gerber et al. 1998). VEGF stimulates Akt activation and over-expression of VEGFR-2 in endothelial cells activates PI3K and Akt (Gerber et al. 1998). PI3K activation appears to be via the association of p85 subunit of PI3K with VEGFR-2 via Y801 and Y1175 phosphorylation (Dayanir et al. 2001). Alternatively, p85 may associate with FAK (focal adhesion kinase) which is recruited to the phosphorylated tyrosine 1214 residue (Qi and Claesson-Welsh 2001). Furthermore, the anti-apoptotic effects of VEGFR-2 appear to be modulated by vascular endothelial (VE)-cadherin as removal of the intracellular VE-cadherin domain impairs Akt and VEGFR-2 coupling (Carmeliet et al. 1999).

1.6 The Tie family of receptors and its ligands

The second subfamily of receptor tyrosine kinases relatively specific to the endothelium and involved in vessel formation is the Tie family. This family comprises of two members, Tie1 and

Tie2. These receptors share a number of structural factors; the extracellular domains consist of two immunoglobulin-like loops, three EGF-like repeats and three-fibronectin type III-like repeats. The intracellular domains contains a characteristic kinase domain with kinase insert (Wilks 1989; Dumont et al. 1992 and 1993; Partanen et al. 1992; Iwama et al. 1993; Maisonpierre et al. 1993; Sato et al. 1993; Ziegler et al. 1993). Figure 1.2 diagrammatically represents the structure of the Tie receptors. Overall amino acid identity between Tie1 and Tie2 is 44% homology at the amino acid level and 76% homology in the intracellular domain (Partanen et al. 1992; Ziegler et al. 1993).

1.6.1 Tie1 function and signalling

Tie1 is a 120kDa transmembrane glycoprotein predominantly expressed on vascular endothelial cells and some haematopoietic cells and is essential for vascular development (Partanen et al. 1992). Targeted gene knockout of the Tie1 gene results in embryonic lethality. Tie1 deficient mice die between midgestation and birth due to extensive haemorrhage and oedema (Puri et al. 1995; Sato et al. 1995). Further analysis of the embryos revealed a lack of endothelial cell integrity and increased hyper-permeability of small vessels. These suggest that Tie1 plays a critical role in late angiogenesis possibly promoting endothelial survival and the maturation of the vasculature (McCarthy et al. 1998). Tie1 expression is first observed at embryonic day 8.5 in angioblasts of the endothelium of the developing mouse embryo (Sato et al. 1993; Korhonen et al. 1994). Furthermore, Tie1 is expressed in adult endothelium and is increased in endothelial cells involved in angiogenesis, such as in wound healing and tumour vascularisation (Korhonen et al. 1992; Hatva et al. 1995).

As yet no ligand has been described for Tie1 and the mechanism for its activation is not known. Little is known about the signalling pathway utilised by Tie1. Tie1 undergoes endoproteolytic cleavage after Protein Kinase C (PKC) stimulation. The cleavage event is mediated by metalloproteinases (MMPs) releasing the extracellular binding domain of Tie1 (McCarthy et al. 1999a). VEGF was also responsible for the release of the Tie1 endodomain, a potential signalling molecule (Tsiamis et al. 2002). Utilising chimeric receptors the ability of Tie1 to undergo phosphorylation was observed to be weak in endothelial cells and kinase activity was negligible compared to Tie2 (Marron et al. 2000a). Tie1 has been shown to interact with Tie2 and there has since been evidence for pre-formed Tie1:Tie2 complexes in endothelial cells (Marron et al. 2000a). Further studies using chimeric receptors have confirmed the receptor is

poorly phosphorylated when expressed in non-endothelial cells (Kontos et al. 2002). Furthermore, the Tie1:Tie2 complex is not disassociated by VEGF mediated cleavage resulting in the Tie1 endodomain, therefore the complex remains as Tie1 endodomain:Tie2 (Tsiamis et al. 2002). These observations suggest that Tie1 may have a role in vessel development by modulating Tie2 signalling. Also, Tie1 has been observed to associate with Shp2 and the p85 subunit of PI3K (Marron et al. 2000b; Kontos et al. 2002). Association with PI3K potentiates the cell survival signalling cascade via Akt inhibiting endothelial cell apoptosis (Kontos et al. 2002), further supporting the hypothesis that Tie1 could regulate microvessel survival.

1.6.2 Tie2 function and signalling

Tie2 is a 135kDa RTK predominantly expressed on vascular endothelial cells and expression of Tie2 is observed at day 7.5 in the mouse embryo in endothelial cells (Dumont et al. 1995). Analysis of mice deficient in the Tie2 receptor reveals a critical requirement for the receptor tyrosine kinase in vascular development. Embryos lacking Tie2 die between embryonic day 9.5 and 12.5 due to impaired cardiac function and vascular haemorrhage (Dumont et al. 1994; Sato et al. 1995; Suri et al. 1996; Maisonpierre et al. 1997). Specific defects include the absence of myocardial trabecular projections, simplified vessel branching and reduction in endothelial cell number (Dumont et al. 1994). Further analysis revealed that Tie2 negative embryos lacked pericytes in regions of deficient vessel branching and possessed defective endothelial cell motility (Patan et al. 1998).

A family of ligands have been identified for the Tie2 receptor, called the angiopoietins (Davis et al. 1996; Maisonpierre et al. 1997; Valenzuela et al. 1999). The angiopoietin family comprises four members all binding Tie2, Ang-1 and Ang-4 activate Tie2 receptor phosphorylation, whereas Ang-2 and Ang-3 can inhibit Ang-1 induced Tie2 phosphorylation.

The best characterised members of the angiopoietins are Ang-1 and Ang-2. Ang-1 was cloned using a novel secretion-trap expression cloning method (Davis et al. 1996). The ligand possesses a hydrophobic secretion signal peptide sequence located at the amino terminus followed by a coiled-coil domain and a carboxyl terminus fibrinogen-like domain. The structure of Ang-1 is referred to as N-C-F and is reminiscent of fibrinogen (Doolittle 1984). Originally thought to bind the Tie2 receptor as a dimer, Davis et al. (2003) reported that a tetramer is the minimum required size for the activation of Tie2 phosphorylation.

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Expression of Ang-1 can be detected in the myocardium of the heart by E8.5 (Davis et al. 1996). Whereas, Tie2 expression is located in the endocardium surrounded by the myocardium, expression patterns of Ang-1 become more widespread by E11.5 locating to the mesenchyme and smooth muscle cells of developing vessels (Maisonpierre et al. 1997). Adult expression of Ang-1 can be observed widely although only low levels are detectable in heart and liver (Maisonpierre et al. 1997).

Mice deficient for the Ang-1 gene exhibit a similar phenotype to those lacking Tie2, although the defects appear less severe (Suri et al. 1996). Transgenic mice appear to be normal up to E10.5 apart from growth retardation in the heart. Later in development embryos become deformed and die by E12.5 (Suri et al. 1996). It appears that mice deficient for Ang-1 or Tie2 both have defective interactions between endothelial cells and mural cells (Patan et al. 1998). Furthermore, over-expression of Ang-1 inhibits microvascular leakage induced by VEGF or inflammatory stimuli (Thurston et al. 1999 and 2000; Gamble et al. 2000). In addition, the ligand enhances lumen formation of patent microvessels when administered with VEGF (Gamble et al. 2000).

Ang-2 was originally isolated by homology screening and shares approximately 55% sequence homology with Ang-1 (Maisonpierre et al. 1997). Ang-2 is a context dependent antagonist or agonist. Ang-2 has been reported to antagonise Ang-1 activation of Tie2 on cultured endothelial cells, but there are several situations where Ang-2 is an agonist. Non-endothelial cells with ectopic expression of Tie2 in fibroblasts are activated by Ang-2 (Maisonpierre et al. 1997). Furthermore, enhanced concentration of Ang-2 increases endothelial cell survival via the PI3K/Akt pathway (Kim et al. 2000b). It does not appear that the differing effects in cultured endothelial cells is due to differential binding of Tie2 as both Ang-1 and Ang-2 bind Tie2 on the first 360 amino acids (Ig-like domain plus EGF-like repeats) (Fiedler et al. 2003).

Transgenic over-expression of Ang-2 exhibits a similar phenotype to mice deficient in Ang-1 or Tie2 giving credence to the hypothesis that Ang-2 antagonises the Tie2/Ang-1 pathway (Suri et al. 1996; Maisonpierre et al. 1997). Whereas Ang-1 expression was observed as being widespread, Ang-2 expression is restricted to the sites of vascular remodelling (Maisonpierre et al. 1997). It has been suggested that Ang-2 acts to destabilise the contacts between endothelial cells and pericytes, and may have an important role in vascular morphogenesis and remodelling (Maisonpierre et al. 1997). In the presence of VEGF and other angiogenic facilitators, Ang-2 is

pro-angiogenic (Lobov et al. 2002) but is anti-angiogenic causing vessel regression in the absence of such growth factors (Goede et al. 1998).

Unlike VEGFR-2, activation of Tie2 does not affect endothelial proliferation, though it has been reported to stimulate endothelial cell motility, monolayer integrity, MMP production and suppression of inflammatory gene expression (Kim et al. 2000c; Master et al. 2001; Hughes et al. 2003).

Several studies have revealed that Ang-1 stimulation of Tie2 results in activation of cell motility including the stimulation of sprouting and tubule formation (Koblizek et al. 1998; Witzenbichler et al. 1998; Hayes et al. 1999; Kwak et al 1999; Papapetropoulos et al. 1999; Teichert-Kuliszewska et al. 2001). Furthermore, the modified Ang-1 molecule (Ang-1*) has been shown to synergize with VEGF in sprouting angiogenesis (Asahara et al. 1998). Ang-2 has also been evident in mediating endothelial cell motility. Although not normally associated with stimulating Tie2 receptor phosphorylation and activation it has been observed in some endothelial cell types leading to capillary tube formation on fibrin and collagen matrices (Teichert-Kuliszewska et al. 2001; Mochizuki et al. 2002).

Several downstream signalling proteins have been shown to associate with Tie2 eliciting cellular functions such as cell survival and migration. The principal tyrosine residues reported to be phosphorylated in Tie2 are Y992 in the activation loop of the kinase and tyrosines 1102, 1108 and 1113 located in the C-terminal tail.

Using the yeast two hybrid method, Jones and Dumont (1998) identified a novel docking protein with sequence homology to p62dok and IRS-3, it was named Dok-R. Dok-R associates with Tie2 via a PTB domain and co-expression with activated Tie2 resulted in tyrosine phosphorylation of Dok-R and co-immunoprecipitation with rasGTPase activating protein (GAP) and Nck. Dok-R also constitutively binds to Crk (Jones and Dumont 1998). Further investigations revealed that Ang-1 activated the phosphorylation of Y1108 mediating recruitment of Dok-R (Murray et al. 2001; Jones et al. 2003). Ang-1 phosphorylation of Dok-R mediates the recruitment of the adapter protein, Nck and Pak (p21-activating kinase) forming a Tie2/Dok-R/Nck/Pak complex (Master et al. 2001). Therefore, endothelial cell migration through Tie2 signalling is mediated by the association of a number of adapter proteins. Ang-1 stimulated endothelial cell motility requires the association, as a complex, of Dok-R, Nck and Pak with phosphorylation of Y1108

potentiating the initial association of Dok-R. Furthermore, the PH domain of Dok-R contributes to the binding of PI3K to the Tie2 receptor.

Again, using the yeast two hybrid system Jones et al. (1999) identified that the p85 subunit of PI3K associated with Tie2 in phosphotyrosine dependent manner via its SH2 domain. Previously, Kontos et al. (1998) using the same method identified the association but also postulated that binding to the receptor was mediated by phosphorylation of the Y1102 residue located in the C-terminus. Mutation of the tyrosine residue to a phenylalanine abrogated activation of PI3K and Akt (Kontos et al. 1998).

PI3K is known to mediate cell survival by phosphorylating the cytosolic protein kinase, Akt via phosphoinositide dependent kinases (PDK1/2) (Downward 1998). Activated PI3K generates inositol phospholipids that are recognized by a particular subset of pleckstrin homology (PH) domains (Vanhaesebroeck and Waterfield 1999; Leevers et al. 1999; Rameh and Cantley 1999). The serine/threonine protein kinase B/Akt was one of first proteins to be identified with a PH domain and it was later shown to bind PI3K lipid products (Coffer et al. 1998; Shepherd et al. 1998). Akt/PKB has been identified in the regulation of cell viability in a number of distinct cellular systems (Dudek et al. 1997; Kauffmann-Zeh et al. 1997; Kennedy et al. 1997; Kulik et al. 1997; Fujio and Walsh 1999). In endothelial cells activation of PI3K and Akt via Tie2 inhibits endothelial cell apoptosis. Evidence is observed by increased apoptosis in the presence of wortmannin and dominant negative Akt (Kim et al. 2000a).

Further studies have discovered that Ang-1 phosphorylates the p85 subunit of PI3K and phosphorylation of Akt (Jones et al. 1999; Fujikawa et al. 1999; Kim et al. 2000a; Papapetropoulos et al. 2000). In the Papapetropoulos et al. study (2000) the inhibitor of apoptosis protein (IAP), survivin or *Birc5* is up-regulated by Ang-1 mediated Akt activation. PI3K has also been suggested to mediate some of the increase in motility following Ang-1 activation of endothelial cells. Inhibitors of PI3K blocked Ang-1 stimulation of migration in cells expressing Tie2 and Ang-2 also mediated motility (Fujikawa et al. 1999; Jones et al. 1999; Mochizuki et al. 2002).

Hughes et al. (2003) identified a novel protein interacting with the intracellular portion of Tie2. Subsequent analysis revealed the protein to be the human A20 binding inhibitor of nuclear factor

κB (NF-κB) activation, ABIN-2. Interaction of ABIN-2 and Tie2 was dependent upon receptor autophosphorylation, although ABIN-2 appeared not to be phosphorylated. Stimulation of Tie2 in endothelial cells by Ang-1 mediated the interaction. ABIN-2 is an inhibitor of NF-κB and may play a role in the anti inflammatory actions of Tie2 (Van Huffel et al. 2001; Hughes et al. 2003). Tie2 appears to play an important role in regulating the inflammatory phenotype of the endothelial cell. Activation of Tie2 has been reported to reduce VEGF and TNF-α induced leukocyte adhesion and TNF-α induced migration of leukocytes across endothelial cell monolayers (Gamble et al. 2000). In addition, Ang-1 stimulation of Tie2 inhibits a number of NF-κB responsive inflammation-associated genes including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, which is induced by VEGF (Kim et al. 2001).

Several other signalling intermediates have been shown to associate with Tie2 including the growth factor receptor bound proteins (Grb) 2, Grb7, Grb14 and the protein tyrosine phosphatase (Jones et al. 1999). The roles played by those molecules in Tie2 functions are yet to be defined. Figure 1.3 summarises the adapter proteins associated with the Tie receptors.

1.6.3 Involvement of Tie2 in endothelial cell:mural cell interaction

Evidence for Tie2 and Ang-1 in endothelial cell survival, cell motility and anti-inflammation as well as observations in the transgenic mice indicates the critical role they play in endothelial cell and mural cell interaction. Patan (1998) observed that Tie2 or Ang-1 deficient mice possessed vessels with poor pericyte support resulting in embryonic lethality. Combine these observations with the knowledge that a mutation of Tie2 causes a venous malformation resulting in irregular and enlarged vessels lacking mural cells (Vikkula et al. 1996) suggesting a crucial role for Tie2 in regulating mural cell acquisition.

It therefore, appears that the Tie receptors, especially Tie2 play a critical role in vessel maturation. The observations discussed in this Chapter establish a role for Tie2 in mediating mural cell acquisition but the exact mechanisms are unknown.
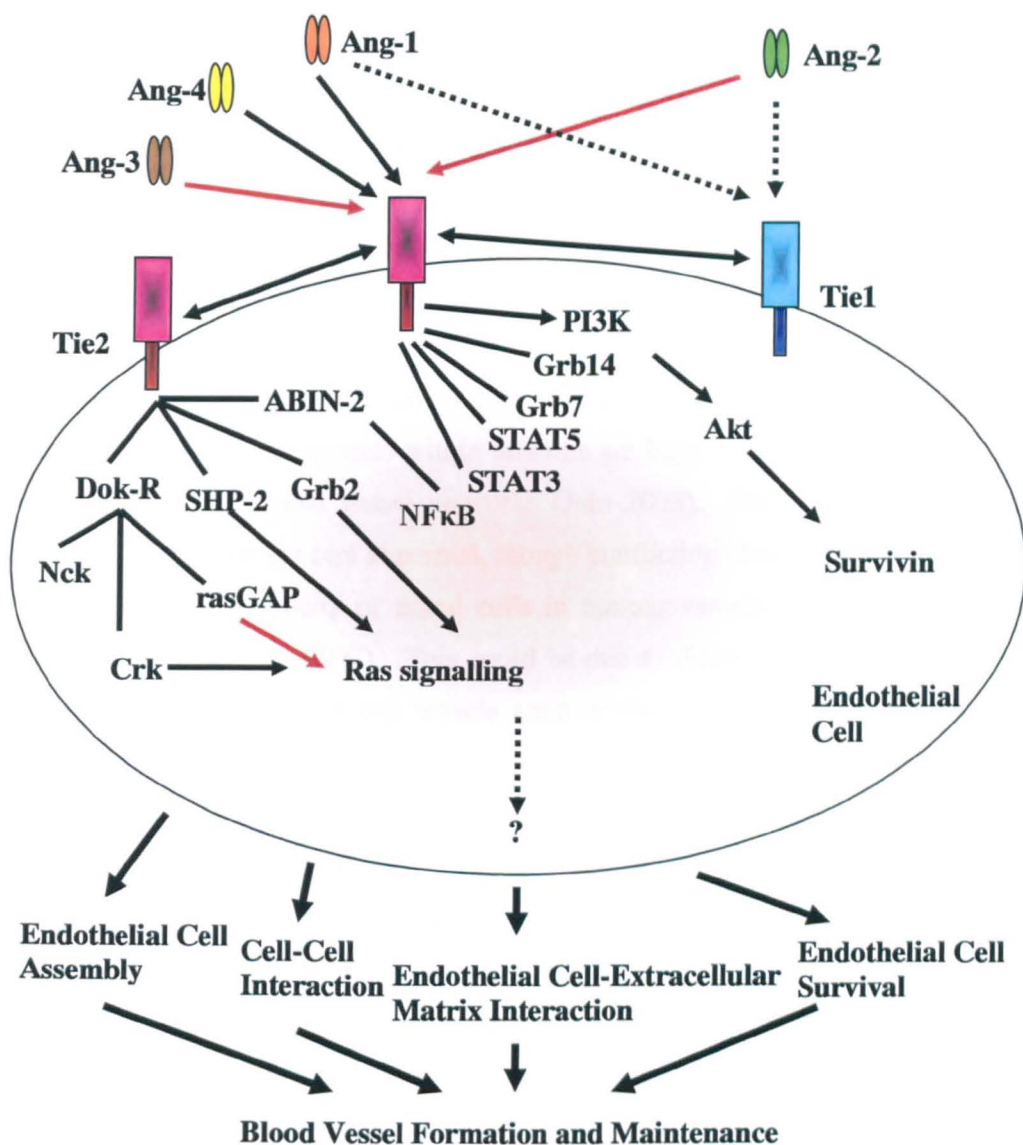
Figure 1.3 **Schematic representations of known downstream targets of the Tie2 receptor.** Tie1 and Tie2 are known to associate with the above signalling intermediates involved in controlling endothelial cell migration, survival, adhesion and anti-inflammation. Adapted from Loughna and Sato 2001. Black arrows – activation; red arrows – inhibition.

1.7 Pathological angiogenesis

Uncontrolled or unregulated angiogenesis has been shown to be associated with several pathological situations including atherosclerosis, arthritis, diabetic retinopathy, tumour growth and tumour metastasis (Blood and Zetter 1990; Paku and Paweletz 1991; Fidler and Ellis 1994; Folkman 1995). In addition to increased angiogenesis in those conditions, the microvessels produced appear in many cases to be defective (Carmeliet 2003).

One example of this is in atherosclerotic plaques of the carotid artery (McCarthy et al. 1999b). In this case numbers of enlarged dysmorphogenic neovessels in the plaques correlated with plaque instability. Another example of dysmorphogenic vessels associated with pathological angiogenesis is in tumour growth. The feature of solid tumours is the abnormal vasculature (Jain 2002; Jain et al. 2002). The vessels within tumours are highly disorganised lacking the normal branching observed in normal vessel networks (Jain 2003). Mural cell coverage within the vasculature of the tumour appears abnormal, though conflicting observations have been reported. Some research shows a paucity of mural cells in tumour vessels whereas others have shown abundance (Morikawa et al. 2002). This could be due to different tumour types or the use of different molecular markers (smooth muscle actin or desmin). A possible explanation is that fibroblasts at the interface of the tumour are stimulated by TGF-β to differentiate into myofibroblasts and then into pericyte-like cells. Pericyte-like cells have been proposed to guide endothelial cell sprouts in tumours (Brown et al. 2001; Morikawa et al. 2002). Research studies using intravital and immunochemical analysis revealed that pericytes in tumours are morphologically abnormal forming weak links with endothelial cells and extracellular matrix. These vessels are leaky and are maintained by the survival factor, VEGF released from pericytes (Fukumura et al. 1998; Brown et al. 2001). The abnormal organisation of the tumour vessels results in disordered blood flow and increased leakiness (Hobbs et al. 1998; Baish and Jain 2000; Hashizume et al. 2000).

Characteristic morphology of pathological vessels lacking support cells with disorganised blood flow and vascular leakage indicates immaturity. Data suggests that defective mural cell association with microvessels in tumours and possibly other pathologies give rise to dysmorphogenic vessels. Lack of mural cell cover could underlie defective microvessel function in other pathologies for example atherosclerotic plaques. Better understanding of how mural cell cover is controlled could lead to ways to reverse defective vessel phenotypes.

1.8 Aims of this study

Firstly, the aim of the study was to test the hypothesis that dysmorphogenic pathological vessels in the atherosclerotic plaque, and myocardial wound healing response, have poor mural cell coverage. Furthermore, the study aims to investigate the hypothesis that mural cell interaction with endothelial cells is controlled by the Tie2/Ang-1 system. This is examined using direct analysis of the Ang-1/Tie2 system in cell to cell interaction, characterising the potential adhesion molecules involved in the interaction. Thirdly, to test the theory that the mutant form of Tie2 associated with venous malformation can modulate endothelial cell interaction with mural cells and other endothelial functions. In particular, focussing upon the survival pathways mediated by the hyperphosphorylated mutant Tie2.

Chapter Two
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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$_2$PO$_4$, pH 7.4.

4% Paraformaldehyde solution – 100ml of PBS heated to 80°C. Remove from heat and stir in 4g Paraformaldehyde. Filter and store at 4°C for 2 weeks maximum.

2.1.1 Microbiological Media Recipes

2.1.1.1 Luria Bertani (LB) broth

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

2.1.1.2 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. Solution was thoroughly mixed and autoclaved for 20 minutes at 120°C. Solution was allowed to cool to approximately 50°C before ampicillin was added to a final concentration of 100µg/ml unless stated otherwise. Approximately 25ml of agar was poured into 100mm petri dishes (Bibby Sterillin). Plates were stored at 4°C.

2.1.1.3 SOB broth

In order 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. This was then autoclaved and allowed to cool before adding 10mM $MgCl_2$ and 10mM $MgSO_4$ to a final volume of 500ml. 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

The following solutions were produced in 50ml aliquots. 10mM Pipes, 55mM $MnCl_2$ plus 15mM $CaCl_2$, and 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 Media recipe was prepared unless otherwise stated. Media was prepared containing 10% FCS, 100units/ml penicillin, 100µg/ml streptomycin and 100µg/ml L-glutamine.

2.2.2 Cell Types

Cell Type	Media	Additives
Bovine Aortic Endothelial (BAE) cells	DMEM	
R849W Tie2 BAE cells	DMEM	500µg/ml G418 sulphate (Duchefa Biochemie)
TrkA/Tie2 BAE cells	DMEM	500µg/ml G418 sulphate
Wild type Tie2 BAE cells	DMEM	500µg/ml G418 sulphate
Chinese Hamster Ovary (CHO) cells	MEM	
Ang-1 3B CHO cells	MEM	500µg/ml G418 sulphate
MF58 (Ang-1*) CHO cells	DMEM	1% non-essential amino acids; 500µg/ml G418 sulphate
Wild type Tie2 CHO cells	MEM	500µg/ml G418 sulphate
Multipotent embryonic (CH310T1/2) cells	DMEM	
Mouse microvascular Heart Endothelial (MHE) cells	DMEM	
Human Umbilical Vein Endothelial (HUVE) Cells	Medium 199	20% FCS; 5µg/ml heparin; 50µg/ml endothelial cell growth supplement; no L-glutamine
Vascular Smooth Muscle (VSM) cells	RPMI 1640	

Cells were obtained from the following sources:

BAE cells provided by Dr Mike Boarder, Department of Physiology and Pharmacology, University of Leicester, UK. All experiments were performed with cells between passages 8 and 13.

R849W Tie2 and **Wild type Tie2 BAE cells** provided by Mr Paul Morris, Department of Surgery, University of Leicester, UK.

TrkA/Tie2 BAE cells provided by Dr David Hughes, Department of Surgery, University of Leicester, UK.

CHO cells purchased from ATCC.

Ang-1 3B CHO cells (refer to Sections 2.8 and 2.9).

MF58 (Ang-1*) CHO cells provided by AstraZeneca, Alderley Park, Macclesfield, Cheshire, UK.

Wild type Tie2 CHO cells provided by Dr Marie Marron, Department of Surgery, University of Leicester, UK.

CH310T1/2 cells purchased from ATCC.

MHE cells provided by Dr. Chiara Urbinati, Universita Degli Studi di Brescia, Italy (Bastaki et al. 1997).

HUVE Cells isolated by Gwyneth Williams, Department of Surgery, University of Leicester, UK. All experiments were performed with cells between passages 2 and 5.

VSM cells isolated by Dawn Croston, Department of Surgery, University of Leicester, UK (White et al. 2000).

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 cells lifted off the flask upon gentle agitation. The trypsin was inactivated by the addition of Complete Media (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 Media. All cell types 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^{TM} transfection

The Superfect^{TM} 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 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 media with no additives and 4μl of Superfect^{TM} per μg of DNA. The mixture was

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incubated for 10 minutes at room temperature to allow a DNA/Superfect^{TM} complex to form. During this time the cells were washed twice with PBS and 2ml of Complete Media was added to the cells (refer to Section 2.2.1). The DNA/Superfect^{TM} 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 Media 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 4mls of serum free media 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 media (no additives) and then the DNA/Targefect complex was added and incubated at 37°C/5% CO_2 for 2 hours. The complex was then removed and Complete Media 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 the wells of a gridded six well plate and transfected with the appropriate DNA constructs and vector encoding green fluorescent protein (GFP) (refer to Chapter Five). Cells were allowed to recover for 48 hours at 37°C/5% CO_2. Cells expressing GFP were visualised under UV light by excitation at wavelength ~385nm, emitting green colour at ~508nm. The numbers of GFP expressing cells were counted in 10 designated areas of the grid before washing and serum starvation for 18 hours at 37°C/5% CO_2. After 18 hours the GFP expressing cells were counted in the same designated areas and the percentage survival was determined.

2.2.6 Apoptosis Assay

In conjunction with the cell survival assay an apoptotic assay was conducted using the nucleic acid specific fluorochrome 4,6-diamidino-2-phenylindole-2-HCl (DAPI) (Sigma). A clear indication of a cell undergoing apoptosis is the characteristic breakdown of the nucleus, comprising of collapse and fragmentation of the chromatin, degradation of the nuclear envelope and nuclear blebbing, resulting in the formation of micronuclei. The DAPI stain attaches to the minor groove of the DNA helix around A-T clusters. Therefore, any apoptotic events that occur, for example, fragmentation within the nucleus, abnormal nucleus shape or cell bursting are visualised by the stain. Examples of apoptotic nuclei are given in Figure 5.8.

Briefly, after cell survival analysis (refer to Section 2.2.5) the cells were fixed with 4% Paraformaldehyde solution (refer to Section 2.1) for 5 minutes. Cells were then washed with DAPI buffer (10mM Tris, pH 7.4, 10mM EDTA, 100mM NaCl) before addition of DAPI buffer containing 0.5% Triton-X-100 to permeabilise the cells. After 5 minutes 0.2μg/ml of DAPI (Sigma) was added to each well and nuclei were observed under a confocal microscope until an appropriate endpoint of cell labelling was reached (approximately 3 minutes). Cells were washed with DAPI buffer before fixation with 4% Paraformaldehyde solution. GFP expressing cells were analysed for nuclear fragmentation and condensation by excitation of the DAPI fluorochrome at ~345nm emitting a blue colour at ~455nm.

2.3 Adhesion Assays

2.3.1 Adhesion Assay

A well of a 96-well plate was coated with vascular smooth muscle cells or substrate for testing endothelial and mural cell adhesion (refer to Section 2.3.2 and Chapters Four and Five). Cells to be investigated were labelled with the fluorescent dye, Calcein AM (Molecular Probes) at a concentration of 5μg/ml for 15 minutes at room temperature. The monolayers of cells were disassociated using trypsin/EDTA treatment (refer to Section 2.2.3) or trypsin in the presence of calcium. Trypsinisation in the presence of calcium is used to preserve the N-cadherin adhesion molecules (Takeichi 1977). For trypsinisation in the presence of Ca^{2+} fluorescently labelled cells

were washed with PBS in the presence of 1mM $CaCl_2$. The cells were disassociated with 0.1% Trypsin, 0.1mM $CaCl_2$ solution and 0.1% trypsin inhibitor was then added.

Following trypsinisation cells were centrifuged at 300g for 6 minutes and resuspended in serum free media. The cell suspension was then plated into the corresponding well of the 96-well plate and incubated for a predetermined time at 37°C/5% CO_2 (refer to Chapters Four and Five). To determine the amount of cells adhered to either cell or substrate, unbound cells were removed by agitation and washing three times with serum free media before fixation with 4% Paraformaldehyde solution. A fluorescent plate reader was used to measure the fluorescent intensity within each well. Cells labelled with Calcein AM emit a green light at ~517nm when excited at ~496nm in UV light conditions. Each assay was done in triplicate.

2.3.2 Coating of 96 well plates

For adhesion to smooth muscle cells, monolayers of mural cells were established using vascular smooth muscle cells. Cells were seeded at a density of 10,000 cells for each well and cultured overnight at 37°C/5% CO_2.

For adhesion to Ang-1 and Ang-1*, conditioned media from Ang-1 3B CHO cells and MF58 (Ang-1*) CHO cells was obtained (refer to Sections 2.9.1 and 2.10) and used at concentrations of 500ng/ml and 200ng/ml, respectively. Conditioned media was plated into wells and incubated overnight at 4°C. Prior to the assay conditioned media was removed and each well was blocked with a 2% BSA solution for 1 hour at 37°C/5% CO_2. Control was conditioned media from CHO cells.

For adhesion to purified Ang-1* (diluted in PBS) (kind donation from AstraZeneca, Alderley Park, Macclesfield, Cheshire, UK) (200ng/ml) or conditioned media from N-cadherin extracellular domain FC (N-cad FC) producing CHO cells (250ng/ml). Constructs were affinity captured by plating 10μg/ml of goat anti-human IgG (FC specific) into the appropriate well and incubating at 37°C/5% CO_2 for 1 hour. This was followed by the addition of a 2% BSA solution to block any non-specific protein binding for 1 hour at 37°C/5% CO_2. Ang-1* and N-cad FC were incubated in the appropriate wells and incubated overnight at 4°C. Controls were Tie-1 FC (200ng/ml) and CHO cell conditioned media, respectively.

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For adhesion to purified Ang-2 (diluted in PBS) (R & D systems) was plated at a concentration of 200ng/ml overnight at 4°C, prior to the assay wells were blocked with a 2% BSA solution for 1 hour at 37°C/5%CO_2. Control was PBS.

2.4 Migration Assays

Lower wells of a 24 well plate were either seeded with a confluent monolayer of MHE cells, conditioned media from BAE cells or serum free media (refer to Chapters Four and Five). The plates were incubated overnight at 37°C/5% CO_2.

8μm pore membrane inserts (Fahrenheit) were inserted into each well. Cells in suspension were diluted to 100,000 cells per aliquot. The cells were aliquoted into each insert and incubated for 2 or 6 hours at 37°C/5% CO_2 (refer to Chapters Four and Five).

Following migration membranes were fixed by placing a few drops of pre-chilled 70% ethanol into each insert and incubated for 1 hour or overnight at –20°C before the membrane was stained. After the fixing process the ethanol was removed and replaced with water. Fresh water was used to wash each insert. Cells were removed gently from the upper surface of the insert using a moistened cotton bud and the insert washed with water. Each insert was serially stained with Harris' Haematoxylin and 0.5% eosin for 45 seconds, washing with water after each stain.

Each membrane was removed from the base of the insert and mounted using glycerol gelatin. At a magnification of x400 the number of cells observed in 10 fields was counted on the underside of the membrane. Migration assays were conducted in duplicate.

2.5 Immunofluorescence

2.5.1 Preparation of Cells

Endothelial cells were plated into six well plates for appropriate transfection treatment. Cells were co-transfected with the appropriate DNA and GFP vector (refer to Section 2.2.4.2 and Chapter Five). After transfection cells were incubated in Complete Media (refer to Section 2.2.1) for 24 hours at 37°C/5% CO_2.

2.5.2 Cleaved Caspase-3 Immunofluorescence Protocol

Media was removed and cells were washed with PBS. Media was replaced with serum free media or maintained in serum for 4 hours at 37°C/5% CO_2. After serum starvation endothelial cells were fixed with 4% Paraformaldehyde solution for 20 minutes at 4°C. Cells were washed three times with TBS (50mM Tris-HCl, pH 7.4, 150mM NaCl) containing 0.2% Triton X-100 (TBS-TX100) for 5 minutes each. Endothelial cells were blocked with 5% BSA diluted in TBS containing 0.2% Triton X-100 for 1 hour at room temperature. Cells were probed for active caspase-3 using an anti-cleaved caspase-3 antibody (Cell Signaling Technology) at a 1 in 50 dilution in 5% BSA (TBS-TX100) overnight at 4°C. Primary antibody was removed and cells were washed 3 times with TBS-TX100. Immunoreactive cells were stained with a rabbit anti-Cy3 secondary antibody (Sigma) enabling the visualisation of the apoptotic protein under UV light (excitation at 512-550nm emitting a red colour between 570-615nm). Dilution was 1 in 100 in 5% BSA (TBS-TX100) for 1 hour at 4°C. Cells were washed three times in TBS-TX100 and a coverslip was placed over the cells with 220mM DABCO, dissolved in 90% glycerol, 10% PBS, pH 8.6.

2.6 Protein Biochemistry

2.6.1 General Reagents

3x Reducing Sample Buffer - 50mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 5mM EDTA.

2x Reducing Sample Buffer - 300μl 3x reducing sample buffer plus 600μl double distilled H_2O, before adding 200mM DTT.

Lysis Buffer – 50mM Tris, pH 7.4, 50mM NaCl, 1mM Na_2VO_4, 1mM NaF, 1mM EGTA. Prior to use 0.1mM AEBSF, 1% Triton-X-100 was added. Keep at 4°C.

Lysis Wash Buffer – As Lysis Buffer except 0.1% Triton-X-100.

Protein Electrophoresis Running Buffer - 192mM glycine, 25mM Tris, 0.5% SDS.

Protein Transfer Buffer - 192mM glycine, 25mM Tris, 20% methanol.

Tris Buffered Saline - 25mM Tris, pH 7.4, 144mM NaCl.

Stripping Buffer - 62.5nM Tris-HCl, 2% SDS.

Tris Acetate Buffer - 40mM Tris-acetate, 20mM Glacial Acetic Acid, 10mM EDTA, pH 8.0.

2.6.2 Whole Cell Lysate Preparation

BAE, CHO, HUVE and VSM cells were grown in 6 well plates (refer to relevant results Chapter). The cells were washed once with PBS and lysed by addition of 50µl of 2x Reducing Sample Buffer containing 200mM DTT. The lysed cells were scraped and pipetted into a fresh microcentrifuge tube. The lysed samples were then sonicated for 10-15 seconds heated to 95°C for 6 minutes and centrifuged at 13,000g for 5 minutes. The proteins in the supernatant were then resolved using SDS-PAGE (refer to Section 2.6.4).

2.6.3 Immunoprecipitation

2.6.3.1 Immunoprecipitation of Tie2

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 on ice. All PBS was removed prior to the addition of 500µl of Lysis Buffer, pH 7.4 (refer to Section 2.6.1). Flasks were gently rocked at 4°C for 10 minutes. Cell lysates were then scraped off and placed into cooled microcentrifuge tubes. The lysates were then vortexed for 30 seconds at 4°C. Cell lysates were centrifuged at 13,000g for 10 minutes at 4°C to remove any insoluble material. 40µl of cell lysate was removed and 10µl of 2x Reducing Sample Buffer was added with 200mM DTT (refer to Section 2.6.1). The remaining supernatant was transferred to a fresh microcentrifuge tube. Tie2 antibody (R & D Systems) was added to final concentration of 4µg/ml and allowed to mix with rotation for 2 hours at 4°C. Approximately 10µg of Protein G:sepharose (Sigma) which had been previously washed with Lysis Wash Buffer (refer to Section 2.6.1) was added to each supernatant and allowed to mix with rotation for a further 2 hours at 4°C. The sepharose beads

were collected by centrifuging at 13,000g for 2 minutes, and washed 4 times using Lysis Wash Buffer. Sepharose beads were resuspended in 50μl 2x Reducing Sample Buffer containing 200mM DTT. Samples were heated for 6 minutes at 95°C disassociating the immunocomplex from the sepharose beads then centrifuged at 13,000g for 5 minutes and 25μl aliquots of supernatant loaded onto SDS-PAGE for resolution of protein (refer to Section 2.6.4).

<u>2.6.3.2 Immunoprecipitation of tyrosine phosphorylated proteins</u>

In order to immunoprecipitate activated Tie2 from BAE cells, immunoprecipitation of tyrosine-phosphorylated proteins was employed. Immunoprecipitation was achieved as described in Section 2.6.3.1 except a monoclonal antibody anti-phosphotyrosine clone PT-66 (Sigma) was used instead of Tie2. Clone PT-66 is directly conjugated to agarose beads, therefore, the addition of Protein G:sepharose is not required. The agarose beads were washed with Lysis Wash Buffer and centrifuged at 13,000g for 2 minutes prior to incubation with the supernatants (refer to Section 2.6.3.1).

2.6.4 Separation of proteins by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was completed using the Bio-Rad microgel kit. Except where indicated the acrylamide gels were prepared using the following recipes:-

	10%
30% Acrylamide/0.8% Biscrylamide	6.7ml
2M Tris, pH 8.8	3.7ml
ddH$_2$O	9.6ml
20% SDS	100μl
10% Ammonium persulphate	134μl
TEMED	14μl

A 5% stacking gel contains:-

30% Acrylamide/0.8% Biscrylamide	3.3ml
1M Tris, pH 6.8	2.5ml
ddH$_2$O	13.7ml
20% SDS	100µl
10% Ammonium persulphate	200µl
TEMED	20µl

For the isolation of proteins with a low molecular weight ProSieve 50 acrylamide gels (Cambrex) were used:-

	14%
ProSeive 50	2.8ml
1.5M Tris, pH 8.8	2.5ml
ddH$_2$O	4.5ml
20% SDS	100µl
10% Ammonium persulphate	100µl
TEMED	4µl

A 5% Stacking gel consists of: -

ProSieve 50	500µl
1M Tris, pH 6.8	650µl
ddH$_2$O	3.75ml
20% SDS	50µl
10% Ammonium persulphate	50µl
TEMED	5µl

Proteins were loaded and separated electrophoretically at 150-200V in Protein Electrophoresis Running Buffer (refer to Section 2.6.1).

2.6.5 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 2.6.1). Proteins were transferred overnight at 120mA. The nitrocellulose membrane was probed using antibodies (refer to Section 2.6.6.3).

2.6.6 Detecting Proteins on a Nitrocellulose Membrane

After transferring, the nitrocellulose membrane was removed from the Protein Transfer Buffer and washed with TBS (refer to Section 2.6.1) containing 0.1% Triton X-100 (TBS-TX100). The membrane was then blocked with blocking buffer (TBS-TX100) containing 5% BSA or semi-skimmed milk for 1 hour at room temperature rocking. Using the relevant primary antibody (details in the relevant results Chapter and Section 2.6.6.3) the nitrocellulose membrane was probed for 1 hour at room temperature. The nitrocellulose membrane was then 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 blocking buffer for 1 hour at room temperature. Again, the membrane was washed 3 times with TBS-TX100 for 10 minutes. Any bound antibody was observed using the enhanced chemiluminescence protocol (Section 2.6.6.1).

2.6.6.1 Enhanced chemiluminescence (ECL) protocol

In order to detect HRP conjugated antibodies or secondary antibodies, which bind to proteins on the nitrocellulose membrane an enhanced chemiluminescence system was used. 22μl of 90mM p-Coumaric acid in DMSO, 50μl of 250mM Luminol (5-amino-2,3-dihydro-1,4-pthalazinedione) in DMSO and 3μl H_2O_2 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. The membrane was then wrapped in clingfilm and exposed to biomax light film (Sigma).

2.6.6.2 Stripping of nitrocellulose membranes

In order to re-probe membranes with different antibodies the nitrocellulose membrane was incubated with Stripping Buffer (refer to Section 2.6.1) and prior to use 100mM β-mercaptoethanol for 1 hour at 65°C with gentle agitation. Excess stripping buffer was removed by washing with TBS (refer Section to 2.6.1) containing 0.1% Triton X-100 before being blocked with the appropriate blocking buffer.

Antibody	Dilution	Blocking Buffer	Conjugated/Secondary Antibody	Source
Akt	1/1000	5% BSA	anti-rabbit HRP	Cell Signaling Technologies
FC-specific	1/2500	5% BSA	HRP conjugate	Sigma
Myc	1/5000	5% Milk	anti-mouse HRP	Invitrogen
N-cadherin	1/200	5% BSA	anti-rabbit HRP	Santa Cruz Biotechnology
Phosphotyrosine	1/1000	5% BSA	anti-mouse HRP	New England Biolabs
Phosphotyrosine	1/1000	5% BSA	anti-mouse HRP	Cell Signaling Technology
Phospho-Akt	1/1000	5% BSA	anti-rabbit HRP	Cell Signaling Technologies
Phospho-Tie2(992)	1/1000	5% BSA	anti-rabbit HRP	Cell Signaling Technologies
Shc	1/1000	5% BSA	anti-rabbit HRP	Cell Signaling Technologies
Tie2	1/1000	5% Milk	anti-goat HRP	R & D Systems
Tie2	1/1000	5% Milk	anti-rabbit HRP	Santa Cruz Biotechnology

Both anti-mouse HRP and anti-rabbit HRP were purchased from Amersham. Anti-goat HRP purchased from Dako Cytomation.

2.7 Molecular Biology Techniques

2.7.1 Competent Cell Production (XL-1 Blues)

Epicurian coli XL-1 Blues were purchased 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 an individual colony and incubated at 37°C overnight in a shaking incubator at 225rpm. 250ml of SOB was inoculated and allowed to grow 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 cool 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 cool 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.

2.7.2 Storage of Bacterial Cultures

Plasmid containing *E. Coli* were stored at -80°C as described by Sambrook et al. 1989. Bacterial cultures of 850μl 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.

2.7.3 DNA Preparation

DNA plasmids were purified in two ways either for DNA sequencing using the Wizard Plus SV miniprep kit (Promega) or for transfection using the DNA plasmid purification mini kit (Qiagen) as directed by the manufacturers protocol.

2.7.3.1 QIAfilter maxi-prep

For DNA in a low-copy plasmid required for transfection, a maxi-prep kit (Qiagen) was used. A single colony was picked from an appropriate agar plate and inoculated into a suitable broth starter culture. Incubated for 8 hours at 37°C with vigorous shaking (300rpm). The starter culture was then diluted 1 in 500 into selective LB broth and grown at 37°C for 16 hours with vigorous shaking (300rpm). Bacterial cells were harvested by centrifugation at 6,000g for 15 minutes at 4°C. The manufacturer's protocol was then followed. The DNA pellet was resuspended in 500μl of nuclease-free water and DNA concentration determined by measuring the absorbance at OD_{260}. To increase the concentration of DNA and reduce the volume required for transfection ethanol precipitation of DNA was performed (refer to Section 2.7.3.3).

2.7.3.2 Spectrophotometric analysis of DNA

DNA concentration was determined by diluting 1μl of DNA solution into 500μl of sterile distilled water. The concentration was estimated using a spectrophotometer by measuring the absorbance at 260nm. The 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 (Sambrook et al. 1989).

2.7.3.3 Ethanol precipitation of DNA

DNA was precipitated with 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.

2.7.4 Agarose Gel Electrophoresis

DNA was separated on 1% to 1.5% 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 agarose to a final concentration of 1μg/ml. The gel was poured into the appropriate tray and a comb was inserted to create wells for DNA loading. The gel was allowed to set before the DNA samples were loaded. DNA samples were prepared in a 6:1 ratio with 6x type IV DNA loading buffer (0.25% bromophenol blue, 40% (w/v) sucrose in water). Gels were run at 100V in TAE buffer until appropriate fragment separation had occurred. The DNA fragments were visualised on an ultra violet light transluminator and images were captured using a Multimage light cabinet. DNA fragment sizes were compared with 1Kb ladder of DNA molecular markers (Roche) run on the same gel.

2.7.5 Restriction Enzyme Digest

Restriction enzyme digests were carried out using Roche restriction endonucleases using the recommended manufacturer's buffer. Briefly, 1μg of DNA was resuspended in digest buffer. 10

units of each restriction endonuclease were added to a final volume of 10μl. Tubes were incubated at 37°C and the reaction was allowed to progress for 1 hour for a single digest or 1 hour 30 minutes for a double digest.

2.7.6 Gel Extraction

After DNA cleavage has occurred using restriction endonucleases, the whole reaction mixture was resolved using agarose gel electrophoresis and visualised using a transluminator. The required DNA fragment was excised from the agarose gel using a scalpel blade. The fragment was placed in 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 the silica membrane column. The silica membrane absorbs the DNA in the presence of high salt concentrations allowing contaminants to pass through by centrifugation. The impurities were washed away and the DNA eluted with 30μl of nuclease-free water.

2.7.7 Ligation

Reaction mixtures were prepared in the following way. 1μl of 10x Ligation buffer, 50ng vector, 19ng PCR product and nuclease free water were added to a final volume of 10μl. T4 DNA ligase was added to the reaction mix and incubated at 14°C overnight.

2.7.8 Transformation

Transformation of plasmid DNA into Epicurian XL-1 Blue supercompetent cells (Stratagene), One Shot or TOP10/P3 ultracompetent cells (Invitrogen) was carried out in accordance with the manufacturer's protocol. Briefly, a 50μl aliquot of supercompetent cells was thawed on ice and then aliquoted into a pre-chilled Falcon 2059 polypropylene tube (Becton Dickinson). 1μl of plasmid DNA was transferred into a separate aliquot of supercompetent cells. The transformation mixture was gently mixed and then incubated on ice for 30 minutes. The transformation mixture was then heat pulsed for 45 seconds at 42°C. The reactions were placed on ice for 2 minutes. The final step was to add 250μl of SOC broth (preheated to 42°C) and incubate the mixture for 1 hour at 37°C shaking at 225rpm. Immediately after transformation 50 and 200μl aliquots were plated onto LB agar (refer to Section 2.1.1.2) containing 100μg/ml

ampicillin. After 18 hours colonies were selected and grown in 10ml of LB broth (refer to Section 2.1.1.1) containing 100µg/ml ampicillin and grown overnight at 37°C shaking at 225rpm; DNA was prepared as indicated in Section 2.7.3.

2.7.9 Site Directed Mutagenesis

Site directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene). Two oligonucleotide primers were manufactured with the appropriate mutation incorporated and termed as oligonucleotide primer #1 (5' to 3') and oligonucleotide primer #2 (3' to 5'). Reaction mixtures were prepared in the following way. 5µl of 10x *Pfu* (reaction) buffer, 10ng, 20ng and 50ng of double stranded DNA template, 125ng oligonucleotide primer #1, 125ng of oligonucleotide primer #2, 1µl dNTP mix and nuclease free water to a volume of 50µl. The volume of oligonucleotide primer added to the PCR reaction is the required concentration (125ng) divided by the concentration of the Sigma Genosys oligo provided. These reactions were set up in thin-walled reaction tubes, and then native turbo *Pfu* DNA polymerase (2.5U/µl) was added. Using a PerkinElmer PCR machine the reactions underwent the polymerase chain reaction. The cycling parameters were as follows: -

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	18	95°C	30 seconds
		55°C	1 minute
		68°C	12 minutes

The PCR reaction was then incubated at 4°C until the next step.

2.7.9.1 Digestion of parental DNA

The digestion of the non-mutated DNA was carried out using *DpnI* restriction enzyme. 1µl of *DpnI* was added to each reaction mixture and was thoroughly mixed by gentle pipetting. The reaction mixtures were incubated at 37°C for 1 hour.

The PCR product was transformed into *E. coli* as indicated in Section 2.7.8. DNA was prepared as indicated in Section 2.7.3.

2.8 Subcloning of Angiopoietin-1 (Ang-1)

The TA cloning vector pCR2.1-TOPO (*Appendix 1*) and expression vector pcDNA3.1A myc/His (*Appendix 2*) were purchased from Invitrogen. The expression vector pFLAG-CMV-2 (*Appendix 3*) was purchased from Sigma.

Ang-1 cDNA obtained from ATCC. Ang-1 was amplified using PCR by Dr. Marie Marron isolated by agarose gel electrophoresis and extracted using QIAquick DNA Gel extraction kit (Qiagen). The DNA was eluted in 25µl of nuclease free water. Estimated DNA concentration was 3.3ng/µl.

To enable efficient ligation an intact A-overhang at the 3' end of PCR product is required. To ensure a 3' A-overhang 19ng of PCR product was added to 5µl of 10x *Taq* reaction buffer, 1µl dNTP mix, 1µl of *Taq* polymerase and nuclease-free water to a volume of 50µl. The reaction mixture was incubated at 72°C for 12 minutes to allow the annealing of adenine bases to the 3' end. The DNA was extracted immediately with an equal volume of phenol-chloroform. The DNA was ethanol precipitated as described in Section 2.7.3.3. The pellet was resuspended in 6µl of nuclease-free water. PCR product coding for Ang-1 was ligated into pCR2.1-TOPO.

After restriction digest analysis of transformants using *EcoRI*, it was concluded that the Ang-1 coding sequence in pCR2.1-TOPO was in the incorrect orientation. The Ang-1 coding sequence was isolated from pCR2.1-TOPO by restriction digestion using *BamHI* and *EcoRV* and ligated into the *BamHI/EcoRV* site in the multiple cloning site of pFlag-CMV-2. This resulted in an in-frame insertion of Ang-1 downstream of the Flag-epitope tag and the correct orientation.

2.8.1 QuikChange Mutagenesis of Ang-1 pFlag-CMV-2

To produce Ang-1 with a C-terminal epitope tag, Ang-1 was subcloned from pFlag-CMV-2 into pcDNA3.1 tagged with myc and poly-histidine.

In order to ensure read through into the DNA coding for the epitope tag the stop codon was removed by introducing a restriction site upstream. This allowed Ang-1 to be sub-cloned into pcDNA3.1 myc/HisA in the correct reading frame. An *XhoI* restriction site was created by designing the following primers for PCR:

1782

    G ATG ATT CGA CCT TTA GAT TTT TGA AAG CGC

1782

    G ATG ATT CGA CCT CTC GAG TTT TGA AAG CGC

                          -- XhoI --

1795   T → C

1797   A → C

1800   T → G

         GATGATTCGACCTCTCGAGTTTTGAAAGCGC

         GCGCTTTCAAAACTCGAGAGGTCGAATCATC

QuikChange mutagenesis was performed as described in Section 2.7.9 and the insertion of the *XhoI* site confirmed by restriction digestion.

cDNA coding for Ang-1 with the mutated stop codon was excised from pFlag-CMV-2 and ligated into pcDNA 3.1 myc/HisA. Sequence analysis was performed by Cambridge Bioscience (Cambridge, United Kingdom) and the mutated sequence is shown in *Appendix 4*. This construct is designated Ang-1 pcDNA3.1 myc/HisA.

2.9 Production of Stable Transfectants

In order, to create a clonal cell line a suitable mode of selectivity needs to be used. The pcDNA3.1 myc/HisA vector contains a coding region for geneticin resistance. Geneticin or G418 disulphate is an antibiotic that inhibits protein synthesis.

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 (MIC) to select for geneticin resistance. The concentration of 500μg/ml appeared to kill approximately 90% of a confluent monolayer of CHO cells.

Transfection of CHO cells with Ang-1 pcDNA3.1 myc/HisA DNA was conducted 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 survived geneticin selection, indicating that the cells had incorporated the transfected angiopoietin-1 DNA, were allowed to grow to confluence. Cells were passaged and plated to allow colonies to form. Twelve 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.

2.9.1 Ang-1 Production

The selected clones were serum starved for 24 hours and the conditioned media was removed for analysis using SDS-PAGE and Western Blotting for Ang-1 expression (refer to Section 2.6). Production of Ang-1 was analysed by probing for the myc tag using a mouse monoclonal anti-myc antibody (refer to Section 2.6.6.3). Suitable clones were selected and grown as described in Section 2.2.3. Conditioned media from selected clones was concentrated as indicated in Section 2.10.

2.10 N-cadherin (extracellular domain) FC Production

DNA encoding N-cadherin (extracellular domain) FC in the pIG 5.2 vector was kindly donated by Dr Patrick Doherty, Molecular Neurobiology Group, GKT School of Medicine, London. TOP10/P3 ultracompetent cells were purchased from Invitrogen. Transformation was carried out as in Section 2.7.8 except LB agar plates contained 15μg/ml ampicillin and 10μg/ml tetracycline. Transformation plates were incubated at 37°C for 16 hours. Plasmid DNA was prepared and transfected into CHO cells using the Superfect protocol (Section 2.2.4.1).

Transfected cells were incubated in 1% low IgG FCS and Complete Media (Section 2.2.1) for 72 hours. The conditioned media was removed from each flask and pooled. This was centrifuged at

300g for 6 minutes to remove any debris. The media was then loaded into a Centricon YM-10 concentrating device (Millipore) in accordance with the manufacturer's instructions. Approximately 15ml of conditioned media was concentrated to a volume of 1ml.

2.10.1 Protein Purification of N-cadherin FC

50mg of Protein A sepharose (Sigma) was added to approximately 10ml of concentrated media, and left to mix overnight at 4°C. The sepharose was centrifuged at 1,000g for 2 minutes, and the supernatant removed. The pellet was washed with PBS and placed in a microcentrifuge tube. The entire pellet was washed three times with PBS centrifuging each time at 13,000g for 30 seconds. The N-cadherin-FC was eluted by mixing with 500µl of 100mM Glycine pH 2.7 and removal of the Protein A sepharose by centrifugation. The supernatant containing eluted protein is added to 50µl of 1M Tris pH 9.0 to neutralise it. The concentration of purified N-cadherin-FC was determined by dot blotting, immunodetection with anti-FC and comparison with known FC-standards (Tie1 FC from R & D Systems).

2.11 Immunohistochemistry

All antibodies and reagents from Dako Cytomation unless stated otherwise.

2.11.1 General Solutions

PBS-Tween – 140mM NaCl, 10mM Na_2PO_4, 1.5mM KH_2PO_4, 0.5×10^{-3}% Tween-20.

10mM Citrate Buffer – 10mM $C_6H_8O_7$, pH 6.0.

Veronal Buffer – 29mM CH_3COONa, 1M NaCl, 50mM $MgCl_2$, 19mM $C_8H_{11}N_2NaO_3$.

2.11.2 Preparation of Tissue

Tissue was removed from the patient and fixed in formalin. Overnight the tissue was automatically dehydrated through alcohol and impregnated with wax. The tissue was then paraffin embedded and serial 4µm sections were cut using a microtone. These sections were then floated onto silane-coated slides ready for immunostaining.

Before fixing in formalin carotid artery tissue was decalcified by incubating in 10% Formic Acid for at least 48 hours.

2.11.3 Detection of Antigen Using Duet/ABC™ Complex Technique

Sections were dewaxed and rehydrated. Antigen retrieval was performed if required (refer to Section 2.11.6). Any endogenous peroxidase activity was suppressed by exposing the tissue to 6% H_2O_2 Solution. The sections were then washed once with water and then with PBS-Tween for 5 minutes.

Any non-specific antibody binding was suppressed by immersing the section in 5% normal goat serum for 10 minutes at room temperature. Excess serum was removed before incubating with primary antibody (refer to Section 2.11.8) for 1 hour at room temperature or overnight at 4°C. Sections were then washed twice in PBS-Tween for 5 minutes. The sections were incubated with biotinylated goat anti mouse/rabbit IgG (1 in 200) for 30 minutes at room temperature. At this point the avidin-biotin complex was prepared allowing at least 30 minutes for the complex to bind. Streptavidin and biotinylated horseradish peroxidase were combined together in PBS-Tween. Sections were then washed in PBS-Tween for 20 minutes at room temperature. To increase the signal from the sections the avidin-biotin complex conjugated to HRP (1 in 200) was added for 30 minutes at room temperature. Slides were washed twice with PBS-Tween for 10 minutes at room temperature before developing with the chromogen, DAB. DAB was prepared to a final concentration of 1mg/ml and then activated with 3% H_2O_2 (refer to Section 2.11.7.1). All sections were counter stained with Haematoxylin and dehydrated before mounting.

2.11.4 Detection of Antigen Using ChemMate™ Envision™ Technique

Sections were dewaxed and rehydrated. Antigen retrieval was performed if required (refer to Section 2.11.6). The section was incubated with the primary antibody (refer to Section 2.11.8) for 1 hour at room temperature. Sections were then washed twice in PBS-Tween. Endogenous peroxidase activity was blocked with ChemMate™ H_2O_2 blocking buffer, twice for 5 minutes at room temperature. Sections were twice washed with PBS-Tween for 5 minutes. The sections were incubated with ChemMate™ secondary complex (streptavidin containing mouse and rabbit immunoglobulins) for 25 minutes at room temperature and then washed twice in PBS-Tween for

10 minutes before developing with DAB. DAB was prepared to a final concentration of 0.5mg/ml (refer to Section 2.11.7.2). All sections were counter stained with Haematoxylin and dehydrated before mounting.

Detection of Tie2 required inclusion of an extra step. After the quenching of endogenous peroxidase activity sections were incubated with rabbit anti-goat immunoglobulins (1 in 100) for 30 minutes at room temperature. Sections were washed twice with PBS-Tween and incubated with ChemMate™ secondary complex.

2.11.5 Dual Staining

Sections were dewaxed, rehydrated and pre-treated with trypsin for 10 minutes at 37°C. Sections were incubated with primary antibodies, rabbit polyclonal anti-von Willebrand Factor (vWF) and mouse monoclonal anti-smooth muscle actin (SMA) for 1 hour at room temperature or overnight at 4°C. Sections were then was washed twice with PBS-Tween for 5 minutes and incubated with a secondary antibody, swine anti-rabbit recognising the anti-vWF antibody (1 in 200) for 30 minutes at room temperature. Sections were washed with PBS-Tween and incubated with a tertiary antibody, rabbit peroxidase anti-peroxidase diluted 1 in 100 for 30 minutes at room temperature. Sections were washed with PBS-Tween and re-incubated with the secondary antibody, swine anti-rabbit for 30 minutes and then washed with PBS-Tween.

Tissue sections were then incubated with the secondary antibody, rabbit anti-mouse biotinylated recognising the anti-smooth muscle actin (1 in 400) for 30 minutes at room temperature. Sections were washed with PBS-Tween and incubated with the tertiary antibodies, rabbit peroxidase anti-peroxidase (1 in 100) and avidin alkaline phosphatase (1 in 400) for 30 minutes at room temperature. Sections were washed with PBS-Tween for 20 minutes at room temperature before developing with DAB to recognise the peroxidase activity of the tertiary antibody, rabbit peroxidase anti-peroxidase (refer to Section 2.11.7.1). Sections were washed with water for 5 minutes and alkaline phosphatase activity was detected using FastRed TR salt (refer to Section 2.11.7.3). FastRed development was checked every 10 minutes and then washed in water. All sections were counter stained with Haematoxylin before aqueous mounting.

2.11.6 Antigen Retrieval

In some cases antigen retrieval was required. The following methods we need for antigen retrieval.

(i) Trypsin

Sections trypsinised in 0.12% Trypsin, 9mM $CaCl_2$, pH 7.8 at 37°C for 10 minutes.

(ii) Microwave Heat Treatment

Sections heat treated in 10mM Citrate Buffer, pH 6.0 in an 800W microwave for 12 minutes on high power. Cooled before starting immunohistochemical technique.

(iii) Pressure Cooker Heat Treatment

Sections heat treated in 10mM Citrate Buffer, pH 6.0 in a pressure cooker for 5 minutes 30 seconds. Cooled before starting immunohistochemical technique.

2.11.7 Development

Two different protocols were used for DAB preparation, dependent on the detection kit that was used.

2.11.7.1 DAB Preparation for Duet/ABC™ Technique

For detection by the duet/ABC kit a frozen tablet of DAB (10mg/ml) was thawed and resuspended in PBS to a final concentration of 1mg/ml. Activating the DAB with 3% H_2O_2 catalysed the peroxidase enzyme of the tertiary antibody. Activated DAB was filtered before use.

2.11.7.2 DAB Preparation for ChemMate™ Envision™ Technique

In the ChemMate™ Envision™ technique the manufacturer's protocol is followed. Briefly, 400µl DAB+ chromogen is added to 20ml Dako Substrate Buffer.

2.11.7.3 FastRed Developer Preparation

The FastRed developer was prepared in the fume cupboard. The levamisole, FastRed TR salt and napthol asB1 phosphate were weighed into separate bijoux. Levamisole and FastRed TR salt were dissolved in veronal acetate buffer. Napthol asB1 phosphate was dissolved in 3-4 drops of dimethylformamide. The two solutions were combined and filtered into a coplin jar.

2.11.8 Primary Antibodies

Antibodies used with each staining technique are detailed below:

(i) Duet/ABC™ and Dual Staining Technique

Antigen	Primary Antibody	Concentration (Ig conc^a unless stated)	Antigen Retrieval	Source
Endothelial cell	Mouse monoclonal anti-CD34	1µg/ml	None	Novocastra
Smooth muscle actin	Mouse monoclonal anti-SMA	0.25µg/ml	None	Dako Cytomation
Macrophage	Mouse monoclonal anti-CD68	2.5µg/ml	Trypsin	Dako Cytomation
Lymphocyte	Mouse monoclonal anti-CD45	13µg/ml	None	Dako Cytomation
Proliferating cell nuclear antigen	Mouse monoclonal anti-PCNA	10µg/ml	Trypsin	Dako Cytomation

		2µg/ml	Microwave	Santa Cruz Biotechnology
VEGF	Rabbit polyclonal anti-VEGF			
Endothelial cell	Rabbit polyclonal anti-vWF	30µg/ml (total protein concentration)	Trypsin	Dako Cytomation

(ii) ChemMate™ Envision™ technique

Antigen	Primary Antibody	Concentration (Ig conc^a unless stated)	Antigen Retrieval	Source
Endothelial cell	Rabbit polyclonal anti-vWF	7µg/ml (total protein concentration)	Trypsin	Dako Cytomation
Smooth muscle actin	Mouse monoclonal anti-SMA	0.15µg/ml	None	Dako Cytomation
Survivin	Rabbit polyclonal anti-survivin	1µg/ml	Pressure Cooker	Abcam
Ki-67	Mouse monoclonal anti-Mib-1	20µg/ml (total protein concentration)	None	Dako Cytomation
Phosphorylated Tie2 receptor	Rabbit polyclonal anti-PYTek	1/1000*	Pressure Cooker	Oncogene Research Products
Tie2 receptor	Goat polyclonal anti-Tie2	10µg/ml	Pressure Cooker	R & D Systems

*Supplier does not provide antibody concentration.

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2.12 Densitometric Analysis

Immunoblots were scanned using an AGFA scanner and then analysed in IPLab software (Scanalytics). Images were saved as TIFF files with no LZW compression and converted to greyscale and then inverted in the IPLab. The band of interest was captured using the Region of Interest (ROI) function and the sum of density in the ROI was measured.

2.13 Statistical Analysis

Analysis of results was conducted using the 'students' t test where three or more samples were available. Data is presented as mean +/-standard deviation. P values ≤ 0.05 were considered statistically significant. Conservative p values were observed due to multiple t tests.

Chapter Three

Characterisation of dysmorphogenic microvessels associated with pathological angiogenesis in atherosclerotic plaques.

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3.1 Introduction

Angiogenesis in pathological situations often give rise to both increased numbers of microvessels and dysmorphogenic microvessels. Angiogenesis is observed in the atherosclerotic plaque (Folkman 1995) and plaque angiogenesis appears to correlate with plaque growth (Moulton et al. 2003). In a study of microvessels associated with atherosclerotic plaques McCarthy et al. (1999b) observed the microvessels retrieved from carotid artery were dysmorphogenic. Specifically, these vessels appeared enlarged and highly irregular in cross-section. Importantly this study found a correlation between the number of dysmorphogenic vessels and patient symptomatology. Patients exhibiting pre-operative transient ischaemic attacks (TIAs), a surrogate marker for plaque instability, had significantly increased numbers of intraplaque dysmorphogenic microvessels (McCarthy et al. 1999b). This study suggests the existence of dysmorphogenic vessels is associated with plaque instability. The reason for dysmorphogenesis of these vessels is not known. One possibility is that the microvessel dysmorphogenesis reflects neovessel immaturity and lack of the regulatory influences normally provided by perivascular mural cells. To address this possibility the mural cell cover of intraplaque microvessels was analysed.

In order, to answer these questions histological analysis and immunohistochemistry was used.

3.2 Immunohistochemical analysis of carotid plaques

Carotid arteries were removed from patients presenting TIAs with informed consent and embedded in paraffin. Sections of paraffin-embedded carotid artery tissue were immunologically stained using primary antibodies in conjunction with the Duet/ABC complex method (Sections 2.11.3 and 2.11.8). All antibodies were supplied by Dako Cytomation except where indicated.

3.2.1 Large, irregular neovessels lack mural cells

Figure 3.1 diagrammatically represents the location of microvessels in the carotid plaque. Microvessels were observed mainly on the shoulder or inside developing fibrous cap of the atherosclerotic plaque (location 1.), as well as in relatively normal artery of the same sample (location 2.). Immunohistochemical analysis using the endothelial cell marker anti-CD34 confirmed the presence of the multi-lobular, irregular vessels observed by McCarthy et al. (1999b) (Figure 3.2). These vessels were observed throughout the excised carotid artery.

The vessels in the sections of the carotid plaque were immunologically labelled with anti-smooth muscle actin (SMA) to determine smooth muscle cell association. Examination of vessels for SMA revealed the large, dilated dysmorphogenic vessels were lacking smooth muscle cell/pericyte association (Figure 3.3). Using a double-blinded analysis carotid plaques from symptomatic patients were scored for the types of vessels in the tissue and their association with smooth muscle cells. Three vessel types were observed and characterised accordingly. Vessels designated Class I were multi-lobular, aberrant vessels; Class II were enlarged vessels, circular or flattened in shape; Class III were the smaller microvessels (Figure 3.4A). Furthermore, a system was devised for scoring the level of SMA staining on the different types of vessels. For substantial SMA staining (75-100%) vessels were marked as +3, moderate staining (50-75%) +2, minimal staining (25-50%) +1 and no smooth muscle cell actin staining (0-25%) 0. In 14 symptomatic patients, presenting all three types of vessels, smooth muscle cell association was quantified using the above scoring system. Irregular vessels had poor SMA staining whereas the enlarged vessels had lower SMA staining than Class III vessels but higher levels of staining than Class I vessels (Smooth muscle association in irregular vessels = 0.14+/-0.36; enlarged vessels = 1.43+/-0.85; microvessels = 2.93+/-0.27, data presented as mean +/-standard deviation) (Figure 3.4B). Statistically, the smaller microvessels had higher levels of SMA staining compared to both the irregular and enlarged vessels ($p \leq 0.0001$ and $p \leq 0.0001$). Enlarged vessels had significantly higher SMA staining than irregular vessels ($p \leq 0.0001$).
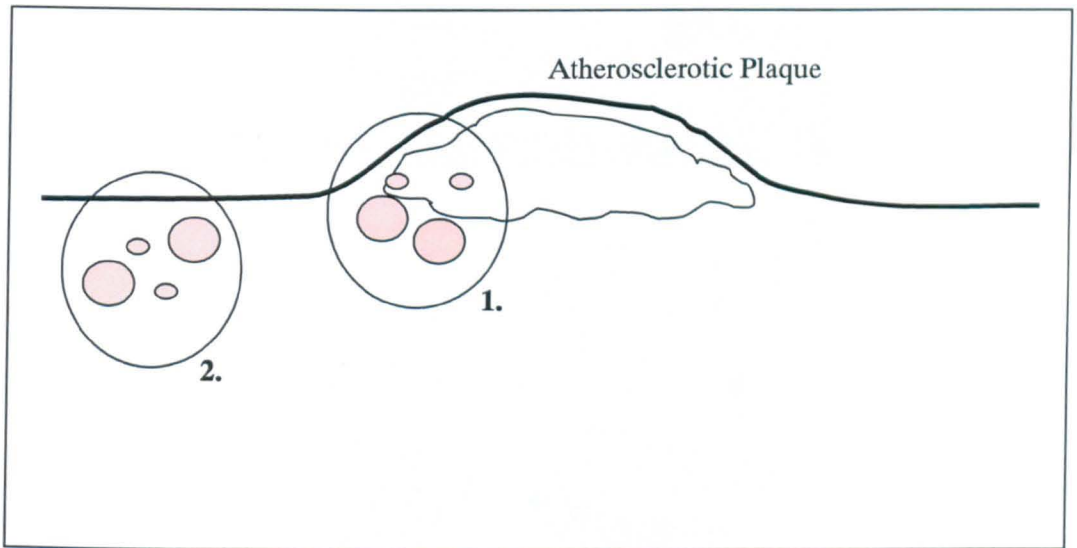
Figure 3.1 **Schematic representations of the locations in which vessel counts were performed.** (**1.**) Microvessels located on the shoulder or inside the developing plaque. (**2.**) Microvessels located in relatively normal artery away from the developing plaque. This is the nomenclature used throughout Chapter Three identifying the location of the microvessels.
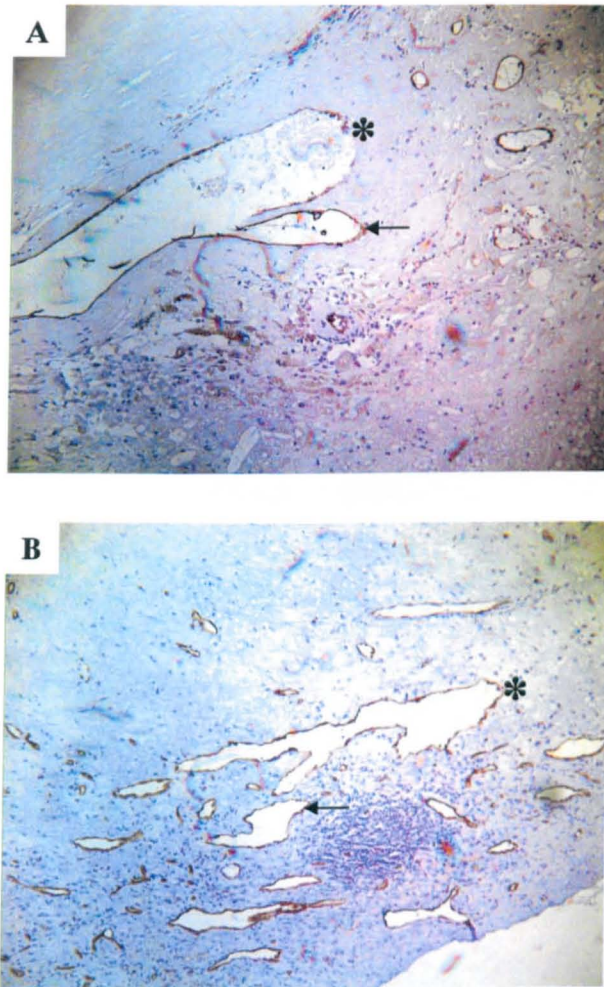
Figure 3.2 **Representative views of microvessels within carotid atherosclerotic plaques.** Sections of paraffin-embedded carotid artery tissue were immunologically stained using an anti-CD34 antibody, an endothelial cell marker. Arrows mark the labelling of endothelial cells using the chromogen, DAB, observed as a brown colour. A heterologous population of microvessels is observed including the large multi-lobular vessels described by McCarthy et al (1999) (indicated by *). **A)** Represents microvessels located at the cusp or inside the developing plaque (location 1.). **B)** Represents microvessels located in relatively normal artery away from the developing plaque (location 2.). Original magnification x100.
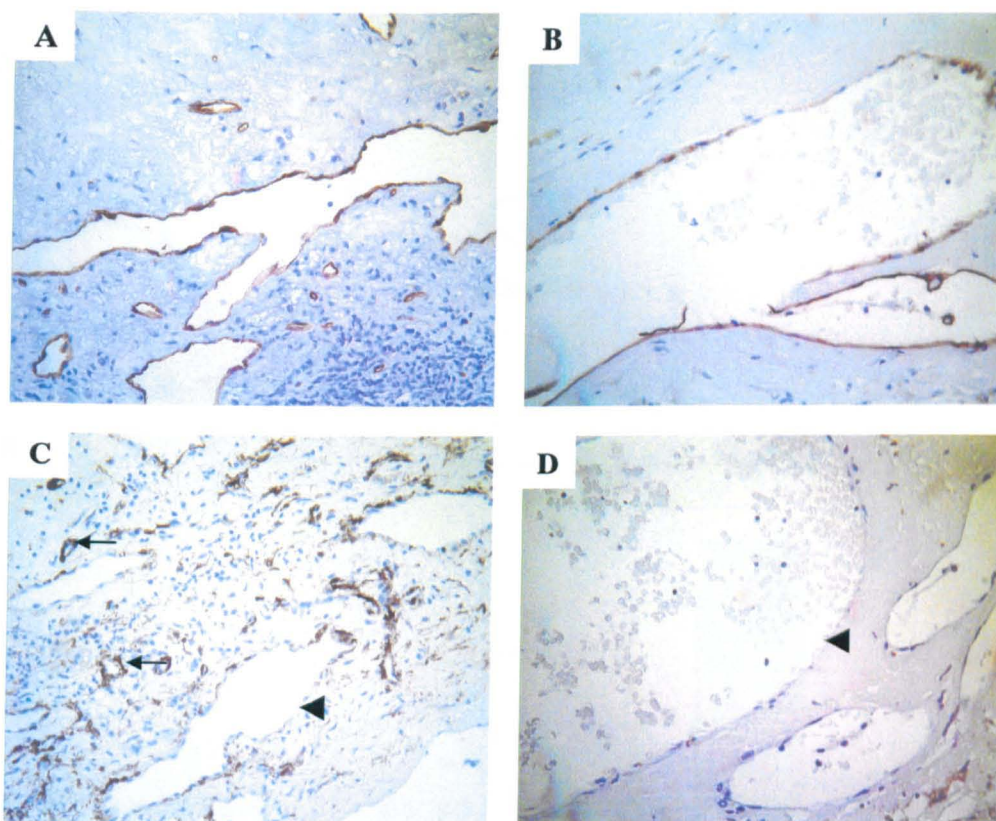
Figure 3.3 **Irregular shaped vessels either lack or have poorly associated smooth muscle cells.** Sections of the carotid artery were cut and immunologically labelled with anti-CD34 and anti-SMA. **A)** and **B)** show endothelial cells are marked with the brown colour in microvessels in relatively normal tissue and near the developing plaque, (location 2.) and (location 1.) respectively in the carotid plaque nomenclature. **C)** and **D)** indicate the respective coverage of smooth muscle cells marked with the brown colour. Irregular shaped microvessels (arrowheads) showed a poor or complete lack of mural cell investment compared to relatively normal vessels (arrows). Microvessels developing at the edge of the plaque often appeared to have no smooth muscle cell coverage whatsoever **(D)**. Original magnification x250.
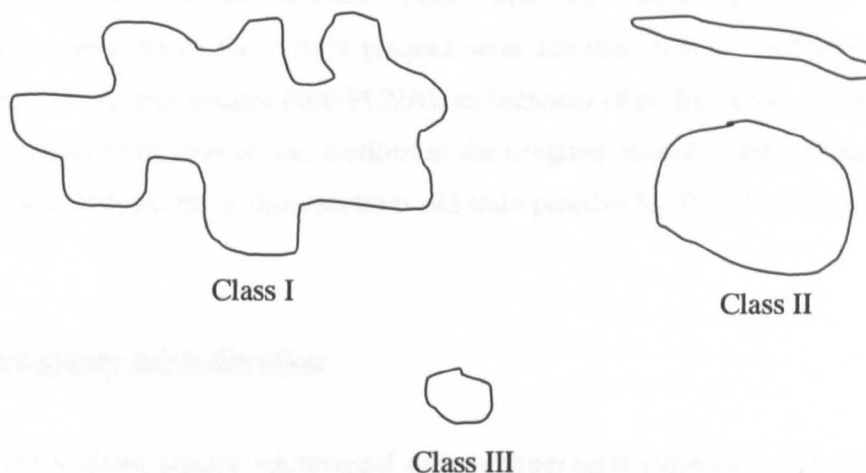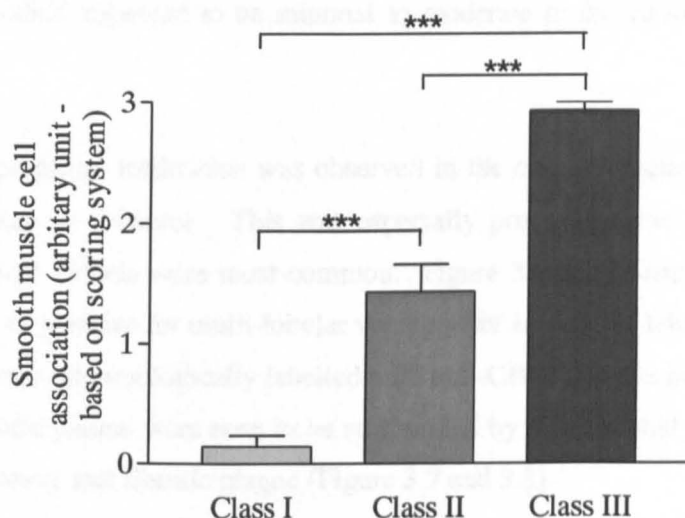
Figure 3.4 **Multi–lobular and enlarged vessels have significantly lower smooth muscle cell coverage than smaller microvessels.** Carotid plaques from 14 patients were scored for the types of vessels in the plaque and their smooth muscle cell coverage using double blinded analysis. **A)** Diagrammatic representation of the types of vessels observed in the carotid plaques. Class I – irregular vessels, multi-lobular; Class II – enlarged vessels, circular or flattened; Class III – smaller microvessels. **B)** Vessels were scored for smooth muscle cell association using the following system. +3 = substantial smooth muscle cell association (75-100%); +2 = moderate association (50-75%); +1 = minimal association (25-50%); 0 = no association (0-25%). Analysis of smooth muscle cell association in the symptomatic patient showed a significant paucity in irregular and enlarged vessels compared to Class III vessels. Data presented as mean +/-standard deviation for 14 patients (n=14). *** p≤0.001 students 't' test.

3.2.2 Endothelial cell proliferation within plaque neovessels

It was hypothesised that the smooth muscle cell poor microvessels may have increased proliferation. Sections of the carotid plaques were labelled with an antibody recognising the proliferating cell nuclear antigen (anti-PCNA), an indicator of proliferation. No proliferation was observed in endothelial cells on the sections in the irregular shaped vessels (Figure 3.5A and B). Some non-endothelial cells in these sections did stain positive for PCNA confirming the antibody worked.

3.2.3 Inflammatory cell infiltration

To determine whether plaque microvessel dysmorphogenesis correlated with inflammatory cell infiltration sections were probed with antibody markers for macrophages (anti-CD68) and lymphocytes (anti-CD45). Lymphocyte infiltration in the carotid plaque where abnormal vasculature was evident appeared to be minimal to moderate in the surrounding tissue (Figure 3.6A and B).

An increase in macrophage infiltration was observed in the carotid plaques where irregular and aberrant vessels were in evidence. This was especially prominent near or in the developing plaque where aberrant vessels were most common. Figure 3.7 shows sections of carotid tissue from three patients all positive for multi-lobular vessels after anti-CD34 labelling (Figure 3.7B, D and F). Sections were immunologically labelled with anti-CD68 and the multi-lobular vessels at the edge of the carotid plaque were seen to be surrounded by a substantial macrophage infiltrate in the surrounding tissue and fibrotic plaque (Figure 3.7 and 3.8).

3.2.4 VEGF expression

Smooth muscle cells associated with microvessels suppress endothelial cell apoptosis and vessel regression (Liu et al. 2000). The relative paucity of smooth muscle cell cover in dysmorphogenic vessels within the plaques therefore suggested the vessels were sustained by other factors. A strong candidate is VEGF a potent initiator of vessel remodelling and associated with the maintenance of the vasculature. Therefore, sections were probed for the presence of VEGF. Sections from three patients positive for multi lobular vessels were labelled with a pan-VEGF antibody recognising the isoforms 121, 165 and 189 (Figure 3.9).
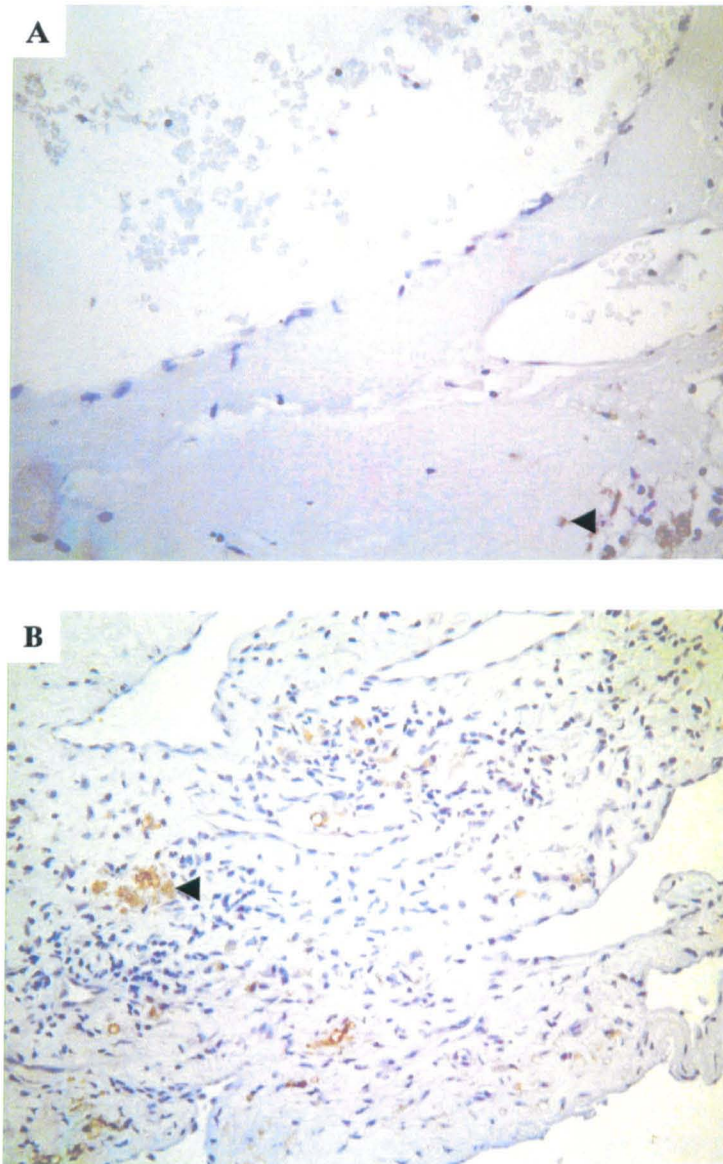
Figure 3.5 **Irregular microvessels do not have higher proliferative rates.** Sections of paraffin-embedded carotid artery tissue were immunologically stained using an anti-PCNA, proliferative cell marker, to determine the level of vessel turnover and possible remodelling of the irregular shaped microvessels. **A)** and **B)** represent the proliferative cells located in (location 1.) and (location 2.), respectively in the carotid plaque nomenclature. No proliferating endothelial cells were observed though PCNA-positive cells (arrowhead) were observed within the plaque. Original magnification x250.
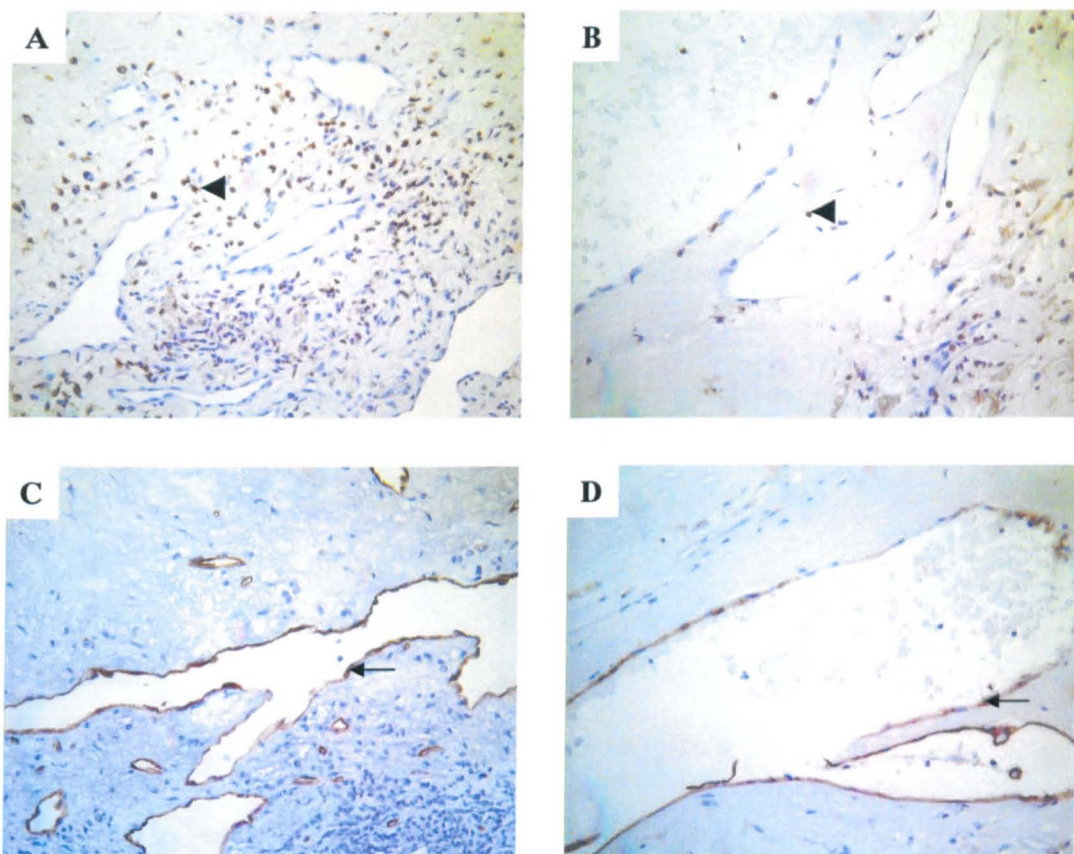
Figure 3.6 **Lymphocyte infiltration increased in areas of irregular vascular development.** Sections of paraffin-embedded carotid artery tissue were immunologically stained using anti-CD45, specific lymphocyte marker. **A)** and **B)** represent the lymphocyte infiltration located in (location 2.) and (location 1.), respectively. **C)** and **D)** anti-CD34 (arrows) indicate the location of lymphocyte infiltration appears to correspond to abnormal microvessels. Arrowheads indicate labelled lymphocytes. Original magnification x250.
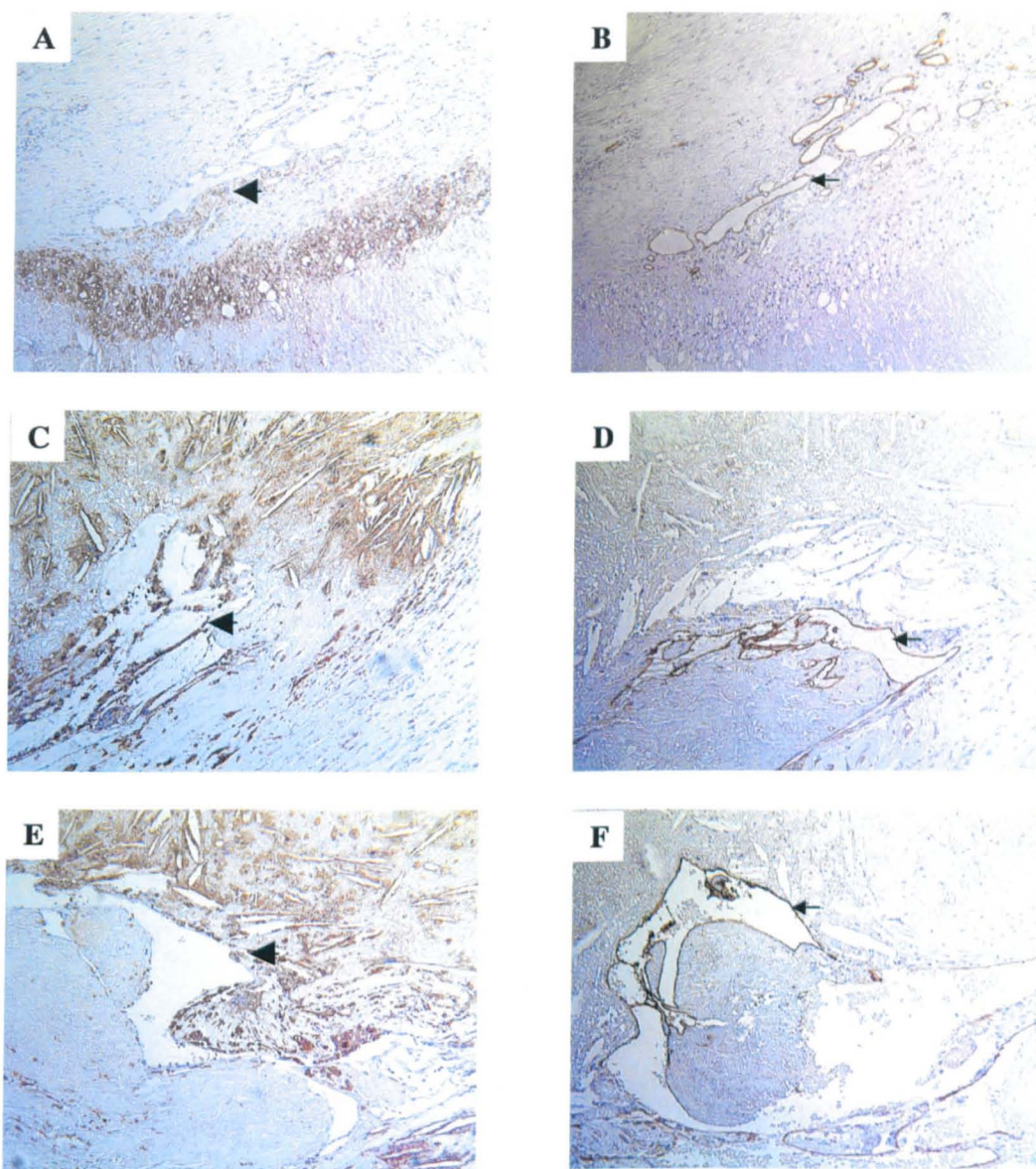
Figure 3.7 **Macrophage infiltration is observed in the tissue surrounding dysmorphogenic vessels.** Sections of carotid plaque tissue were stained with anti-CD68, macrophage marker (**A, C, E**) or anti-CD34, endothelial cell marker (**B, D, F**). Substantial macrophage infiltration (indicated by the brown colour and arrowheads) was evident around the dysmorphogenic vessels (arrows) and in the fibrotic plaque (**A, C, E**). Original magnification x100.
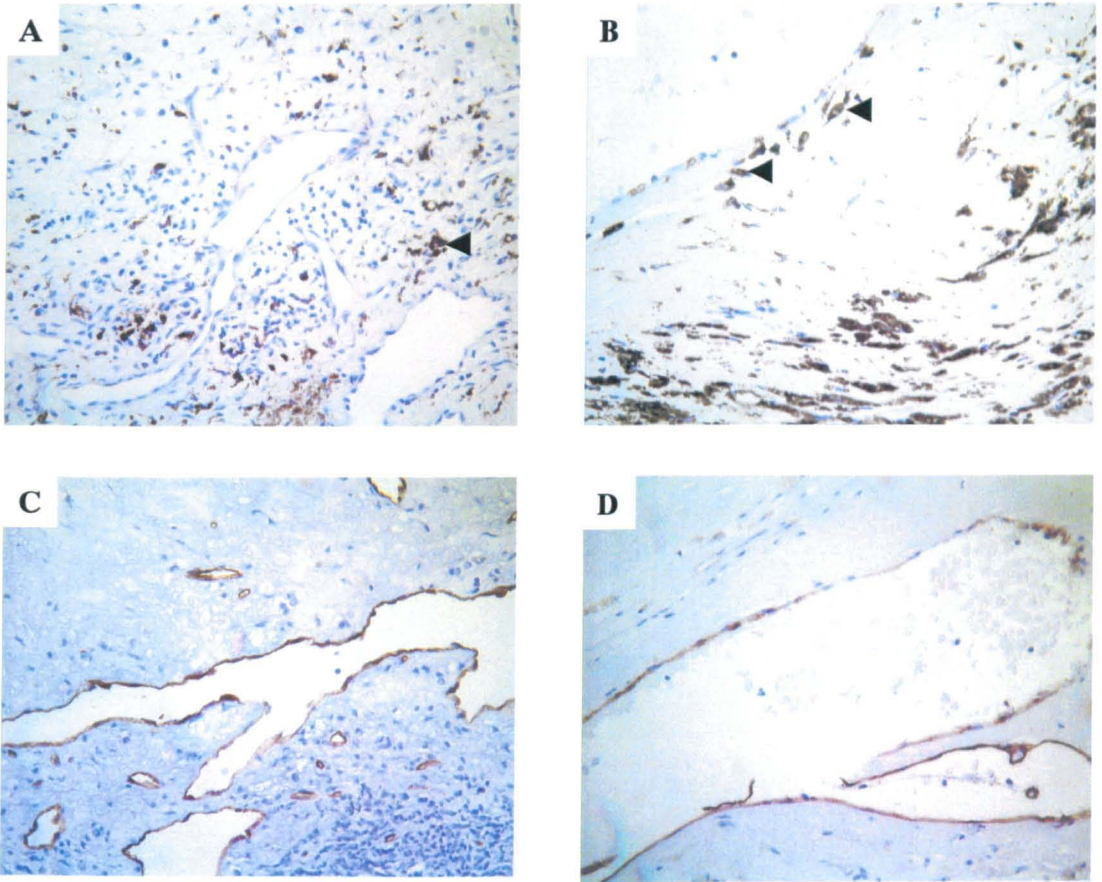
Figure 3.8 **Increased macrophage infiltration at the site of irregular microvessels lacking smooth muscle cells.** Sections of paraffin-embedded carotid artery tissue were immunologically labelled with anti-CD68, a macrophage specific marker. **A)** and **B)** show the macrophage infiltration located in (location 2.) and (location 1.), respectively in the carotid plaque nomenclature. Macrophages were observed near or directly next to irregular vasculature. **C)** and **D)** show the dysmorphogenic vessels labelled with anti CD34 in the areas (location 2.) and (location 1.) of the carotid nomenclature, respectively. Arrowheads indicate labelled macrophages next to a dysmorphogenic vessel. Original magnification x250.
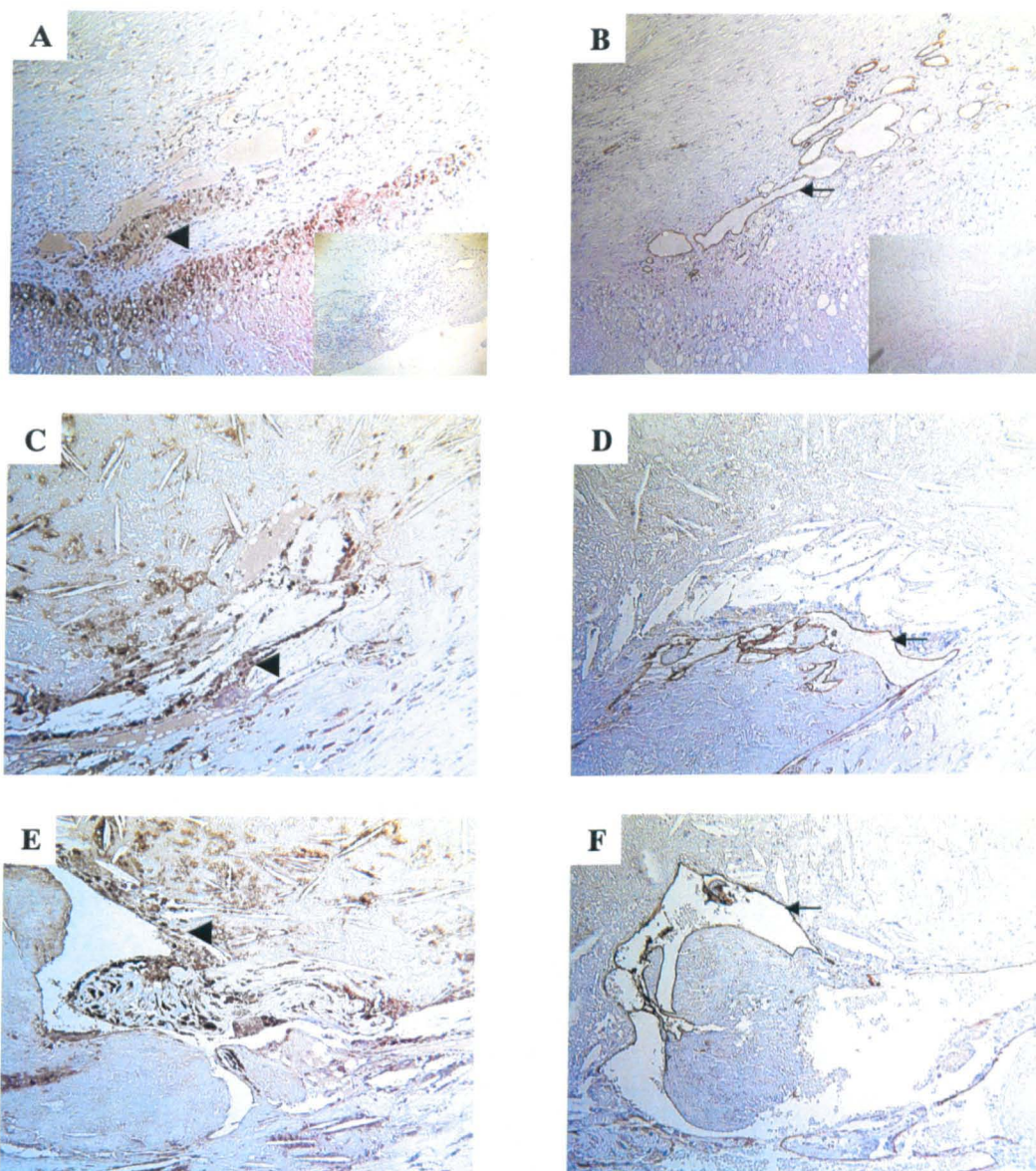
Figure 3.9 **VEGF expression is observed in the tissue surrounding dysmorphogenic vessels.** Sections of carotid plaque tissue were labelled with an anti-pan VEGF antibody (**A, C, E**) or anti-CD34 (**B, D, F**). Substantial VEGF expression (indicated by the brown colour and arrowheads) was evident around the dysmorphogenic vessels and in the fibrotic plaque. Multi-lobular vessels labelled with CD34 and indicated by arrows were observed. **A)** Sections were also stained in the presence of the peptide to which the antibody was raised to confirm specificity (inset). **B)** Negative labelling shows the specificity of the Duet/ABC complex protocol used throughout the carotid plaque study (inset). Original magnification x100.

On closer inspection of another patient VEGF was expressed in the surrounding tissue and in the endothelium of both normal and abnormal vessels (Figure 3.10). The presence within the surrounding tissue coincided with the increase in macrophage infiltration (Figure 3.11).

In order to determine the specificity of anti-VEGF binding the protocol was also carried out in the presence of a blocking peptide for anti-VEGF (APMAEGGGQNHHEVVKFMDV) (Santa Cruz Biotechnology). Insets on Figure 3.9A and B demonstrate the specificity of the VEGF antibody and negative immunostaining confirming the specificity of the Duet/ABC technique, respectively.

Overall, these data demonstrate the dysmorphogenic neovessels within the carotid plaque lacked smooth muscle cell association. There was an increase in macrophage cell infiltration which was associated with an increase in VEGF expression in the surrounding tissue and endothelium of the abnormal vasculature.

3.3 Percutaneous myocardial revascularisation (PMR)

During the course of the carotid artery study a further example of pathology-associated angiogenesis became available. This was in the form of myocardial wound healing following percutaneous myocardial revascularisation.

Percutaneous myocardial revascularisation (PMR) is a procedure which involves delivering a holmium:YAG laser into the left ventricular cavity via a catheter under fluoroscopic guidance. The laser would then deliver controlled bursts of energy to create small channels (~1.75 mm diameter) extending from the endocardial surface into the myocardial wall (Cotton et al. 2002). Preliminary studies have suggested that PMR provides relief from the symptoms of myocardial ischaemia (Lauer et al. 1999). However, a recent randomised trial has shown little benefit to patients (Leon). At the time of the present study it was hypothesised that one mechanism whereby PMR may aid relief of ischaemia symptoms could be by stimulating myocardial angiogenesis (Bridges 2000; Owen and Stables 2000). It was of interest therefore to determine whether areas treated by PMR exhibited increased microvessel density and if so to examine the nature of the vessels.
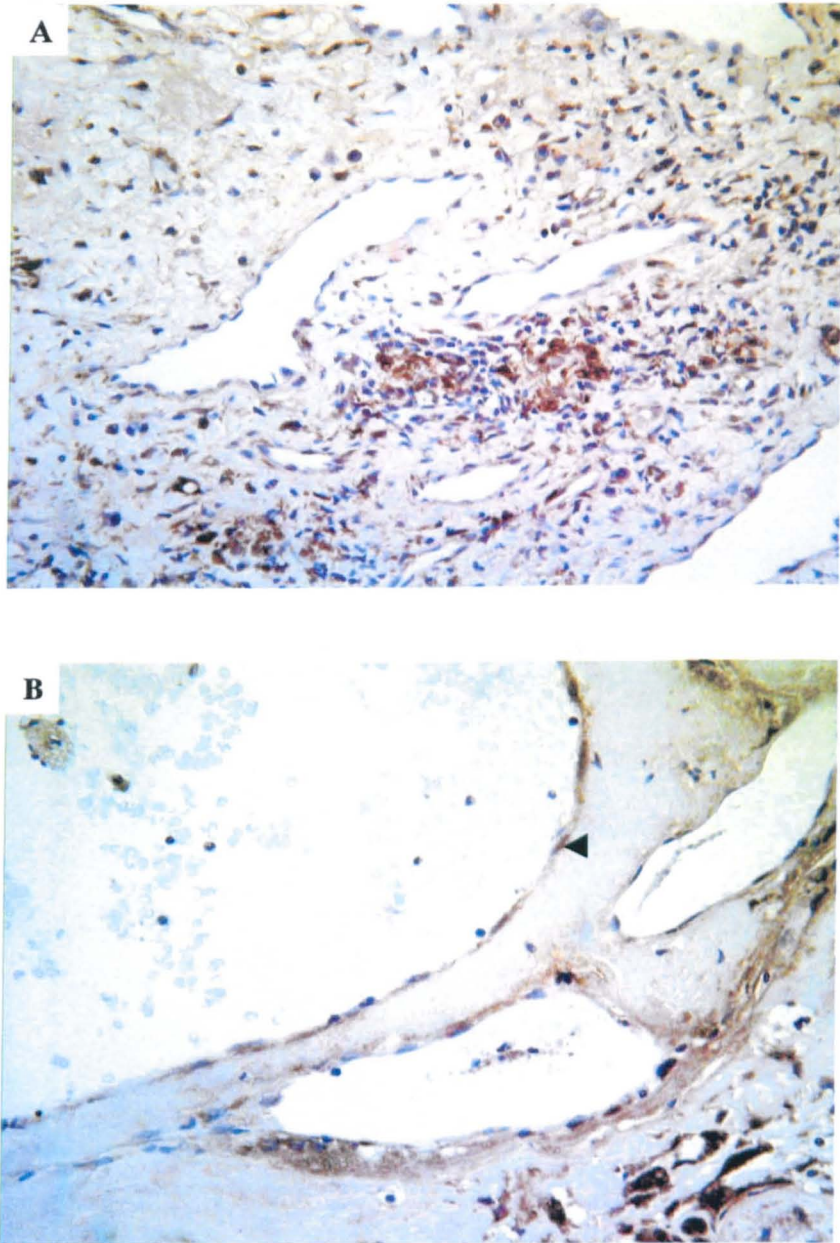
Figure 3.10 **VEGF expression is observed close to dysmorphogenic vessels.** Sections of the carotid artery were cut and immunologically labelled with anti-VEGF. **A)** and **B)** show microvessels in (location 2.) and (location 1.), respectively in the carotid plaque nomenclature. VEGF expression can be clearly seen in the endothelial cells of the irregular microvessels (arrowhead). Original magnification x250.
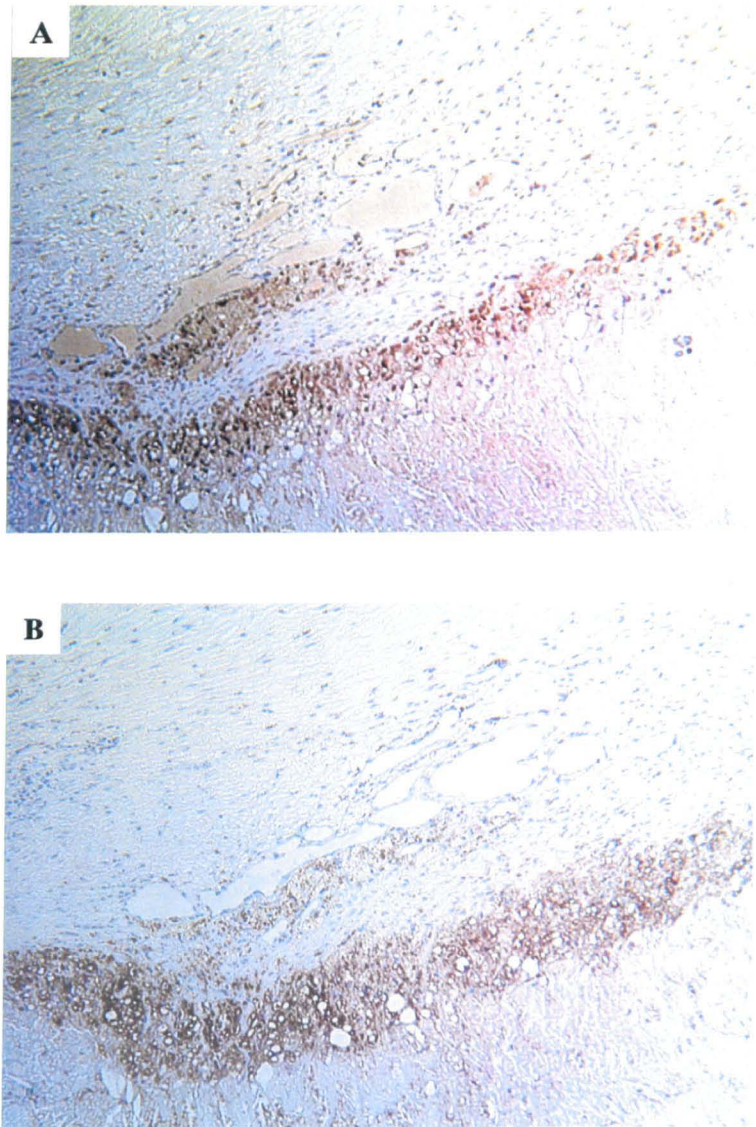
Figure 3.11 **VEGF expression corresponds to macrophage infiltration.** Sections of carotid plaque tissue were labelled with an anti-pan VEGF antibody (**A**) and anti-CD68 macrophage marker (**B**). Multi lobular vessels and the fibrotic carotid plaque showed similar labelling patterns for VEGF and CD68. Original magnification x100.

Samples of PMR treated and non-treated left ventricles were kindly provided by Professor Shah, Department of Cardiology, King's College Hospital, London, UK. These samples were taken from two patients, one who died at 8 weeks and one at 52 weeks post treatment. Both patients died of causes unrelated to myocardial infarction. Ventricle samples were fixed, paraffin embedded, and 4μm sections cut as described in Section 2.11.2. Sections were stained with antibodies recognising endothelial cells to examine vessel morphology, as well as anti-SMA.

3.3.1 PMR induces microvessels

Patients had responded positively after surgery and there appeared to be a symptomatic benefit. Fibrotic scarring was observed in treated areas compared with relatively normal tissue taken from a non-treated area of the left ventricle (Figure 3.12). Within the zones of fibrosis there was an increase in microvessel density indicated by the positive staining by the endothelial cell marker, anti-vWF (Figure 3.12). Microvessels in the fibrotic zones were enlarged and irregular. Furthermore, the microvessels in the fibrotic zones contained blood cells, suggesting the vessels had been perfused (Figure 3.12).

3.3.2 PMR induced microvessels lack smooth muscle cell support

The mural cell investment of the microvessels was investigated. A dual staining technique was devised to stain for vWF and the smooth muscle cell marker, SMA (Section 2.11.5).

In the non-treated left ventricle tissue from the 8 week patient the vessels possessed a tight smooth muscle cell coating (Figure 3.13A). Microvessels in the treated section in the areas lacking fibrotic scarring were invested with smooth muscle cells and appeared to be relatively normal. However, the microvessels in the fibrotic zones possessed no smooth muscle cells or poorly associated smooth muscle cells, these neovessels were abnormally shaped similar to those observed in the carotid artery study (Figure 3.13B). In both patients large, abnormal vessels lacking a smooth muscle cell investment were observed and these were particularly prominent in the 52-week patient (Figure 3.14).
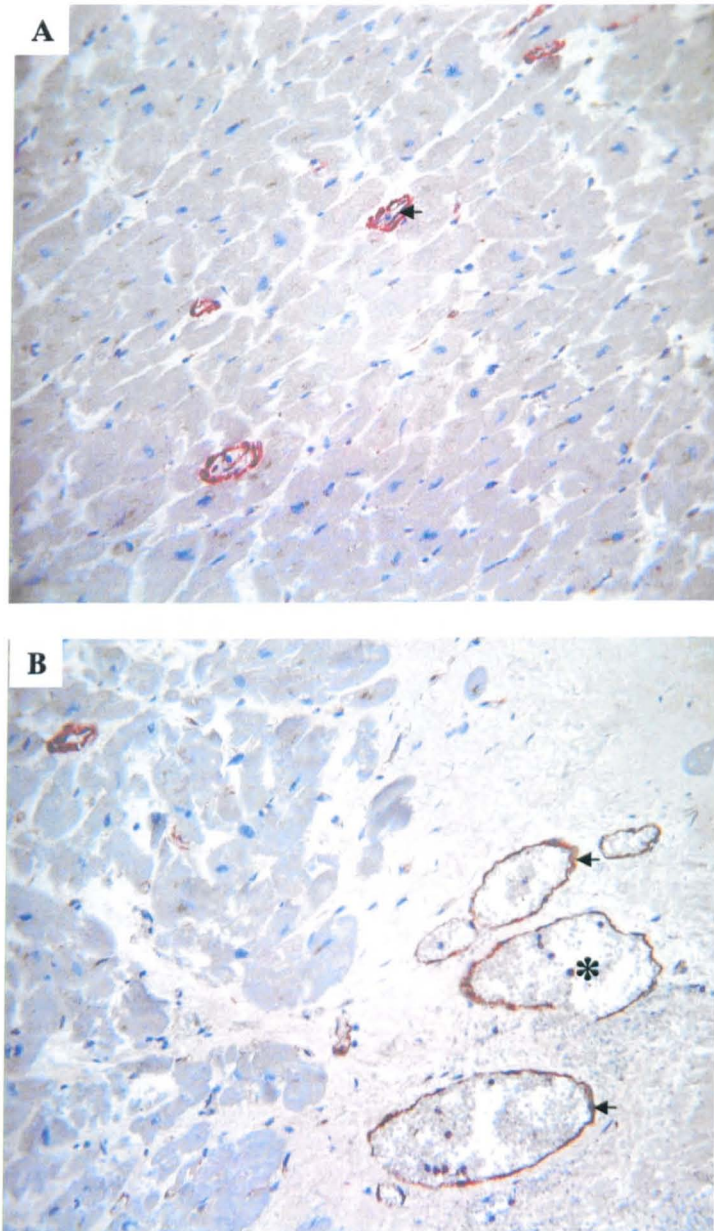
Figure 3.12 **Comparison of treated and non-treated areas of myocardium after PMR treatment.** A section from the 8 week PMR patient was immunologically labelled with anti-vWF (brown). **A**) Shows the relatively healthy left ventricle from the same patient. **B**) Indicates the fibrotic scarring possibly from a previous myocardial infarction and the PMR treatment. It also represents an increase in microvessel density (arrow) and the labelling of blood cells provides evidence for perfusion (*). Original magnification x100.
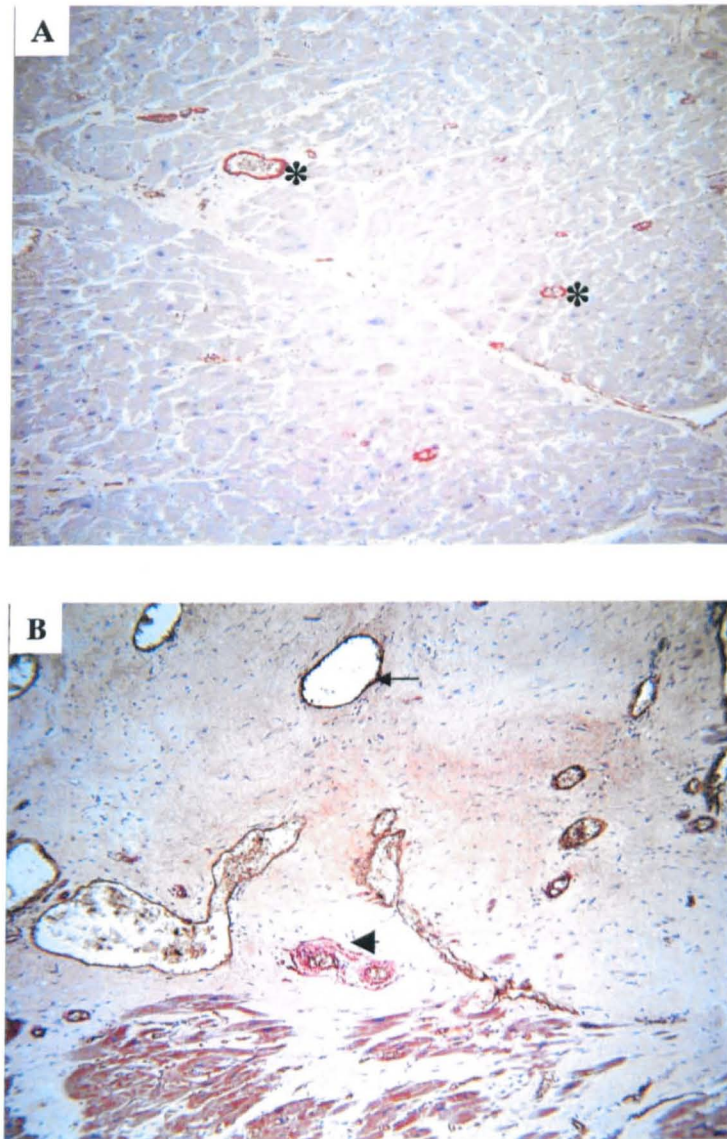
Figure 3.13 **Dysmorphogenic vessels either lack or have a loose smooth muscle cell investment compared to relatively normal vessels.** Section from an 8-week PMR patient was immunologically labelled with anti-vWF (brown) and anti-SMA (pink). **A**) Relatively healthy left ventricle exhibits normal vessels with a tight smooth muscle cell association (indicated by *). **B**) The ventricle treated by PMR exhibited larger vessels lacking smooth muscle cells (arrows) or with loose smooth muscle cell investment (arrowheads). Original magnification x100.
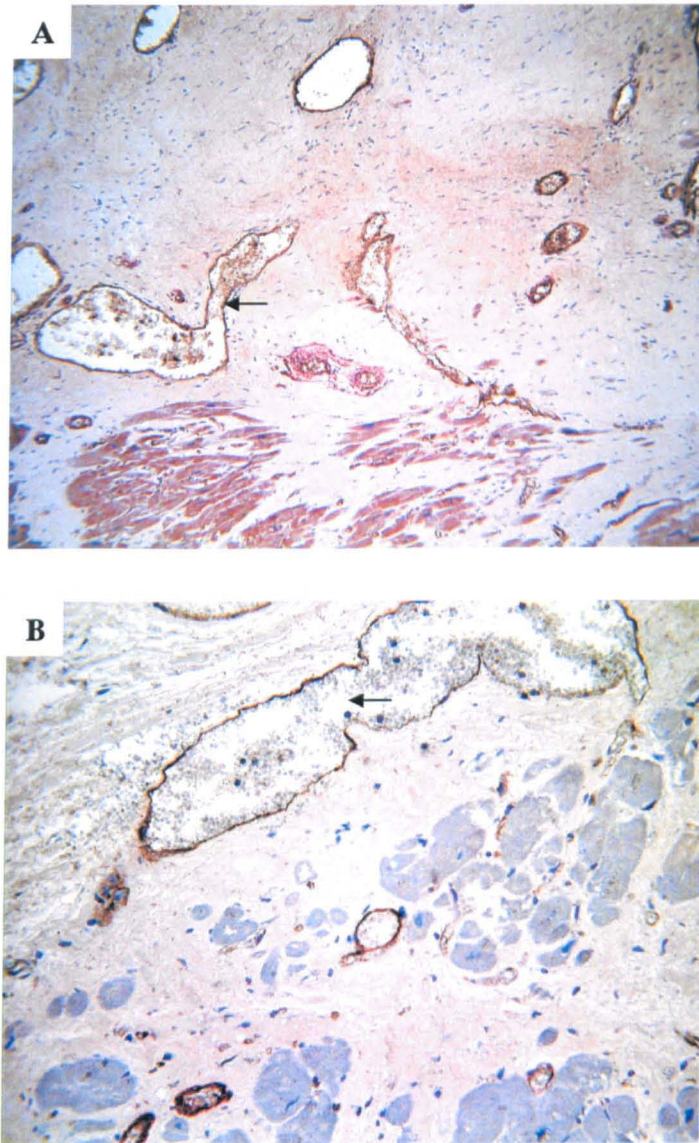
Figure 3.14 **Dysmorphogenic vessels appear larger in the section taken from the patient 52 weeks after PMR treatment.** Sections from the (**A**) 8 and (**B**) 52-week PMR patients were immunologically dual labelled with anti-vWF (brown) and anti-SMA (pink). The microvessels of the 52 week patient appear substantially larger (arrows). Original magnification x100.

3.3.3 PMR treated myocardium possessed elevated levels of VEGF expression

To determine whether PMR treated areas of the ventricles expressed VEGF sections were probed with a VEGF antibody that recognises the isoforms 121, 165 and 189. VEGF expression was increased in the PMR treated sections. In the control sections, cardiac myocytes and the microvessels containing a smooth muscle investment expressed VEGF. In sections from PMR treated ventricles, fibrotic areas showed little or no VEGF expression, although smooth muscle cells surrounding microvessels in this area did exhibit expression (Figure 3.15).

Although tissue from only two patients was available the immunohistochemistry did show PMR increased the number of microvessels within the fibrotic areas of the ventricle after treatment. The microvessels created were dysmorphogenic, either lacking or poorly associated with smooth muscle cells. Again, these vessels were associated with an increase in VEGF expression. VEGF expression was associated with the myocardium containing relatively normal vessels. Although this study is based upon observations made in two patients precluding quantitative statistical analysis there are clear trends in both patients.

3.4 Discussion

This chapter has described studies aimed at characterising the dysmorphogenic microvessels associated with atherosclerotic plaque neovascularisation. In addition, preliminary data is reported on microvessel morphology and mural cell coverage in PMR-treated human myocardium. A primary finding is dysmorphogenic microvessels generally lack smooth muscle cells. This raises a number of questions, why do these vessels lack mural cells? How the neovessels are maintained? and does the lack of smooth muscle cells make the neovessels dysmorphogenic?

Potential prosurvival factors that could prevent regression of these mural cell poor vessels include VEGF. Certainly VEGF could maintain these vessels as was observed by Benjamin et al. (1999) where xenografted glioma tumour vessels lacking smooth muscle cells were ablated upon the removal of chronic VEGF expression. Furthermore, research has shown that chronic over-expression of VEGF resulted in malformed and hyper-perfused vessels (Drake and Little 1995). In the present study expression of VEGF was seen in the surrounding tissue adjacent to the dysmorphogenic mural cell poor vessels in both the carotid plaque and PMR studies.
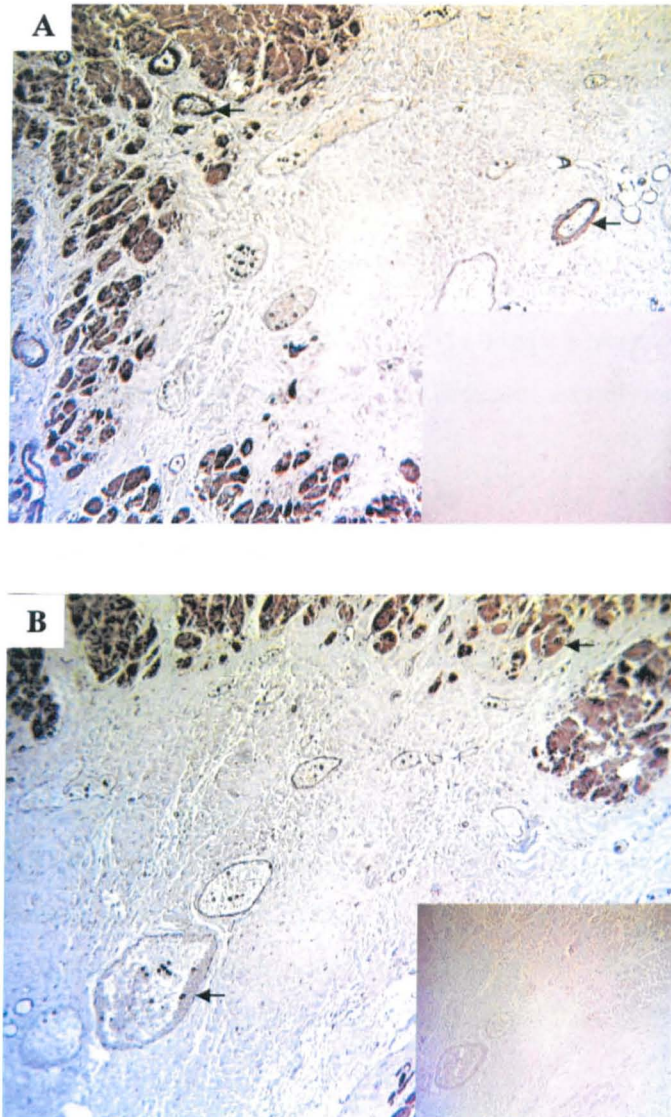
Figure 3.15 **VEGF expression is found in myocytes and smooth muscle cells.** Sections from the 8-week PMR patient were cut and immunologically labelled with an anti-VEGF antibody. **A)** and **B)** show VEGF expression in both the fibrotic area and relatively normal myocytes (arrows). **A)** Sections were also stained in the presence of the peptide to which the antibody was raised to confirm specificity (inset). **B)** Negative labelling shows the specificity of the Duet/ABC complex protocol used throughout the carotid plaque study (inset). Original magnification x100.

In physiological vessel formation PDGF has a key role in mural cell differentiation and recruitment (Lindahl et al. 1997; Crosby et al. 1998; Hirschi et al. 1998; Lindahl et al. 1998; Hirschi et al 1999). Mice deficient for either PDGF-B or PDGFR-β are either lacking or have poorly associated smooth muscle cells/pericytes in several vascular beds causing the development of microaneurysms (Lindahl et al. 1997; Hellstrom et al. 1999). Another molecule involved in promoting stable vascular development is TGF-β. Mice deficient in TGF-β present a vascular phenotype lacking smooth muscle cells. In hereditary haemorrhagic telangiectasia (HHT1) vessels exhibit a lack of smooth muscle cells due to the loss of function of the TGF-β binding protein, endoglin. Mice deficient in endoglin die at embryonic day 11.5 due to defective vascular development from poor vascular smooth muscle cell growth (Li et al. 1999). Furthermore in mice deficient in the activin receptor-like kinase 1 (ALK1), a type I receptor for TGF-β, vascular defects were characterised by poor differentiation and deficient recruitment of vascular smooth muscle cells (Oh et al. 2000).

It is possible; therefore, that the lack of mural cell cover of microvessels in the plaques and PMR could reflect impairment of PDGF mediated recruitment of smooth muscle cells or TGF-β induced differentiation of the mesenchyme. It will be necessary to examine PDGF/PDGFR and TGF-β/TGF-β receptor status of plaques and PMR microvessels to test the hypothesis.

As discussed earlier it is a realistic possibility that the dysmorphogenic vessels of the carotid plaque and PMR treated myocardium are maintained by VEGF. Increased VEGF expression was observed in both pathological situations but this not conclusive evidence for dysmorphogenic vessel maintenance. In order to demonstrate this, investigations into the blockage of VEGF and regression of dysmorphogenic vessels in the carotid plaque would have to be undertaken.

As observed in the pathological situations of this study it is possible that dysmorphogenic vessels manifest due to the lack of pericytes/smooth muscle cells. The possible causes of vessel dysmorphogenesis could be explained by the following mechanisms. Firstly, irregular vessels develop as a result of no physical limit on vessel expansion. Secondly, the mural cell poor vessels lack negative regulators such as TGF-β thus allowing endothelial cell proliferation and migration. Thirdly, adhesion molecule function and signalling could be impaired resulting in defective mural cell attachment. In the carotid plaque and PMR treated myocardium dysmorphogenic vessels were observed with poorly associated mural cells.

From the observations in this study and research conducted by McCarthy et al. (1999b) it is possible that dysmorphogenic vessels contribute to pathology in the carotid plaque. Indeed, direct correlation has been observed between vessel dysmorphogenesis and symptoms (plaque instability) (McCarthy et al. 1999b). These dysmorphogenic vessels could be 'activated' and leaky resulting in increased inflammatory cell recruitment. Potentially, enhanced expression of inflammatory mediators could lead to increased levels of matrix metalloproteinases (MMPs), the main physiological regulator of the extracellular matrix (Dollery et al. 1995; Loftus et al. 2000). Studies have shown increased expression of MMPs in atherosclerotic plaques. Increased MMP-2 expression has been observed in coronary atherosclerotic plaques and increased MMP-9 expression has been correlated with an increase in carotid plaque instability of highly symptomatic carotid plaque patients (Loftus et al. 2000).

The identification of mural cell poor vessels resulting in dysmorphogenic vessels generates a possible therapeutic target. The dysmorphogenic vessels of the atherosclerotic plaque could be stimulated to regress if the growth factor or cytokine maintaining these vessels was known. Therefore, knowledge of the factors involved in normal and pathological vascular development is critical.

Interaction between endothelial cells and smooth muscle cells in vessel formation is so far poorly understood though clearly of importance. The Tie2 receptor has been implicated in controlling endothelial:mural cell interaction. In the next Chapter studies will be conducted aimed at defining mechanisms, particularly those involving Tie2, in mural cell acquisition.

Chapter Four
Angiopoietin-1 increases adhesion between endothelial and mural cells.

Chapter Four

Angiopoietin-1 increases adhesion between endothelial and mural cells.

4.1 Introduction

In the previous Chapter vessel dysmorphogenesis in the carotid artery of the symptomatic patient was associated with poor smooth muscle/pericyte coverage. This suggests paucity of smooth muscle cell coverage in plaque microvessels may contribute to plaque instability as detailed in Section 3.4. A lack of smooth muscle cells therefore could result in microvessels that are activated, promoting recruitment of inflammatory cells into the plaque, which in turn could contribute to plaque fragility via protease production by inflammatory cells. Furthermore, dysmorphogenic vessels that are thin walled potentially could contribute to intraplaque haemorrhage further destabilising the plaque. In addition, the dysmorphogenesis of the plaque vessels, and the finding that microvessels lacking mural cell coverage are also dysmorphogenic in other pathological situations such as the PMR treated myocardium; suggest mural cells may also possess a role in promoting normal vessel morphology.

Therefore, smooth muscle cell interaction with endothelial cells and recruitment of smooth muscle cells or pericytes to newly formed vessels is important in normal vascular formation.

A possible role for the Tie2 receptor and angiopoietins in mural cell:endothelial cell interaction has already been suggested from observations in gene knockout mice (Suri et al. 1996; Patan 1998). In particular, deficiency of the Ang-1 gene reduced the association of mural cells with microvessels, and the vessels were poorly formed failing to branch and remodel (Patan 1998). These data indicate Ang-1 may have a role in regulating heterotypic cell interaction between endothelial cells and smooth muscle cells.

The aim of the studies described in this Chapter was to test directly whether Ang-1 affects smooth muscle cell recruitment and adhesion to endothelial cells in a simplified cell culture model.

4.2 Angiopoietin-1 phosphorylates Tie2 in endothelial cells

Ang-1* is a readily purified recombinant form of Ang-1 previously used in most studies examining Tie2 activation and function (Maisonpierre et al. 1997; Asahara et al. 1998). This was kindly provided by AstraZeneca. Ang-1* is different to the physiological molecule as the complete native Ang1 signal sequence is replaced with that of the Ang-2 ligand (amino acid residues 1-73 inclusive), throughout this Chapter two forms of Ang-1* were used. As well as the purified form (Ang-1*), serum free conditioned media from Ang-1* secreting cells was produced as described in Section 2.9 (this is designated Ang-1*cond). In addition to Ang-1* the native form of Ang-1 was also produced as described in Section 2.9 and this was utilised as serum free conditioned media in some experiments (this is designated Ang-1). Controls for each form of Ang-1 was as follows – for Ang-1* control was PBS, for Ang-1 and Ang-1*cond control was conditioned media from serum starved CHO cells.

To confirm Ang-1* and Ang-1 were biologically active the ability of the ligand to stimulate tyrosine phosphorylation of Tie2 was studied. BAE cells were serum starved for 2 hours and then pre-treated with 10mM Na_3VO_4 for 15 minutes, suppressing the ability of phosphatases to reduce protein phosphorylation. Cells were stimulated with Ang-1* at 200ng/ml or PBS for 15 minutes at 37°C/5% CO_2. Cells were then lysed as in Section 2.6.3.1. Tyrosine phosphorylated proteins were immunoprecipitated using a mouse monoclonal antibody specific for tyrosine phosphorylated proteins (Section 2.6.3.2) and tyrosine phosphorylated proteins were detected by immunoblotting with a mouse monoclonal phosphotyrosine antibody (New England Biolabs). Levels of Tie2 receptor were identified using an anti-Tie2 antibody (Santa Cruz Biotechnology). As shown in Figure 4.1, increased levels of Tie2 were immunoprecipitated by the anti-phosphotyrosine antibody from cells treated with Ang-1* and the immunoprecipitated Tie2 corresponded to an increased phospho-tyrosine immunoreactivity. These data confirm the ability of Ang-1* to activate phosphorylation of Tie2 receptor (Figure 4.1A). Similar levels of Tie2 expression were confirmed in whole cell lysates of Ang-1* treated and control cells.

The ability of native Ang-1 conditioned media (500ng/ml) was also determined in parallel experiments using the same method for Ang-1* (Figure 4.1B). As shown in Figure 4.1B stimulation of cells with Ang-1 resulted in increased levels of Tie2 immunoprecipitated by anti-phosphotyrosine antibody and increased tyrosine phosphorylation immunoreactivity. These data confirm Ang-1 can activate tyrosine phosphorylation of Tie2.
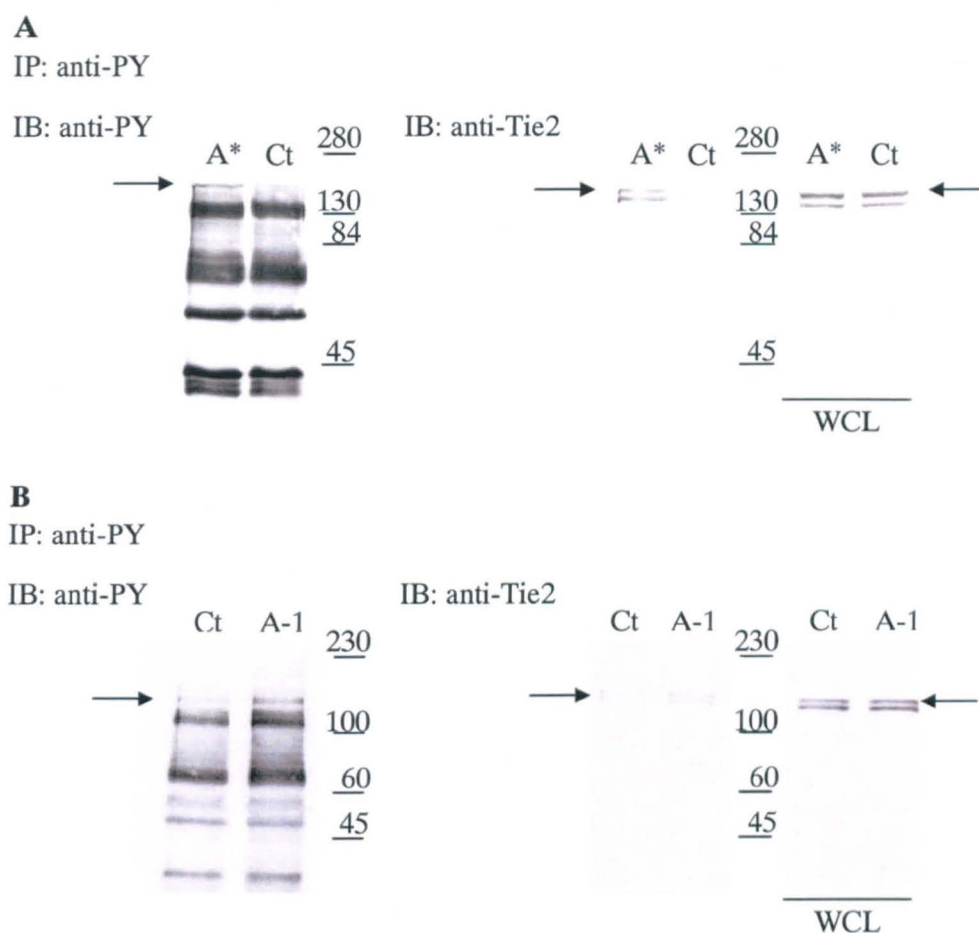
Figure 4.1 **Ang-1 activates Tie2 receptor phosphorylation.** BAE cells were stimulated with **A**) Ang-1* (200ng/ml) (**A***) or **B**) Ang-1 (~500ng/ml) (**A-1**), respectively, for 15 minutes prior to lysis and immunoprecipitation using an anti-phosphotyrosine antibody (Section 2.6.3.2). Samples were fractionated using (**A**) 10% or (**B**) 14% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Membranes were probed with antibodies that specifically recognise tyrosine phosphorylated proteins (**anti-PY**) and the Tie2 receptor (**anti-Tie2**). Arrows represent the Tie2 receptor. **Ct** – control; **IP** – immunoprecipitation; **IB** – immunoblots; **WCL** – whole cell lysate.

4.3 Angiopoietin-1 stimulates mesenchymal cell migration towards endothelial cells

Mural cell acquisition by a newly forming vessel will be influenced by the ability of endothelial cells to recruit mural cells and mural cell precursors, retention of mural cells and the influence on differentiation, and proliferation of recruited mural cells. In this study the effects of Ang-1 on recruitment of mural cells and their adhesion was investigated.

CH310T1/2 is a multipotent mouse embryonic cell line that can be induced to differentiate into a smooth muscle cell phenotype on contact with endothelial cells in culture and *in vivo* (Hirschi et al. 1998). This cell line was used as a presumptive mural cell precursor to examine the effects of Ang-1 on endothelial cell directed mural cell migration.

Preliminary experiments were conducted to determine whether the endothelial cells released chemoattractants for 10T1/2 cells. To do this, lower wells of a 24 well plate were left unseeded or seeded with mouse heart endothelial (MHE) cells to form a confluent monolayer. After 24 hours the wells were filled with serum free medium. After another 24 hours 10T1/2 cells (10^5 cells) were added to the upper chamber of the transwell tissue culture insert (8μm pore size) and placed in each well. 10T1/2 cells were allowed to migrate towards the endothelial cells or serum free media for a period of 6 hours at 37°C/5% CO_2. Migration was stopped and inserts were fixed and the presence of 10T1/2 cells on the lower surface of the insert counted following staining with haematoxylin and eosin. For details of migration assays refer to Section 2.4.

The migration of 10T1/2 cells was increased in the presence of endothelial cells confirming the release of pro-migratory factors by endothelial cells (average cell count for control = 4.4+/-2.35 compared to the presence of MHE cells = 85.3+/-15.85 data presented as mean +/-standard deviation for 20 fields) (Figure 4.2).
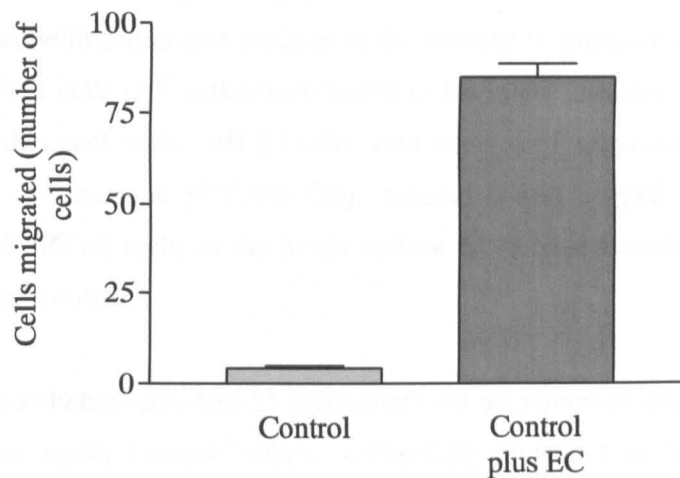
Figure 4.2 **Migration of 10T1/2 cells is increased by the presence of endothelial cells.** Lower chambers of a 24 well plate were seeded to 100% confluence with MHE cells or serum free media. Serum free media was placed in lower chambers for 24 hours and then 10T1/2 cells (10^5 cells) were placed into the upper chambers of the insert and incubated for 6 hours. The number of 10T1/2 cells present on the lower surface of the insert were counted following staining with haematoxylin and eosin. Data is presented as mean +/-standard deviation for 20 fields. **EC** – MHE cells.

To examine the effects of Ang-1* on MHE directed migration of 10T1/2 cells a confluent monolayer of MHE cells was seeded into the lower chamber of a 24 well plate and serum starved for 6 hours in the presence or absence of Ang-1* (200ng/ml). Transwells were then inserted into each well and 10T1/2 cells were placed into each insert (10^5 per well). 10T1/2 cells were allowed to migrate towards the endothelial cells for a period of 2 hours at 37°C/5% CO_2. As shown in Figure 4.3A there was a small but statistically significant increase in the number of 10T1/2 cells migrating towards the Ang-1* treated MHE cells compared with the control treated MHE cells (126.4+/-7.58% as a percentage of control, data presented as mean +/-standard deviation for three experiments, p≤0.05; Figure 4.3A).

To test whether this effect of Ang-1* required the presence of MHE cells, lower wells of a 24 well plate were filled with serum free medium in the absence or presence of Ang-1* (200ng/ml). After 24 hours 10T1/2 cells (10^5 cells) were added to the upper chamber of the transwell tissue culture insert placed in each well. 10T1/2 cells were allowed to migrate towards the serum free media for a period of 6 hours at 37°C/5% CO_2. Migration was stopped and inserts were fixed and the presence of 10T1/2 cells on the lower surface of the insert counted following staining with haematoxylin and eosin.

In the absence of endothelial cells Ang-1* (200ng/ml) did not stimulate migration of 10T1/2 cells compared to control media (control cells = 4.40+/-2.35 compared to Ang-1* treated cells = 1.20+/-2.74 data presented as mean +/-standard deviation for 20 fields; Figure 4.3B).

The assay was also conducted in the presence of VEGF (100ng/ml). VEGF did not significantly effect the migration of 10T1/2 cells to endothelial cells compared with mock treated cells (113.7+/-21.11% as a percentage of control data presented as mean +/-standard deviation for three experiments; Figure 4.4).

These data indicate that Ang-1* has the ability to cause a small increase in mural cell precursor migration towards endothelial cells whereas VEGF has no significant effect upon MHE cell directed migration of 10T1/2 cells.

A

B
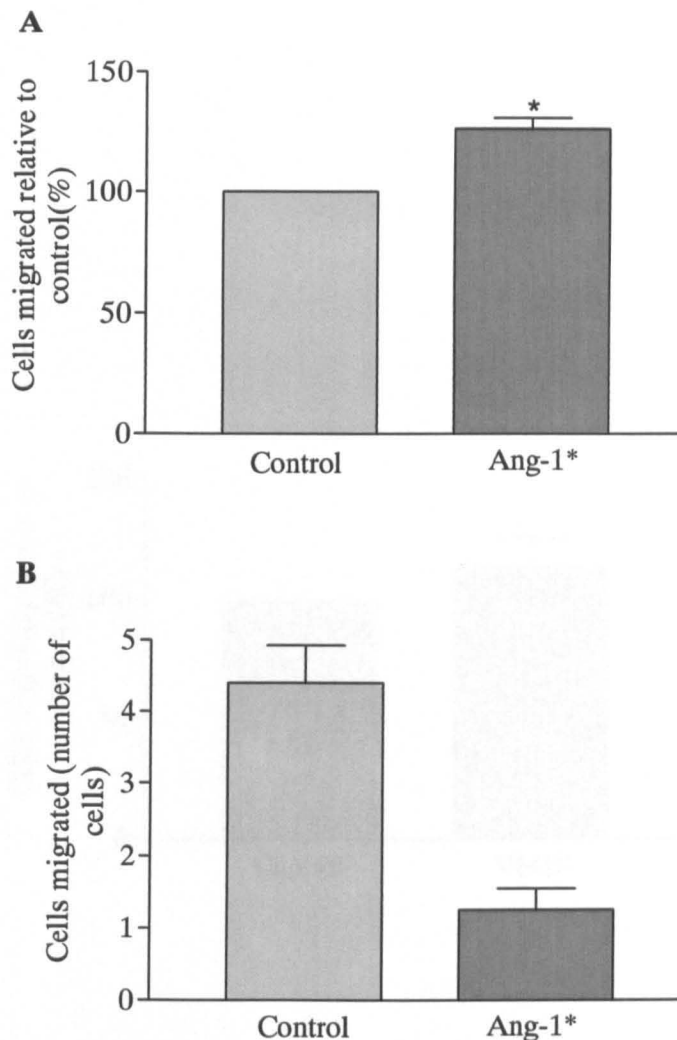
Figure 4.3 **10T1/2 cell migration towards an endothelial cell stimulus is significantly increased in the presence of Ang-1. A**) Confluent monolayers of MHE cells were maintained in the lower chambers of a 24 well plate. Prior to insertion of transwells MHE cells were serum starved in the presence or absence of Ang-1* (200ng/ml) for 6 hours. 10T1/2 cells (10^5 cells) were then added to an 8μm porous membrane transwell and incubated for 2 hours. The number of 10T1/2 cells present on the lower surface of the insert were counted following staining with haematoxylin and eosin. Data presented as mean +/-standard deviation for three experiments (n=3). * p≤0.05 students 't' test. **B**) To test the effect of Ang-1* above on 10T1/2 migration, lower chambers of a 24 well plate were left unseeded and serum free medium in the absence (control) or presence of Ang-1* (200ng/ml) was added. 10T1/2 were then added to the upper wells of the insert and incubated for 6 hours. The number of 10T1/2 cells present on the lower surface of the insert were counted following staining with haematoxylin and eosin. Data presented as mean +/-standard deviation for 20 fields.
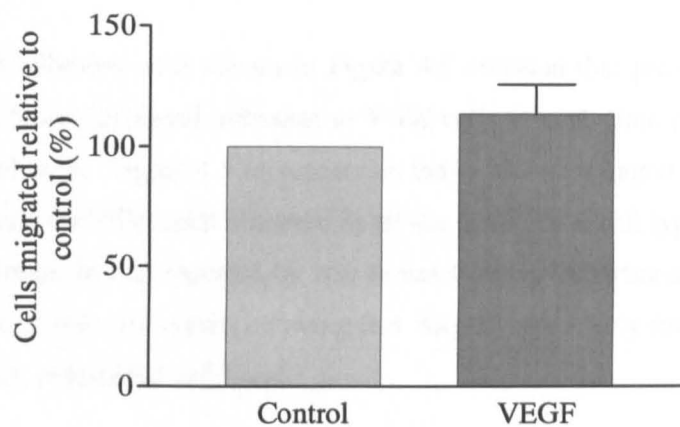
Figure 4.4 **10T1/2 cell migration is not affected by VEGF.** Confluent monolayers of MHE cells were maintained in the lower chambers of a 24 well plate. Prior to insertion of transwells MHE cells were serum starved in the presence or absence of VEGF (100ng/ml) for 6 hours. 10T1/2 cells (10^5 cells) were then added to an 8μm porous membrane transwell and incubated for 2 hours. The number of 10T1/2 cells present on the lower surface of the insert were counted following staining with haematoxylin and eosin. Data presented as mean +/-standard deviation for three experiments (n=3).

4.4 Angiopoietin-1 stimulates increased adhesion between endothelial cells and vascular smooth muscle cells

In order to test whether Ang-1 influences adhesion between endothelial cells and smooth muscle cells a simple adhesion assay was devised. These experiments were conducted with human vascular smooth muscle (VSM) cells and bovine aortic endothelial (BAE) cells. A monolayer of VSM cells were grown to confluence and maintained overnight in Complete Media in a 96 well plate. BAE cells were pre-labelled for 15 minutes with a fluorescent dye, 5μg/ml of Calcein AM, washed and brought into suspension as described in Section 2.3.1 and then plated onto the monolayer of VSM cells and incubated at 15 minute intervals for up to 1 hour in the presence of Ang-1*cond (200ng/ml) or control.

A time course of adhesion as is shown in Figure 4.5 revealed that pre-treatment of endothelial cells with Ang-1*cond increased adhesion to VSM cells at each time point compared to mock treated endothelial cells. Figure 4.5 represents an individual experiment that is illustrative of the increase in adhesion on VSM cells observed in all the endothelial cell types utilised in this study. These data are similar to that reported by Mr. James Clover, Department of Surgery, University of Leicester (refer to relevant thesis) showing that Ang-1* effectively increased adhesion in both arterial and venous endothelial cell lines.

Further experiments confirmed adhesion of endothelial cells to vascular smooth muscle cells was shown to be statistically significant after 15 minute prestimulation with Ang-1*cond (Figure 4.6). BAE cells were pre-labelled for 15 minutes with a fluorescent dye, 5μg/ml of Calcein AM, washed and brought into suspension as described in Section 2.3.1 and then incubated in the presence or absence of Ang-1*cond (200ng/ml) for 15 minutes at 37°C/5% CO_2. Endothelial cells were then plated onto the monolayer of VSM cells and incubated for 15 minutes at 37°C/5% CO_2. Adhesion between endothelial cells and smooth muscle cells was significantly increased in the presence of Ang-1* (152.92+/-29.35% as a percentage of control, data presented as mean +/- standard deviation for three experiments, $p \leq 0.05$; Figure 4.6).
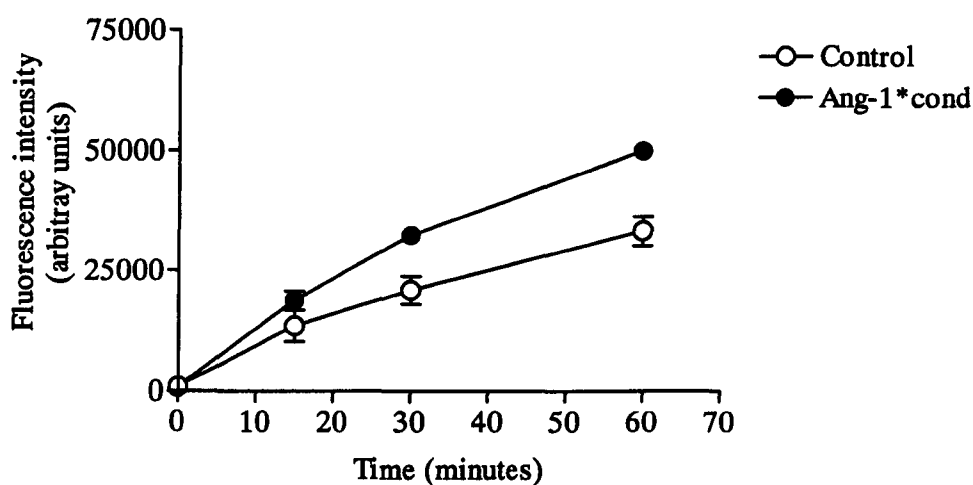
Figure 4.5 **Ang-1 increases adhesion between endothelial cells and vascular smooth muscle cells.** BAE cells were fluorescently labelled with Calcein AM (5μg/ml) and incubated in the presence or absence of Ang-1*cond (200ng/ml) prior to seeding onto a confluent monolayer of VSM cells and incubation for 15 minutes at 37°C/5% CO_2. BAE cells were incubated with VSM cells at 15 minute intervals for 1 hour in the presence or absence of Ang-1*cond (Figure representative of one experiment that is consistent with observations made within the Department of Surgery, University of Leicester).
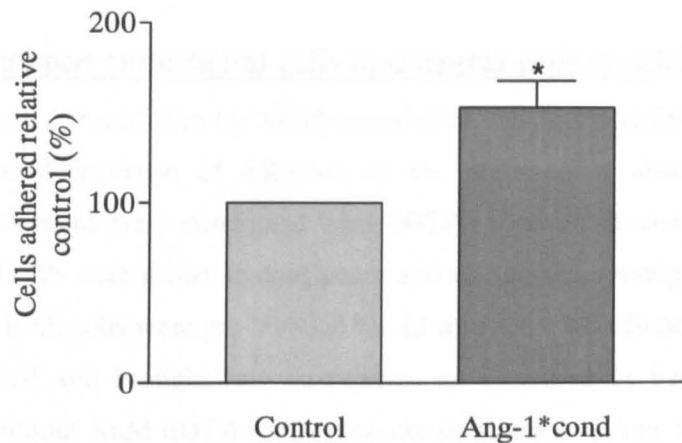
Figure 4.6 **Ang-1* increases endothelial cell adhesion to vascular smooth muscle cells.** Confluent monolayers of VSM cells were seeded into wells of a 96 well plate. BAE cells were fluorescently labelled using a 5μg/ml of Calcein AM for 15 minutes and then washed. Resuspended BAE cells were incubated with control or Ang-1*cond for 15 minutes. Cells were plated onto VSM cell monolayers and incubated for 15 minutes at 37°C/5% CO_2. Endothelial cells stimulated with Ang-1* adhered to vascular smooth muscle cells preferentially. Data presented as mean +/-standard deviation for three experiments (n=3). * p≤0.05 students 't' test.

To determine whether the effects of Ang-1 on heterotypic adhesion to smooth muscle cells was specific to endothelial cells the adhesion experiments were conducted with CHO cells. A monolayer of VSM cells were grown to confluence and maintained overnight in Complete Media in a 96 well plate. CHO cells were pre-labelled for 15 minutes with a fluorescent dye, 5µg/ml of Calcein AM, washed and brought into suspension as described in Section 2.3.1 and then plated onto the monolayer of smooth muscle cells and incubated for 15 minutes with Ang-1* (200ng/ml) or control at $37°C/5\%$ CO_2. Adhesion was not affected by Ang-1 (85.02+/-19.70% as a percentage of control, data presented as mean +/-standard deviation for three experiments; Figure 4.7). Therefore the ability of Ang-1 in increasing adhesion to vascular smooth muscle cells does appear to be cell type specific, though a more comprehensive survey of cell types would be required to conclude endothelial cell specificity.

4.5 Adhesion between endothelial cells and mural cells is calcium dependent

To gain insight into the mechanism by which endothelial cell and vascular smooth muscle cells adhere the calcium dependence of adhesion in the presence or absence of Ang-1* was investigated. Experiments were conducted with EDTA, a potent divalent cation chelator. A monolayer of VSM cells were grown to confluence and maintained overnight in Complete Media in a 96 well plate. BAE cells were pre-labelled for 15 minutes with a fluorescent dye, 5µg/ml of Calcein AM, washed and brought into suspension as described in Section 2.3.1 and then incubated with or without 5mM EDTA in the presence or absence of Ang-1*cond (200ng/ml) for 15 minutes at $37°C/5\%$ CO_2. Endothelial cells were then plated onto the monolayer of smooth muscle cells and incubated for 15 minutes at $37°C/5\%$ CO_2.

As previously described, adhesion between endothelial cells and smooth muscle cells was significantly increased in the presence of Ang-1* (152.92+/-29.35% as a percentage of control, data presented as mean +/-standard deviation for three experiments, $p \leq 0.05$; Figure 5.8). Adhesion was inhibited in both Ang-1* and control treated cells by EDTA (6.16+/-5.38% and 11.76+/-12.70% as a percentage of control, data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$ and $p \leq 0.001$, respectively; Figure 4.8). These data demonstrate calcium dependence in adhesion between endothelial cells and vascular smooth muscle cells in absence and presence of Ang-1*.
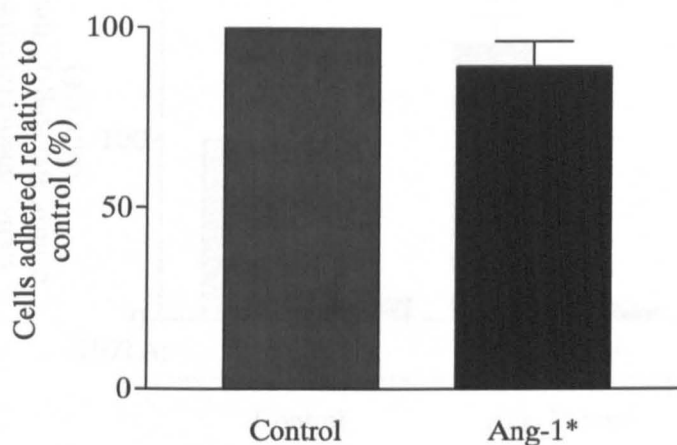
Figure 4.7 **Ang-1* stimulated adhesion to vascular smooth muscle cells is not observed in CHO cells.** CHO cells were fluorescently labelled with Calcein AM (5μg/ml) and incubated in the presence or absence of Ang-1* (200ng/ml) prior to seeding onto a confluent monolayer of VSM cells and incubation for 15 minutes at 37°C/5% CO_2. Adhesion to smooth muscle cells was observed but no increase in the presence of Ang-1* was witnessed. Data presented as mean +/- standard deviation for three experiments (n=3).
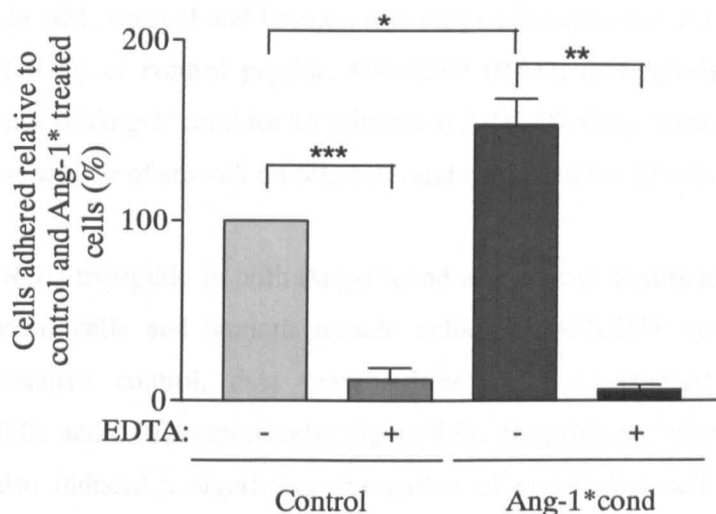
Figure 4.8 **Basal and Ang-1* stimulated adhesion between endothelial cells and vascular smooth muscle cells is calcium dependent.** Confluent monolayers of VSM cells were seeded into wells of a 96 well plate. BAE cells were fluorescently labelled using a 5µg/ml of Calcein AM for 15 minutes. Suspended BAE cells were incubated in the presence or absence of Ang-1*cond, plus or minus 5mM EDTA at 37°C/5% CO_2 and then incubated with VSM cell monolayers for 15 minutes at 37°C/5% CO_2. EDTA inhibited adhesion of both Ang-1 stimulated and unstimulated BAE cells to vascular smooth muscle cells significantly. Data presented as mean +/-standard deviation for three experiments (n=3). * p≤0.05; ** p≤0.01; *** p≤0.001 students 't' test.

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4.6 Angiopoietin-1 induced adhesion is inhibited by the integrin blocking peptide, RGD

The possibility that endothelial cell and vascular smooth muscle cell adhesion could involve the integrins was investigated using RGD peptide. The peptide, Arg-Gly-Asp serves as a primary integrin recognition site in extracellular matrix proteins, and peptides containing this sequence can either mimic the activities of the matrix proteins or inhibit the binding of integrin adhesion molecules (Pierschbacher and Ruoslahti 1984; Brooks et al. 1994; Rupp and Little 2001).

A monolayer of VSM cells was grown to confluence and maintained overnight in Complete Media in a 96 well plate. BAE cells were pre-labelled for 15 minutes with a fluorescent dye, 5µg/ml of Calcein AM, washed and brought into suspension (Section 2.3.1) prior to incubation with GRGDSP (RGD) or control peptide, GRADSP (RAD) (250µg/ml) (Calbiochem) in the presence or absence of Ang-1*cond for 15 minutes at $37°C/5\%$ CO_2. Endothelial cells were then plated onto the monolayer of smooth muscle cells and incubated for 15 minutes at $37°C/5\%$ CO_2.

Treatment with RGD tripeptide in both Ang-1*cond and control significantly reduced adhesion between endothelial cells and smooth muscle cells (4.15+/-3.25% and 5.82+/-5.49% as a percentage of relative control, data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$ and 0.01, respectively; Figure 4.9). Surprisingly, treatment with the control peptide, RAD also induced a significant abrogation of endothelial cell and vascular smooth muscle cell adhesion (46.04+/-2.71% and 47.62+/-13.77% as a percentage of relative control, data presented as mean +/-standard deviation for three experiments, $p \leq 0.05$ and 0.05, respectively; Figure 4.9). The suppression of adhesion was greater for RGD peptide than RAD peptide (in both treatments, $p \leq 0.01$ and 0.01; Figure 4.9).

These data suggest RGD-dependent integrins may be involved in adhesion between endothelial cells and smooth muscle cells. However, because RAD peptide also caused some inhibition of binding further experiments would be required to confirm the specificity of the peptide effects.
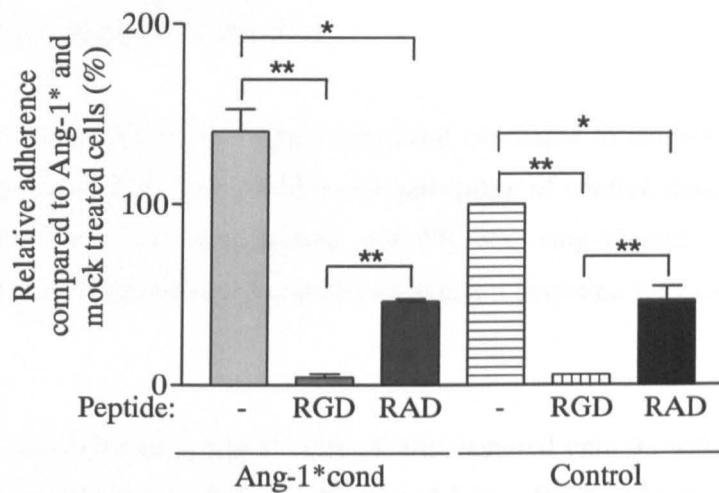
Figure 4.9 **Basal and Ang-1* increased adhesion of endothelial cells and vascular smooth muscle cells is inhibited by integrin blocking peptides (RGD).** BAE cells were fluorescently labelled with Calcein AM (5µg/ml) and brought to suspension prior to incubation in the presence or absence of Ang-1*cond, plus RGD, RAD (both 250µg/ml) or PBS. BAE cells were seeded onto confluent monolayers of VSM cells and incubated for 15 minutes. Inhibition of endothelial cell and vascular smooth muscle cell adhesion was observed in the presence of Ang-1* and control for both active tripeptide, RGD and control peptide, RAD. Difference in inhibition between the two peptides was significant for RGD. Data presented as mean +/-standard deviation for three experiments (n=3). * p≤0.05; ** p≤0.01 students 't' test.

4.7 Direct adhesion of endothelial cells to angiopoietin-1

Increased adhesion between endothelial cells and smooth muscle cells could occur as a result of Ang-1 stimulation of a Tie2-mediated signalling pathway or through direct adhesion of each cell type to Ang-1. Indeed in the course of the project it was reported endothelial cells can adhere directly to Ang-1 (Carlson et al. 2001). Confirmation of direct endothelial cell adhesion to Ang-1 was tested by replacing the vascular smooth muscle cells in the 96 well plate with immobilised Ang-1.

Ang-1*cond or Ang-1 were immobilised into the wells of a 96 well plate and blocked with a 2% BSA solution (Section 2.3.2). BAE cells were pre-labelled for 15 minutes with a fluorescent dye, 5μg/ml of Calcein AM, washed and brought into suspension as described in Section 2.3.1. Endothelial cells were then plated onto the immobilised Ang-1*cond, Ang-1 or control and incubated for 30 minutes at 37°C/5% CO_2.

Cells adhered preferentially to the Ang-1 treatment compared to control. This adherence was observed in both Ang-1 (156.75+/-30.63% as a percentage of control, data presented as mean +/-standard deviation for four experiments, p≤0.05) and Ang-1*cond (161.5+/-23.04% as a percentage of control, data presented as mean +/-standard deviation for four experiments, p≤0.05; Figure 4.10).

In further experiments purified Ang-1* was affinity captured onto 96 well plates using a human FC specific goat monoclonal antibody (Section 2.3.2). BAE cells were pre-labelled for 15 minutes with a fluorescent dye, 5μg/ml of Calcein AM, washed and brought to suspension (Section 2.3.1). Endothelial cells were then plated onto the immobilised Ang-1* (200ng/ml) or Tie1 FC (200ng/ml) and incubated for 30 minutes at 37°C/5% CO_2. Tie1 FC was used as an experimental control to determine that adhesion of endothelial cells was not due to the FC portion of the Ang-1*. Figure 4.11 shows endothelial cells adhere preferentially to Ang-1* compared with Tie1 control (167.55+/-25.50% as a percentage of control, data presented as mean +/-standard deviation for four experiments, p≤0.05). These data demonstrate that endothelial cells can bind to Ang-1 confirming previous observations (Carlson et al. 2001).

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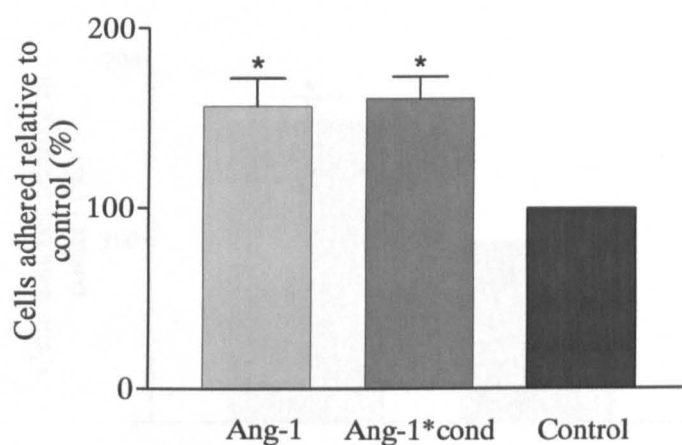
Figure 4.10 **Endothelial cells significantly adhere to both native Ang-1 and Ang-1*.** Native Ang-1 (500ng/ml), Ang-1*cond (200ng/nl) and control were immobilised into wells of a 96 well plate overnight at 4°C. Prior to fluorescent labelling of BAE cells with Calcein AM (5µg/ml) wells were blocked with a 2% BSA solution. Endothelial cells were brought to suspension and incubated on the immobilised protein for 30 minutes at 37°C/5% CO_2. Endothelial cell adhesion was increased to both Ang-1 and Ang-1*cond compared with control-conditioned media. Data presented as mean +/-standard deviation for four experiments (n=4). * p≤0.05 students 't' test.
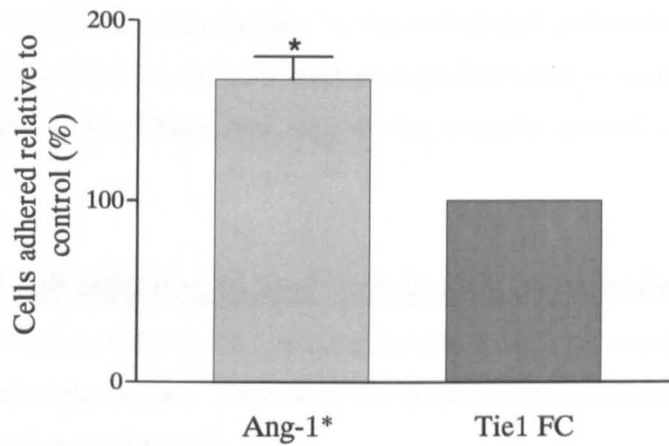
Figure 4.11 **Endothelial cells adhere to purified Ang-1*.** Ang-1* (200ng/ml) or Tie1 endodomain (200ng/ml) were affinity captured into the wells of a 96 well plate by the use of a human FC specific goat monoclonal antibody. Prior to the seeding of the fluorescently labelled BAE cells (using Calcein AM, 5μg/ml) protein binding sites were blocked by a 2% BSA solution. After fluorescent labelling, endothelial cells were seeded onto the immobilised protein for 30 minutes at 37°C/5% CO_2. Endothelial cells adhered preferentially to Ang-1* compared to the Tie1 FC. Data presented as mean +/-standard deviation for four experiments (n=4). * p≤0.05 students 't' test.

4.8 Vascular smooth muscle cells adhere to angiopoietin-1

The possibility that Ang-1 also promoted adherence of vascular smooth muscle cells was investigated. VSM cells were pre-labelled for 15 minutes with a fluorescent dye; 5µg/ml of Calcein AM then washed and re-suspended (Section 2.3.1). The cells were plated onto immobilised Ang-1*cond or control and incubated for 30 minutes at 37°C/5% CO_2. Vascular smooth muscle cells bind to Ang-1*cond preferentially (252.63+/-37.64% as a percentage of control, data presented as mean +/-standard deviation for three experiments, p ≤0.05; Figure 4.12).

In additional experiments, VSM cells were pre-labelled for 15 minutes with a fluorescent dye, 5µg/ml of Calcein AM and then plated onto immobilised Ang-1* (200ng/ml) or Tie1 FC (200ng/ml) and incubated for 30 minutes at 37°C/5% CO_2. Increased adhesion to purified Ang-1* was also observed in the same manner as the endothelial cells although this did not reach statistical significance (212.01+/-55.21% data presented as mean +/-standard deviation for three experiments; Figure 4.13). These data suggest that vascular smooth muscle cells can adhere directly to Ang-1*.

4.9 Endothelial cell adhesion to angiopoietin-1 is calcium dependent

Adhesion between endothelial cells and vascular smooth muscle cells was shown to be dependent upon presence of divalent cations. Therefore, the divalent cation dependence of endothelial cell adherence to Ang-1 was investigated.

BAE cells were pre-labelled for 15 minutes with a fluorescent dye, 5µg/ml of Calcein AM, washed and resuspended with or without 5mM EDTA for 15 minutes at 37°C/5% CO_2 prior to seeding on Ang-1* (Section 2.3.2). Prior to seeding of endothelial cells the wells were blocked with 2% BSA solution for 1 hour. Endothelial cells were then plated onto the immobilised Ang-1*cond (200ng/ml) or control and incubated for 30 minutes at 37°C/5% CO_2.

Endothelial cell adhesion to Ang-1* was increased consistent with observations made earlier (141.73+/-16.27% as a percentage of control, data presented as mean +/-standard deviation for four experiments, p≤0.05; Figure 4.14). Ang-1* adhesion in the presence of the cation chelator, EDTA, was completely abolished (1.51+/-0.70% as a percentage of Ang-1* treated cells, data presented as mean +/-standard deviation for four experiments, p≤0.01; Figure 4.14).
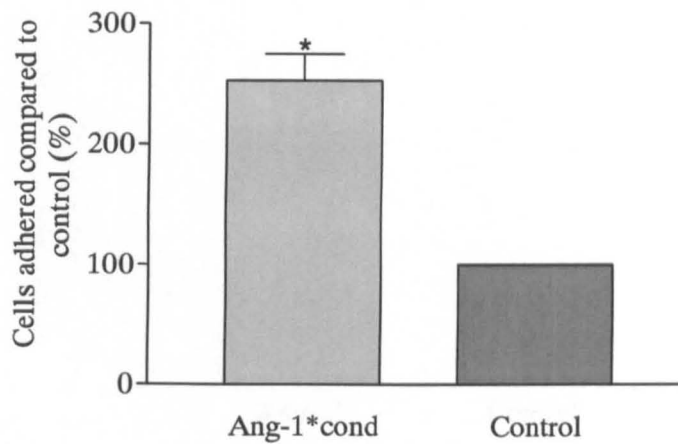
Figure 4.12 **Vascular smooth muscle cells adhere to Ang-1*.** Ang-1*cond (200ng/nl) and control were immobilised into wells of a 96 well plate overnight at 4°C (Section 2.3.2). Prior to fluorescent labelling of VSM cells with Calcein AM (5µg/ml) protein binding sites were blocked with a 2% BSA solution. VSM cells were brought to suspension and seeded onto the immobilised protein for 30 minutes at 37°C/5% CO_2. Vascular smooth muscle cell adhesion was increased to Ang-1* conditioned media compared with control-conditioned media. Data presented as mean +/- standard deviation for three experiments (n=3). * p≤0.05 students 't' test.
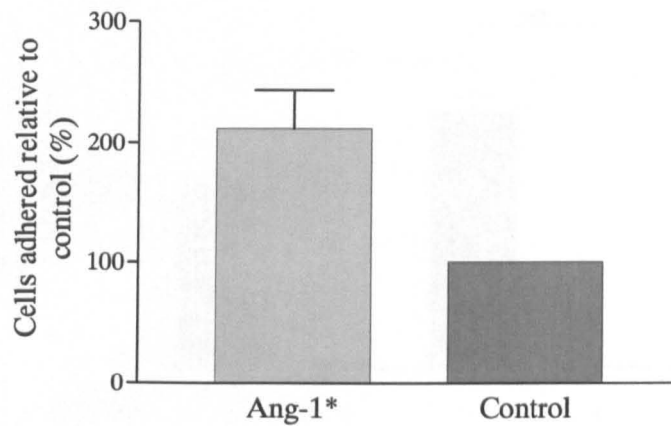
Figure 4.13 **Vascular smooth muscle cells adhere to immobilised to Ang-1*.** Ang-1* (200ng/ml) or control, Tie1 FC (200ng/ml) were affinity captured into the wells of a 96 well plate by the use of a human FC specific goat monoclonal antibody (Section 2.3.2). Prior to the seeding of the fluorescently labelled VSM cells with Calcein AM (5µg/ml) protein binding sites were blocked by a 2% BSA solution. VSM cells were brought to suspension and seeded on to the immobilised protein and incubated for 30 minutes at 37°C/5% CO_2. Data presented as mean +/- standard deviation for three experiments (n=3).
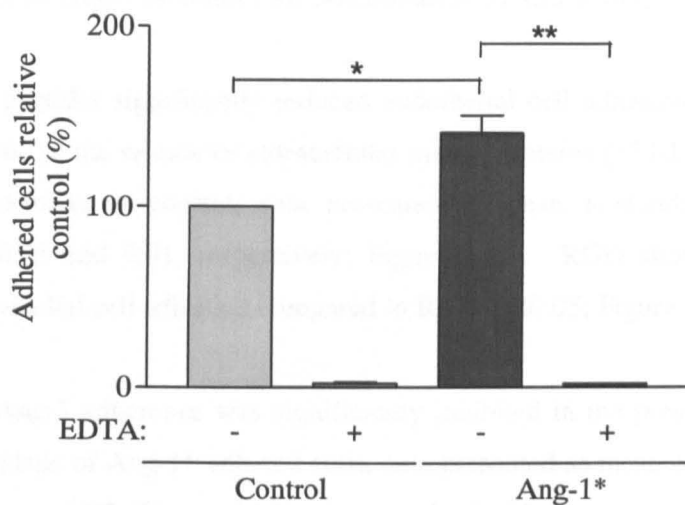
Figure 4.14 **Endothelial cell adhesion to Ang-1* is inhibited by the removal of divalent cations.** Ang-1*cond (200ng/nl) and control were immobilised into wells of a 96 well plate overnight at 4°C. Prior to fluorescent labelling of BAE cells with Calcein AM (5µg/ml) protein binding sites were blocked with a 2% BSA solution. Before seeding onto the immobilised protein BAE cells were treated for 15 minutes with 5mM EDTA or PBS and then incubated for 30 minutes at 37°C/5% CO_2. Endothelial cell adhesion to Ang-1*cond and control was abolished in the presence of EDTA. Data presented as mean +/-standard deviation for four experiments (n=4). * $p \leq 0.05$; ** $p \leq 0.01$ students 't' test.

4.10 Endothelial adhesion to angiopoietin-1 is partially inhibited in the presence of integrin blocking peptides

Adhesion between endothelial cells and vascular smooth muscle cells was found to be partially inhibited by RGD peptide suggesting involvement of integrins (Section 4.6). The possible effects of RGD peptide in endothelial cell adhesion to Ang-1 was investigated using the inhibitory integrin peptide, RGD and RAD control peptide.

Wells of a 96 well plate were coated with Ang-1 (500ng/ml) or control and blocked for 1 hour with a 2% BSA solution (Section 2.3.2). BAE cells were fluorescently labelled with 5µg/ml Calcein AM for 15 minutes, washed and re-suspended prior to incubation with RGD, RAD (both 250µg/ml) or PBS for 15 minutes at 37°C/5% CO_2 (Section 2.3.1). The cells were then seeded onto the immobilised Ang-1 or control for 30 minutes at 37°C/5% CO_2.

RGD and RAD peptides significantly reduced endothelial cell adhesion to control conditioned media possibly due to the release of extracellular matrix proteins (17.81+/-18.00% and 66.26+/-5.66% as a percentage of control, data presented as mean +/-standard deviation for three experiments, $p \leq 0.05$ and 0.01, respectively; Figure 4.15). RGD showed significantly more inhibition of endothelial cell adhesion compared to RAD ($p \leq 0.05$; Figure 4.15).

Endothelial cell:Ang-1 adherence was significantly inhibited in the presence of RGD (42.25+/-5.42% as a percentage of Ang-1* adhered cells, data presented as mean +/-standard deviation for three experiments, $p \leq 0.05$; Figure 4.15). Unlike the RGD treatment, RAD peptides partially suppressed adhesion (69.03+/-2.58% as a percentage of Ang-1* adhered cells, data presented as mean +/-standard deviation for three experiments) though not statistically significant (Figure 4.15). The RGD peptide inhibited adhesion to Ang-1 to a greater extent than RAD peptide ($p \leq 0.05$; Figure 4.15). As there was significant RGD/RAD-dependent adhesion to control wells, and the control RAD peptide provided some inhibition of adhesion, further experiments would be required to definitely test whether binding to angiopoietin-1 is RGD-dependent.

101
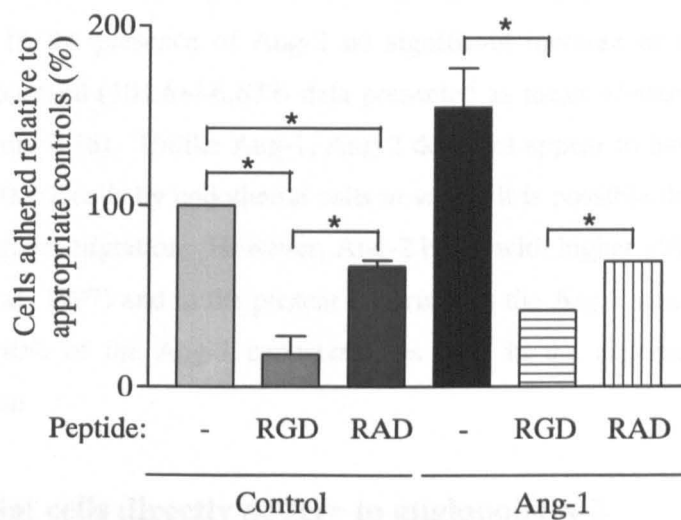
Figure 4.15 **Endothelial cells adhere to Ang-1 preferentially. Both basal and Ang-1 adhesion is partially blocked in the presence of an integrin blocking peptide, RGD.** BAE cells were fluorescently labelled with Calcein AM, 5μg/ml for 15 minutes then brought to suspension, and incubated with RGD, RAD (250μg/ml) or PBS for 15 minutes at 37°C/5% CO_2. The cells were then seeded onto immobilised Ang-1 (500ng/ml) or control for 30 minutes at 37°C/5% CO_2 after the wells were blocked for 1 hour with a 2% BSA solution. Basal adhesion was inhibited by both active and control tripeptide. RGD peptide did inhibit adhesion significantly. Ang-1 adhesion was significantly inhibited by RGD but not RAD. Data presented as mean +/- standard deviation for three experiments (n=3). * p≤0.05 students 't' test.

4.11 Angiopoietin-2 does not affect endothelial cell directed 10T1/2 cell migration

The focus of this Chapter has been the potential role of the Tie2 receptor agonist, Ang-1 in the recruitment of mural cells and modulating the interaction of endothelial cells and vascular smooth muscle cells. A possible role for the antagonistic ligand, Ang-2 in regulating the recruitment of mural cells by the endothelium and binding endothelial cells was investigated.

Migration assays were conducted using Ang-2 in the same manner as the Ang-1* experiment (Section 4.3). Confluent monolayers of MHE cells in the lower chambers of a 24 well plate were serum starved for 6 hours in the presence or absence of purified Ang-2 (200ng/ml). Transwells were then inserted into each well and 10T1/2 cells were placed into each insert (10^5 cells). 10T1/2 cells were allowed to migrate towards the endothelial cells for a period of 2 hours at 37°C/5% CO_2. In the presence of Ang-2 no significant increase or decrease in 10T1/2 cell migration was observed (101.6+/-6.67% data presented as mean +/-standard deviation for three experiments; Figure 4.16). Unlike Ang-1, Ang-2 does not appear to have role in regulating the recruitment of 10T1/2 cells by endothelial cells *in vitro*. It is possible that higher concentrations of Ang-2 could affect migration. However, Ang-2 binds with higher affinity to Tie2 than Ang-1 (Maisonpierre et al. 1997) and in the present experiments the Ang-2 concentration was the same as Ang-1* and 40% of the Ang-1 concentrations used in the experiments examining Ang-1 effects on adhesion

4.12 Endothelial cells directly adhere to angiopoietin-2

The possibility that Ang-2 was able to act as an adhesive substrate for endothelial cells was also investigated. Ang-2 adhesion experiments were conducted as previously described for Ang-1. Briefly, purified Ang-2 was immobilised onto the wells of a 96 well plate and blocked with a 2% BSA solution (Section 2.3.2). BAE cells were pre-labelled for 15 minutes with a fluorescent dye, 5µg/ml of Calcein AM washed and brought to suspension (Section 2.3.1). Endothelial cells were then plated onto the immobilised Ang-2 and incubated for 30 minutes at 37°C/5% CO_2. Adhesion of endothelial cells was significantly higher to Ang-2 than control treated wells (2236.04+/-473.39% as a percentage of control, data presented as mean +/-standard deviation for four experiments, p≤0.05; Figure 4.17). These data demonstrate that, like Ang-1, Ang-2 can also act as an adhesive substrate for endothelial cells. Similar observations were made by Carlson et al. (2001).
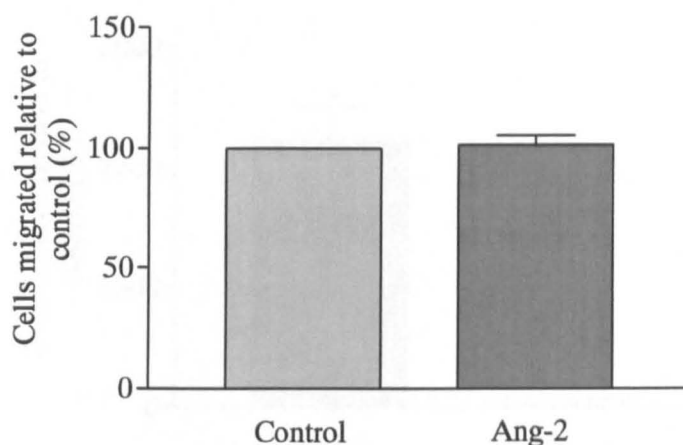
Figure 4.16 **Ang-2 does not affect endothelial-directed 10T1/2 cell migration.** Confluent monolayers of MHE cells were maintained in the lower chambers of a 24 well plate. Prior to insertion of transwells MHE cells were serum starved in the presence or absence of Ang-2 (200ng/ml). 10T1/2 cells (10^5) were then added to an 8μm porous membrane transwell and incubated for 2 hours at 37°C/5% CO_2. The number of 10T1/2 cells present on the lower surface of the insert were counted following staining with haematoxylin and eosin. Data presented as mean +/-standard deviation for three experiments (n=3).
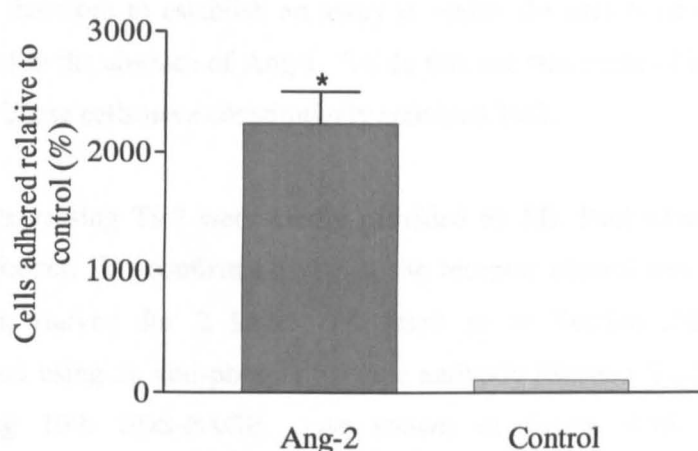
Figure 4.17 **Endothelial cells adhere to Ang-2.** Ang-2 (200ng/ml) or PBS were incubated in wells of a 96 well plate. Prior to the seeding of the fluorescently labelled BAE cells (using Calcein AM, 5μg/ml) protein binding sites were blocked by a 2% BSA solution. BAE cells were brought to suspension and added to Ang-2 coated or control coated wells for 30 minutes at 37°C/5% CO_2. BAE cells adhere preferentially compared to control cells. Data presented as mean +/-standard deviation for four experiments (n=4). * p≤0.05 students 't' test.

Data presented in Sections 4.4 – 4.10 show that Ang-1 increases adhesion between endothelial cells and smooth muscle cells. In addition, endothelial cells and smooth muscle cells were both able to adhere to Ang-1. It is possible that Ang-1 could act to increase apparent heterotypic cell adhesion by acting as an adhesive substrate between the two cell types. Alternatively, the pro-adhesive activity of Ang-1 may require activation of Tie2 signalling. To test whether increased signalling by Tie2 could lead to increased adhesion, endothelial cells expressing constitutively activated Tie2 were examined.

4.13 Constitutively active Tie2 increases endothelial cell adhesion

Use of Ang-1 to activate Tie2 signalling would complicate examination of the effects of Tie2 activation on adhesion because of the potential for the Ang-1 to act also as an adhesive substrate. It was necessary therefore to establish an assay in which the effects of active Tie2 signalling could be examined in the absence of Ang-1. To do this use was made of BAE cells stably over-expressing Tie2. These cells have constitutively activated Tie2.

BAE cells over-expressing Tie2 were kindly provided by Mr. Paul Morris, Dept. of Surgery, University of Leicester. To confirm activity of the receptor control and over-expressing BAE cells were serum starved for 2 hours and lysed as in Section 2.6.3.1. Lysates were immunoprecipitated using an anti-phosphotyrosine antibody (Section 2.6.3.2) and then proteins fractionated using 10% SDS-PAGE. As shown in Figure 4.18 increased Tie2 was immunoprecipitated from over-expressing cells by an anti-phosphotyrosine antibody indicating the receptor is tyrosine phosphorylated or associated with a tyrosine-phosphorylated protein. Blots were re-probed with an anti-Tie2 antibody to confirm the Tie2 protein is tyrosine phosphorylated.

To examine adhesion of cells with activated Tie2 versus basal Tie2 activity, VSM cell monolayers were seeded into wells of a 96 well plate to 100% confluence and maintained overnight in Complete Media. BAE and BAE/Tie2 cells were fluorescently labelled using 5µg/ml of Calcein AM for 15 minutes, washed and brought into suspension and then plated onto the monolayer of VSM cells and incubated for 30 minutes at 37°C/5% CO_2 (Section 2.3.1). Fluorescence was measured in each well before washing off non-adherent cells and the measurement of the remaining fluorescent cells. The fraction of cells adhering to VSM cells was calculated for control and BAE/Tie2 over-expressing cells.

A

C   W

IB: anti-PY

→

250

125

B

IB: anti-Tie2

→

250

125
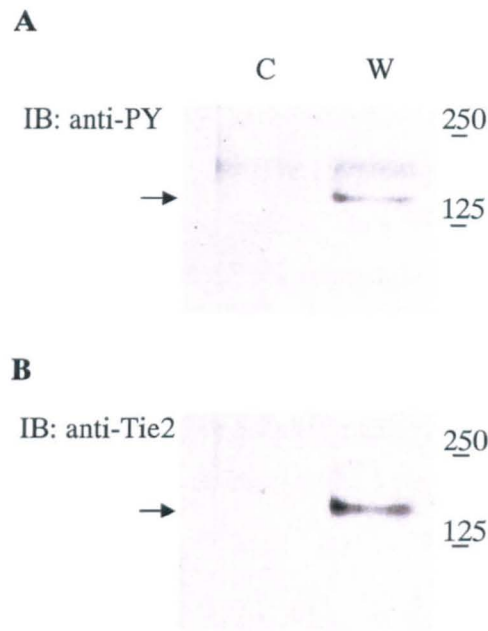
Figure 4.18 **Tie2 is constitutively phosphorylated in cells over-expressing the receptor.** BAE cells and cells expressing the wild type Tie2 were grown to 100% confluence in 80cm^2 flasks and then serum starved for 2 hours. Cells were lysed and phosphorylated tyrosine proteins were immunoprecipitated using an anti-phosphotyrosine antibody (Section 2.6.3.2). Samples were fractionated upon 10% SDS-PAGE. Phosphorylated tyrosine proteins and the Tie2 receptor upon the immunoblots were identified using specific antibodies (**anti-PY** and **anti-Tie2**) **A**) and **B**). Tie2 is phosphorylated in cells over-expressing the receptor (indicated by arrows). Endogenous levels of Tie2 in BAE cells are not recognised by the antibodies due to species specificity. **C** – control; **W** – wild type Tie2 expressing BAE cells; **IB** – immunoblot.

Adhesion of over-expressing cells was higher than control cells (318.59+/-124.24% as a percentage of control, data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$) (Figure 4.19).

4.14 The intracellular domain of Tie2 increases endothelial cell:mural cell adhesion

To confirm that any effects on adhesion were not the result of the elevated levels of Tie2 extracellular domain expressed on the cells, additional experiments were performed with cells expressing chimeric receptors. These receptors comprised of the extracellular domain of the nerve growth receptor, TrkA, fixed to the transmembrane and intracellular domain of Tie2. Over-expression of these receptors results in constitutive autophosphorylation of the Tie2 intracellular domain and it's signalling. BAE cells stably expressing TrkA/Tie2 chimeric receptors were kindly provided by Dr. David Hughes, Dept. of Surgery, University of Leicester. To confirm receptors were reactive in TrkA/Tie2 over-expressing cells, whole cell lysates of BAE cells and BAE cells expressing the TrkA/Tie2 (BAEclone8) were prepared as described in Section 2.6.2. Samples were fractionated using 10% SDS-PAGE and tyrosine-phosphorylated proteins were immunoblotted using an anti-phosphotyrosine antibody (New England Biolabs) (Figure 4.20A). The over-expressed chimeric receptor was identified with an anti-Tie2 antibody re-probe (R & D systems) (Figure 4.20B).

To examine adhesion of these cells, monolayers of VSM cells were grown to confluence and maintained overnight in Complete Media in a 96 well plate. Control BAE cells and the clonal cell line over-expressing TrkA/Tie2, BAEclone8 cells were fluorescently labelled for 15 minutes with 5µg/ml Calcein AM, washed and re-suspended prior to seeding upon the confluent monolayer of smooth muscle cells and incubated for 30 minutes at 37°C/5% CO_2 (Section 2.3.1). Fluorescence was measured in each well before and after removal of non-adherent and the fraction of adherent cells measured for control and BAEclone8.
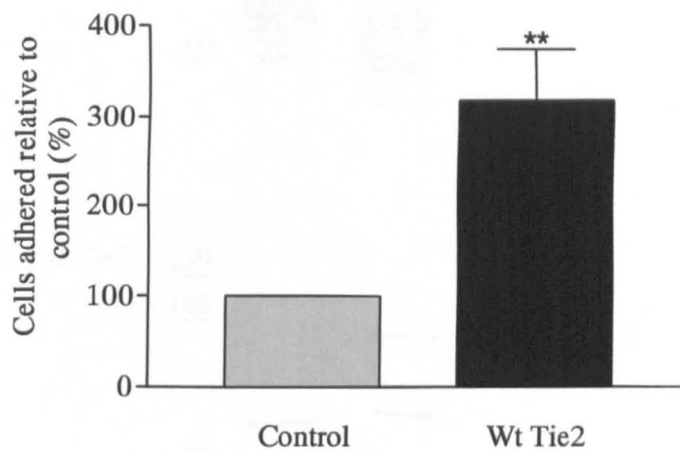
Figure 4.19 **Endothelial cells over-expressing the Tie2 receptor adhere to mural cells.** BAE cells or BAE over-expressing Tie2 cells were fluorescently labelled with Calcein AM (5μg/ml), washed and brought to suspension prior to seeding onto a confluent monolayer of VSM cells and incubation for 30 minutes at 37°C/5% CO_2. The fraction of cells adhering was calculated for each cell type and normalised between experiments by setting BAE adhesion to 100%. Adhesion of the BAE cell lines was expressed as a percentage of the total amount of cells. BAE cells over-expressing the Tie2 receptor adhered preferentially to vascular smooth muscle cells compared to control BAE cells. Data presented as mean +/- standard deviation for three experiments (n=3). ** $p \leq 0.01$ students 't' test.
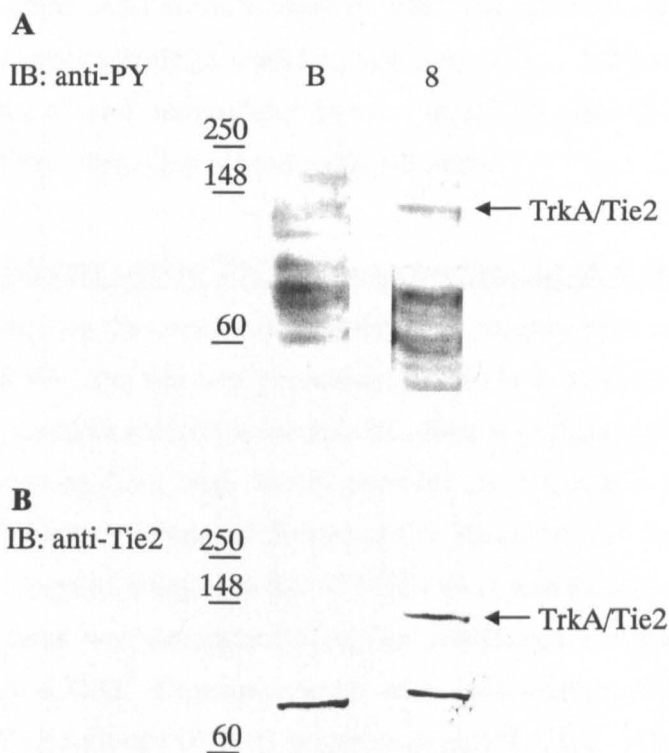
Figure 4.20 **Phosphotyrosine levels of endothelial cell lines expressing a chimeric TrkA/Tie2 receptor.** BAEclone8 are cells expressing a TrkA/Tie2 chimera construct. Whole cell lysates from BAE cells and BAEclone8 cells were treated as detailed in Section 2.6.2. Samples were separated using 10% SDS-PAGE. **A)** Tyrosine phosphorylated proteins were identified using an anti-phosphotyrosine antibody (**anti-PY**). Phosphotyrosine containing protein corresponding to Tie2 was observed in cell lysates from clone 8. **B)** An anti-Tie2 re-probe confirmed the expression of the chimeric receptor. Due to species specificity endogenous levels of the Tie2 receptor were not observed. **B** – BAE cells; **8** – BAEclone8; **IB** – immunoblot.

The cell line over-expressing TrkA/Tie2 exhibited increased adhesion to smooth muscle cells compared with control (192.44+/-34.39% as a percentage of control, data presented as mean +/- standard deviation for three experiments, p≤0.05; Figure 4.21). Taken together these data suggest BAE cells with constitutively activated Tie2 show elevated levels of adhesion to smooth muscle cells. These data are consistent with increased Tie2 signalling stimulating adhesion between endothelial cells and smooth muscle cells. However, it is also a possibility that the elevated levels of Tie2 intracellular domain could, in some way, increase adhesion via a mechanism not involving their constitutively increased signalling capacity. More experiments, utilising kinase inactive mutants of Tie2 intracellular domain, would be required to definitively demonstrate involvement of Tie2 signalling in heterotypic adhesion.

4.15 Constitutively active Tie2 increases adhesion of a non-endothelial cell line

CHO cells expressing the constitutively active Tie2 receptor were used to determine whether a non-endothelial cell line that was previously found not to respond to Ang-1 treatment in the adhesion assay could be induced to increase its adhesion to mural cells (Section 4.4). Clonal cell lines over-expressing Tie2 were kindly provided by Dr. Marie Marron, Dept. of Surgery, University of Leicester. Whole cell lysates of the different clones were prepared as described in Section 2.6.2, separated using 10% SDS-PAGE and immunoblotted. Tyrosine phosphorylation of cellular proteins was determined using an anti-phosphotyrosine antibody (New England Biolabs) (Figure 4.22A). Expression levels of the over-expressed Tie2 receptor were verified using an anti-Tie2 antibody (R & D systems) (Figure 4.22B). The different clonal cell lines expressed different levels of Tie2 and phosphorylated receptor. Two different clones, CHOTie2 1A and CHOTie2 7A were tested for adhesion to smooth muscle cells as they expressed differing levels of tyrosine phosphorylation and receptor activation (Figure 4.22A).

Monolayers of VSM cells were grown to confluence and maintained overnight in Complete Media in a 96 well plate. CHO, CHOTie2 1A and CHOTie2 7A cells were fluorescently labelled for 15 minutes with 5µg/ml Calcein AM, washed and brought to suspension prior to seeding upon the monolayer of smooth muscle cells and incubated for 30 minutes at 37°C/5% CO_2 (Section 2.3.1). Fluorescence was determined in each well before and after removal of non-adherent cells. The fraction of cells adhering was determined for control and over-expressing cells.
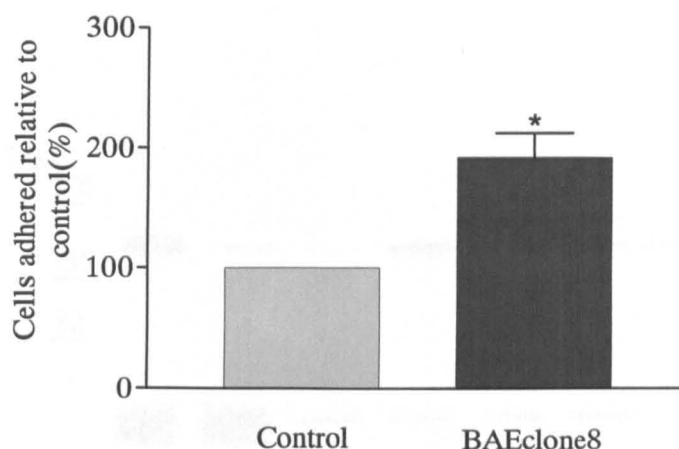
Figure 4.21 **Endothelial cell lines over-expressing the TrkA/Tie2 receptor preferentially adhere to smooth muscle cells.** BAE cells and BAE cells over-expressing Trk/Tie2 (**BAEclone8**) were fluorescently labelled with Calcein AM (5µg/ml), washed and brought to suspension prior to seeding onto a confluent monolayer of VSM cells and incubation for 30 minutes at 37°C/5% CO_2. The fraction of cells adhering was calculated for each cell type and normalised between experiments by setting BAE adhesion to 100%. Adhesion of the BAE cell lines was expressed as a percentage of the total amount of cells. BAE cells over-expressing the TrkA/Tie2 chimera adhered preferentially to VSM cells compared to control BAE cells. Data is presented as mean +/-standard deviation for three experiments (n=3). * $p \leq 0.05$ students 't' test.

A

IB: anti-PY   1A   2A   3A   4A   7A   9A

290

135   ←pTie2

82

B

IB: anti-Tie2

290

135   ←Tie2

82

53
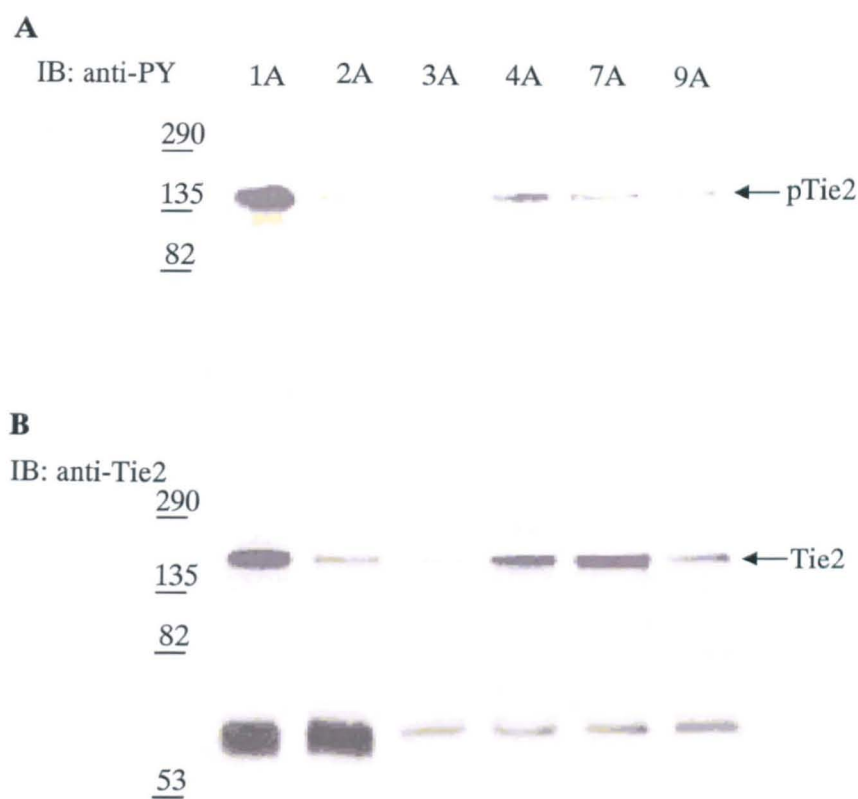
Figure 4.22 **Phosphotyrosine levels of the Tie2 receptor in a non-endothelial cell line.** Clonal CHO cell lines expressing different levels of the Tie2 receptor were provided by Dr. Marie Marron. Whole cell lysates were prepared as previously described (Section to 2.6.2) and resolved on 10% SDS-PAGE. **A)** Phosphorylated protein corresponding to Tie2 receptor in the clones was identified using an anti-phosphotyrosine antibody (**anti-PY**) and **B)** an anti-Tie2 antibody re-probe confirming the expression of the full-length receptor. Clones 1A and 7A were used in the adhesion assay due to their differing levels of receptor phosphorylation. **IB** – immunoblot.

Both cell lines adhere preferentially, but the more highly phosphorylated cell line, clone 1A, adhered significantly more than for control CHO cells (164.34+/-17.07% as a percentage of control, data presented as mean +/-standard deviation for three experiments, $p \leq 0.05$; Figure 4.23). Clone 7A did not present statistically significant higher binding than control cells (133.20+/-29.96% data presented as mean +/-standard deviation for three experiments; Figure 4.23). It is noteworthy that the cell line exhibiting increased adhesion had the highest level of phospho-Tie2 and Tie2.

4.16 Endothelial cell adhesion to extracellular matrix (ECM) proteins is not affected by over-expression of Tie2

To determine whether the increased adhesion of endothelial cells over-expressing Tie2 to smooth muscle cells reflects a generally increased adhesion, their ability to adhere to ECM proteins was tested. Parallel assays were conducted with wells containing monolayers of smooth muscle cells for direct comparison of the relative adhesion between endothelial cells and ECM and, endothelial cells and smooth muscle cells. Fibronectin and vitronectin (1µg/ml) (Sigma) were immobilised onto wells of a 96 well plate for 1 hour at 37°C/5% CO_2, and confluent monolayers of VSM cells were plated into parallel wells. BAE, BAE-Tie2 Wt cells were fluorescently labelled with 5µg/ml Calcein AM, for 15 minutes washed and brought to suspension before plating onto a confluent monolayer of smooth muscle cells or extracellular matrix proteins and incubated for 30 minutes at 37°C/5% CO_2. The fraction of cells adhering to ECM proteins and smooth muscle cells was determined as previously described.

Endothelial cell adhesion to the extracellular matrix proteins was compared to adhesion to VSM cells. As previously described, BAE cells expressing wild type Tie2 receptor increased adhesion to vascular smooth muscle cells. The percentage of cells relative to the total amount of cells before washing was 3.90+/-1.84% and 11.80+/-4.66% for BAE and BAE-Wt Tie2, respectively, data presented as mean +/-standard deviation for five experiments, $p \leq 0.05$ (Figure 4.24). Endothelial cell adhesion was increased to both fibronectin and vitronectin for the BAE and BAE-Wt Tie2 expressing cells. BAE cells adhesion to fibronectin and vitronectin was 26.93+/-12.20% and 29.82+/-15.30%, respectively, data presented as mean +/-standard deviation for five experiments, $p \leq 0.05$ and 0.05, respectively (Figure 4.24). Adhesion of the Tie2 expressing cells to fibronectin was increased although not statistically significant (32.59% +/-16.39 data presented as mean +/-standard deviation for five experiments).
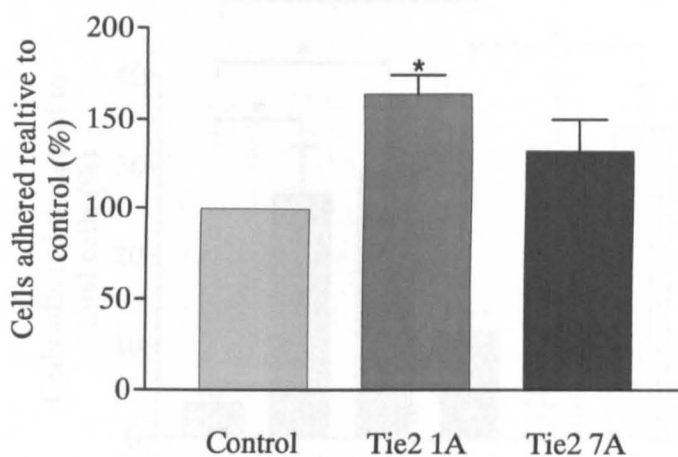
Figure 4.23 **A non-endothelial cell line over-expressing the Tie2 receptor preferentially adheres to smooth muscle cells compared to control cells.** Monolayers of VSM cells were grown to confluence. CHO, CHOTie2 1A and CHOTie2 7A cells were fluorescently labelled using Calcein AM (5μg/ml) for 15 minutes washed and brought into suspension prior to seeding onto the monolayer of smooth muscle cells and incubated for 30 minutes at 37°C/5% CO_2. The fraction of cells adhering was calculated for each cell type and normalised between experiments by setting BAE adhesion to 100%. Adhesion of the BAE cell lines was expressed as a percentage of the total amount of cells. Clone 1A adherence to smooth muscle cell monolayer was significantly increased compared with control CHO cells. However, clone 7A with low phosphotyrosine activity did not bind significantly more than the control cell line. Data presented as mean +/-standard deviation for three experiments (n=3). * $p \leq 0.05$ students 't' test.
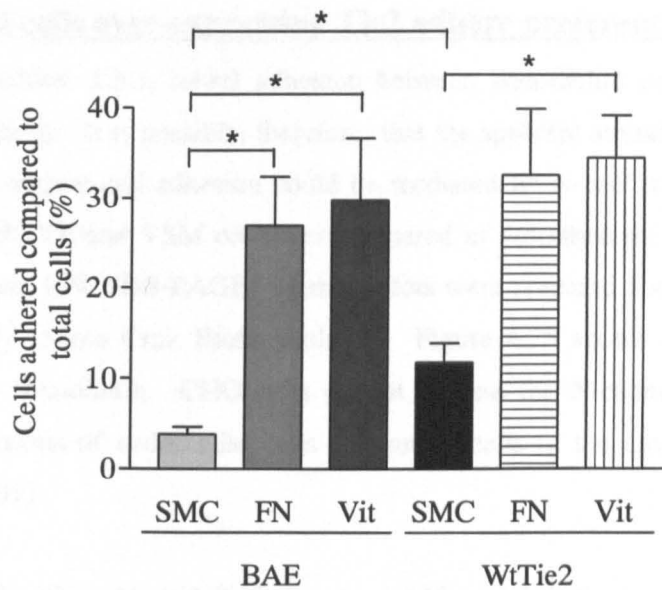
Figure 4.24 **Endothelial cell adhesion is increased to extracellular matrix proteins compared to vascular smooth muscle cells.** BAE cells or BAE over-expressing Tie2 (**WtTie2**) cells were fluorescently labelled with Calcein AM (5µg/ml), washed and brought to suspension prior to seeding onto a confluent monolayer of VSM cells or dishes coated with fibronectin (**FN**) or vitronectin (**Vit**) and incubated for 30 minutes at 37°C/5% CO_2. The fraction of cells adhering was calculated for each cell type and normalised between experiments by setting BAE adhesion to 100%. Adhesion of the BAE cell lines was expressed as a percentage of the total amount of cells. Data presented as mean +/- standard deviation for five experiments (n=5). * p≤0.05 students 't' test.

However, adhesion to vitronectin was significantly increased (34.54+/-10.45%, data presented as mean +/-standard deviation for five experiments, p≤0.05; Figure 4.24).

Over-expression of Tie2 in BAE cells did not significantly increase adhesion to either fibronectin or vitronectin compared to control BAE cells (119.57+/-18.64% and 128.06+/-30.69%, respectively, as a percentage of control cells, data presented as mean +/-standard deviation for five experiments). Therefore, both cell types appeared to adhere preferentially to the extracellular matrix proteins but there was no significant difference due to the over-expression of Tie2. Constitutive activation of the Tie2 receptor only causes an increase in endothelial cell adhesion to smooth muscle cells and not a general increase in endothelial cell activity.

4.17 Endothelial cells over-expressing Tie2 adhere preferentially to N-cadherin

As described in Section 1.3.3, initial adhesion between endothelial cells and mural cells is mediated by N-cadherin. It is possible, therefore, that the apparent stimulatory effect of Tie2 on endothelial:smooth muscle cell adhesion could be mediated by N-cadherin. Whole cell lysates from BAE, CHO, HUVE and VSM cells were prepared as described previously (Section 2.6.2) and fractionated upon 10% SDS-PAGE. Immunoblots were prepared and probed using an anti-N-cadherin antibody (Santa Cruz Biotechnology). Figure 4.25 shows that BAE, HUVE and VSM cells express N-cadherin. CHO cells do not express the N-cadherin seen fluorescently labelled at the junctions of endothelial cells and mural cells in the developing chick embryo (Hellstrom et al. 2001).

To investigate whether the activated Tie2 receptor could modulate the heterotypic interaction via N-cadherin the adhesion assay was modified. A DNA construct containing the extracellular domain and an FC domain provided by Professor Patrick Doherty, Molecular Neurobiology Group, GKT School of Medicine, London (Utton et al. 2001) was transfected into CHO cells to produce conditioned media (Sections 2.2.4.1 and 2.10) and affinity captured at a concentration of 250μg/ml into the wells of a 96 well plate (Section 2.3.2) and compared to control. BAE cells and BAE cells over-expressing the Tie2 receptor were fluorescently labelled for 15 minutes with 5μg/ml of Calcein AM, and then washed and brought to suspension in the following manner. Before incubation upon the affinity captured extracellular domain of N-cadherin the cells were trypsinised as in Section 2.3.1.
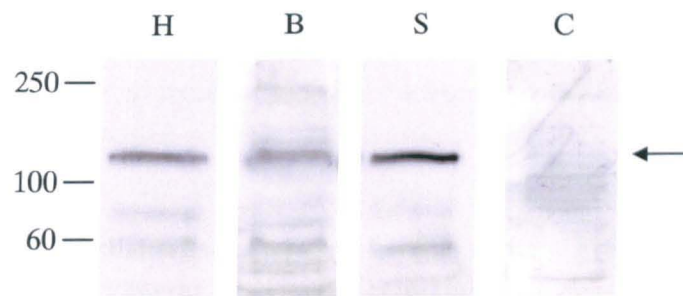
Figure 4.25 **N-cadherin expressed in endothelial cells, vascular smooth muscle cells but not Chinese hamster ovary cells.** HUVE, BAE, VSMC and CHO cells were grown to confluence in 80cm^2 flasks. Cells were lysed (Section 2.6.2) and samples were fractionated using 10% SDS-PAGE. Samples were immunoblotted with an anti-N-cadherin antibody. N-cadherin was expressed in endothelial cells and vascular smooth muscle cells but not Chinese hamster ovary cells (indicated by arrow). **H** – HUVE cells; **B** – BAE cells; **S** – VSM cells; **C** – CHO cells.

Both BAE cells and BAE cells expressing the Tie2 receptor adhered to the N-cadherin extracellular domain (Figure 4.26). BAE cells over-expressing Tie2 showed a slightly increased level of adhesion to N-cadherin compared with control cells (163.29+/-17.52% data presented as mean +/-standard deviation for three experiments, $p \leq 0.05$; Figure 4.26). Therefore it can be concluded that phosphorylation and activation of the Tie2 receptor increases endothelial cell adhesion to the extracellular domain of N-cadherin.

4.18 Discussion

In this Chapter the possibility that recruitment and association of mural cells with endothelial cells is regulated by angiopoietins/Tie2 was tested directly in cell culture-based assays. Ang-1 was found to stimulate endothelial-directed migration of putative mural cell precursors. In addition the ligand was found to increase adhesion between endothelial cells and smooth muscle cells.

PDGF-B released by endothelial cells is known to have an important role in recruitment of mural cells and the differentiation and recruitment of mesenchymal cells to functional mural cells (Holmgren et al. 1991; Lindahl et al. 1997; Hirschi et al. 1998). It is possible; therefore, that Ang-1 could stimulate PDGF-B release by endothelial cells, thereby increasing recruitment. To investigate this, additional experiments in which the effects of the Tie2 ligand on expression and release of PDGF-B by endothelial cells would be required. In a recently published study using rat aorta model it was reported that Ang-1 could have direct pro-migratory effects on a subset of mesenchymal cells that were found to express Tie2 (Iurlaro et al. 2003). This is not the case in the present study where Ang-1* was found to have no direct stimulatory effects on 10T1/2 cells.

The finding that Ang-1 can stimulate adhesion between endothelial cells and smooth muscle cells is the first direct demonstration of the pro-adhesive activity of the ligand in heterotypic cell interaction. To gain insight into the adhesion molecules that are maybe involved in this interaction the cation-dependence was tested using the chelator, EDTA. Basal and Ang-1 stimulated adhesion was inhibited by EDTA, suggesting possible involvement of integrins and/or cadherins. A possible role of integrins is strengthened by the finding that adhesion was inhibited by RGD peptide. However, further work would be required to test this conclusion, as the RAD control peptide also exhibits some inhibitory effects.
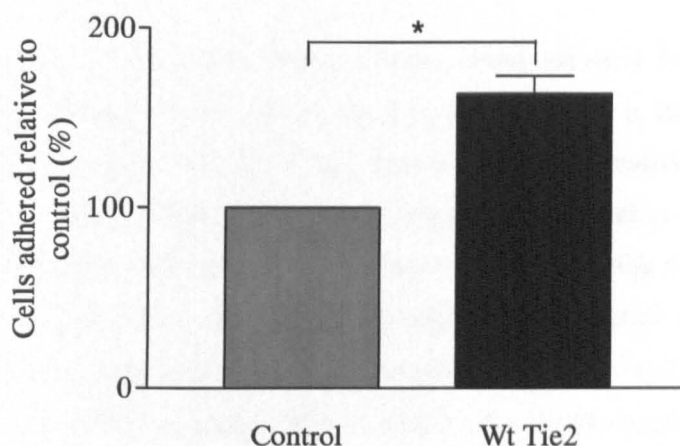
Figure 4.26 **Endothelial cells adhere preferentially to N-cadherin, the adhesion is increased by the presence of constitutively active Tie2.** N-cadherin was affinity captured at a concentration of 250μg/ml into the wells of a 96 well along with control. BAE cells and BAE cells over-expressing the Tie2 receptor (**WtTie2**) were fluorescently labelled for 15 minutes with Calcein AM, 5μg/ml and then washed and brought into suspension using the modified trypsinisation protocol (Section 2.3.1). Endothelial cells incubated upon the immobilised N-cadherin for 1 hour at 37°C/5% CO_2. The fraction of cells adhering was calculated for each cell type and normalised between experiments by setting BAE adhesion to 100%. Adhesion of the BAE cell lines was expressed as a percentage of the total amount of cells. Data presented as mean +/-standard deviation for three experiments (n=3). * p≤0.05 students 't' test.

In addition, use of blocking antibodies for specific integrins may help define which integrins are involved. The way in which integrins could mediate heterotypic cell adhesion was not investigated.

One possibility is that ECM proteins associated with one cell type could provide an adhesion substrate for integrins expressed on the other cell. Another intriguing possibility is that the angiopoietin itself could act as an intercellular adhesive substrate between the two cell types. This possibility arose from the observation, during the course of the present study that endothelial cells bind directly to Ang-1 via integrins (Carlson et al. 2001). As shown in this Chapter, smooth muscle cells can also adhere to Ang-1, consistent with a model of adhesion involving the ligand acting as an intercellular pro-adhesive substrate.

It was also of importance to determine whether Ang-1 could influence heterotypic adhesion via activation of Tie2 signalling. Clearly use of Ang-1 to stimulate Tie2 in these assays is precluded because of the direct adhesive effects of this ligand. As an alternative approach cells over-expressing Tie2 were used. In these cells ligand-independent activation of Tie2 occurs, due to crowding of receptors at the cell surface. Adhesion of endothelial cells over-expressing Tie2 or the chimeric receptor TrkA/Tie2 was elevated compared with control cells. These data are consistent with increased signalling from Tie2 enhancing endothelial cell adhesion. Additional studies to confirm this would require over-expression of a kinase-negative form of Tie2. In addition to endothelial cells a small, but significant, increase in adhesion was observed in non-endothelial CHO cells over-expressing Tie2. It is possible that endothelial specific signalling intermediates and/or adhesion molecules are required for a more robust increase in adhesion in response to activated Tie2.

Additional experiments were conducted to determine whether N-cadherin could act as an adhesive protein for endothelial cells. The identification of N-cadherin in endothelial cells although not involved in endothelial cell:cell junctions (Navarro et al. 1998) could be implicated in mediating a further increase in mural cell and endothelial cell association. Further evidence by Gilbertson-Beadling et al. 1993 and Gerhardt et al. 2000 revealed expression of N-cadherin in endothelial cells and smooth muscle cells of the developing vasculature in several animal models. N-cadherin was detected in endothelial and mural cells by Western blotting. In addition endothelial cells could adhere directly to N-cadherin extracellular domain when immobilised in tissue culture wells. Furthermore, adhesion of endothelial cells expressing constitutively active

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Tie2 was approximately 1.6 fold that of control endothelial cells. These data suggest N-cadherin maybe involved in Tie2-activated endothelial adhesion to N-cadherin, and therefore potentially smooth muscle cells. Further experiments will be required to confirm this and establish mechanisms by which activated Tie2 could increase N-cadherin mediated adhesion.

Taken together the data presented in this Chapter show that Ang-1/Tie2 could increase mural cell recruitment and association with endothelial cells. At least two mechanisms maybe involved in adhesion, direct interaction of each cell type with angiopoietin and increased adhesion triggered by a Tie2 initiated signalling cascade. In addition to the *in vivo* data from transgenic studies implicating Ang-1/Tie2 in mural cell association with endothelial cells (Sato et al. 1996; Patan 1998) a vascular defect in which mural cell poor vessels are formed has recently been mapped to a mutation in Tie2 (Vikkula et al. 1996). To gain further insight into Tie2 involvement in mural cell acquisition and vessel stabilisation the functional effects of this mutation in Tie2 will be examined in the next Chapter.

Chapter Five

R849W mutant Tie2 associated with venous malformations acts to increase endothelial cell survival

Chapter Five

R849W mutant Tie2 associated with venous malformations acts to increase endothelial cell survival

5.1 Introduction

As described previously in Chapter One, Ang-1/Tie2 has been implicated in regulating mural cell acquisition and endothelial:mural cell interaction *in vivo* (Suri et al. 1996; Patan et al. 1998). Chapter Four provides some evidence that mechanisms involved in this are likely to involve Tie2-independent adhesive activity of Ang-1 as well as an undefined mechanism involving Tie2 activated endothelial cell and smooth muscle cell adhesion. To gain further insight into the mechanisms by which the Ang-1/Tie2 system controls mural cell acquisition a mutant of Tie2 that is associated with the formation of dysmorphogenic mural cell poor vessels was examined.

Inherited vascular diseases that do not result in developmental lethality are useful tools to provide insight into postnatal vessel maintenance and vascular remodelling. Common vascular anomalies present after birth are the venous malformations which manifest as a vascular mass composed of dilated channels lined by endothelial cells (Mulliken and Glowacki 1982; Mulliken and Young 1988). Lesions are normally located on the skin or oral mucosa, and can also develop or enlarge in adult life (Pasyk et al. 1984; Mulliken and Young 1988; Gallione et al. 1995). The dilated vessels have an abnormally low smooth muscle cell to endothelial cell ratio causing a functionally low resistance vessel (Mulliken and Young 1988; Vikkula et al. 1996). Venous malformations are usually sporadic, although they can be inherited (Mulliken and Glowacki 1982; Mulliken and Young 1988). Venous malformation, multiple cutaneous and mucosal (VMCM; OMIM number 600195) is an autosomal dominant inherited disorder where the same C2545T transition has been observed in the coding sequence of the Tie2 receptor located on the short arm of chromosome 9 in two unrelated families (Boon et al. 1994; Gallione et al. 1995; Vikkula et al. 1996). A third unrelated family was found to have the same C2545T substitution making the transition the single most common mutation detected so far in inherited venous malformations (Calvert et al. 1999). The transition C2545T in the coding sequence of the Tie2 receptor results in the substitution of an arginine for a tryptophan at amino acid 849 of the receptor's kinase domain (R849W). Expression of the R849W mutant in insect cells resulted in hyperphosphorylation and increased tyrosine kinase activity in comparison to the wild type receptor (Vikkula et al. 1996). The role of the mutant Tie2 receptor in the development of

venous malformations is yet to be defined. It has been suggested that expression of the R849W mutant in endothelial cells could result in the defective recruitment and/or interaction of mural cells by interfering with the normal function of Tie2 (Vikkula et al. 1996). These data further implicate the Tie2 receptor in mural cell acquisition with the mutant form of Tie2 associated with apparent defects in mural cell acquisition. Study of this mutant form of Tie2 therefore may provide further insight into how Tie2 controls mural cell recruitment. In this Chapter the possible mechanisms by which R849W Tie2 mutant could lead to venous malformations was examined. The characteristics of venous malformation vessels and phosphorylation status of Tie2 were examined using immunohistochemistry. In addition, studies were performed with endothelial cells expressing mutant Tie2 to test its effects on endothelial cell function.

5.2 Immunohistochemical analysis of venous malformation vessels

Previously, studies in NIH3T3 and COS cells suggests that R849W Tie2 hyperphosphorylates due to an increase in kinase activity (Vikkula et al. 1996). This has been suggested to be responsible for the defects seen in venous malformations. However, venous malformations are focal lesions therefore suggesting that expression of mutant Tie2 alone is not sufficient to result in vessel defects. Alternatively it has been suggested that expression of mutant Tie2 needs to reach a critical level before vessel defects are observed and this level is achieved in the lesions (Vikkula et al. 1996). It was of interest therefore to examine the phosphorylation status of Tie2 in venous malformation vessels and non-dysmorphogenic vessels. Immunohistochemistry was performed on venous malformation tissue to examine morphology, smooth muscle cell association, endothelial cell proliferation and Tie2 phosphorylation status.

Biopsy tissue from a venous malformation was obtained with informed consent from an individual with the Tie2 R849W mutation (Calvert et al. 1999). Tissue was paraffin embedded, cut to a thickness of 5μm and kindly donated by Dr. Douglas Marchuk, Department of Genetics, Duke University Medical Centre, Durham, North Carolina, USA. All immunohistochemistry was undertaken on the serial tissue sections using the ChemMate™/Envision™ kit and antibodies from DakoCytomation except where indicated (Sections 2.11.4 and 2.11.8.2).

5.2.1 Morphology of vessels in venous malformation

Microvessel morphology was determined by staining with an endothelial cell marker, anti-vWF (Figure 5.1A). Large, irregular structures were observed consistent with the observations made by Vikkula et al. (1996). Both arterial and venous vessels are present within tissue extracted from the venous malformation patient indicated by R (arterial) and V (venous) (Figure 5.1A). As previously described, the dilated vessels of venous malformations lack abluminal support cells, therefore, the mural cell status of the dysmorphogenic vessels was investigated using the smooth muscle cell marker, anti-smooth muscle actin (SMA). As observed by Vikkula et al. (1996) and as seen in the other pathological situations in this project the large, irregular vessels revealed a poor smooth muscle cell investment (Figure 5.1B).

Additional sections showing SMA staining are shown in Figure 5.2. As can be seen segments of the lesional vessels are devoid of mural cells whereas in other areas mural cell association is disorganised (Figure 5.2A and B). Furthermore, non-dysmorphogenic vessels located on the same tissue appeared tightly associated with smooth muscle cells (Figure 5.2C and D). As described by both Mulliken and Young (1988) and Vikkula et al. (1996) the ratio of smooth muscle cells in relation to endothelial cells of the lesional vessels was low in the venous malformation. To confirm specificity of staining a serial tissue section was probed in the same way as other sections but lacking the primary antibody (Figure 5.1A inset).

5.2.2 Proliferation of endothelial cells in the venous malformation

The aberrant vessels of the venous malformation were investigated for proliferation. As previously indicated, venous malformation tissue was paraffin embedded and serial sections were immunostained for endothelial cells using the endothelial cell marker, anti-CD34 and for proliferating cells using anti-Mib-1. Figure 5.3A indicates the endothelial cells surrounding the dilated channels dominating the morphology of the venous malformation. Mib-1 expression was observed in the tissue but not in the endothelial cells of the dilated channels (Figure 5.3B). As seen in Figure 5.3C Mib-1 staining was evident in the positive control sections, confirming the antibody is capable of detecting the antigen. It is possible that the proliferating cells observed in the venous malformation tissue could be either inflammatory cells or haematopoietic progenitor cells. These data suggest that the lesional vessels are not undergoing any extensive remodelling.
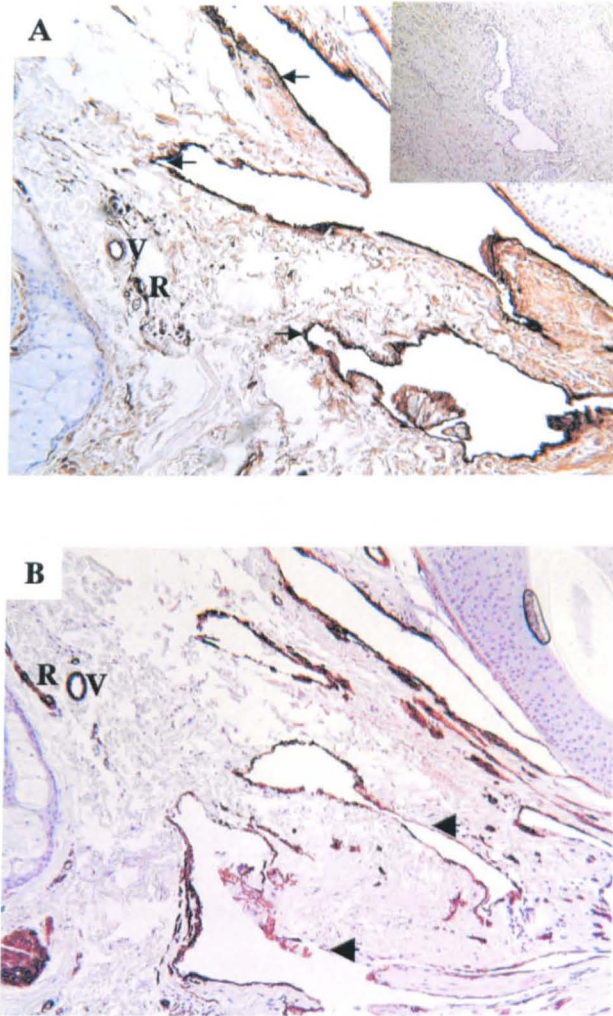
Figure 5.1 **Venous malformations are large dilated structures poorly invested with smooth muscle cells.** Dermal venous malformation tissue was fixed in formalin and embedded in paraffin. Sections were probed with the endothelial cell marker, anti-vWF (**A**) and the smooth muscle cell marker, anti-SMA (**B**). **A**) Illustrates apparently normal arterial (**R**) and venous (**V**) vessels as well as highly dilated endothelial lined venous malformation vessels indicated by arrows. **B**) Dysmorphogenic vessels are poorly associated with smooth muscle cells indicated by arrowheads. **A**) Inset is negative control using the ChemMate[TM] protocol (Section 2.11.4). Original magnification x100.
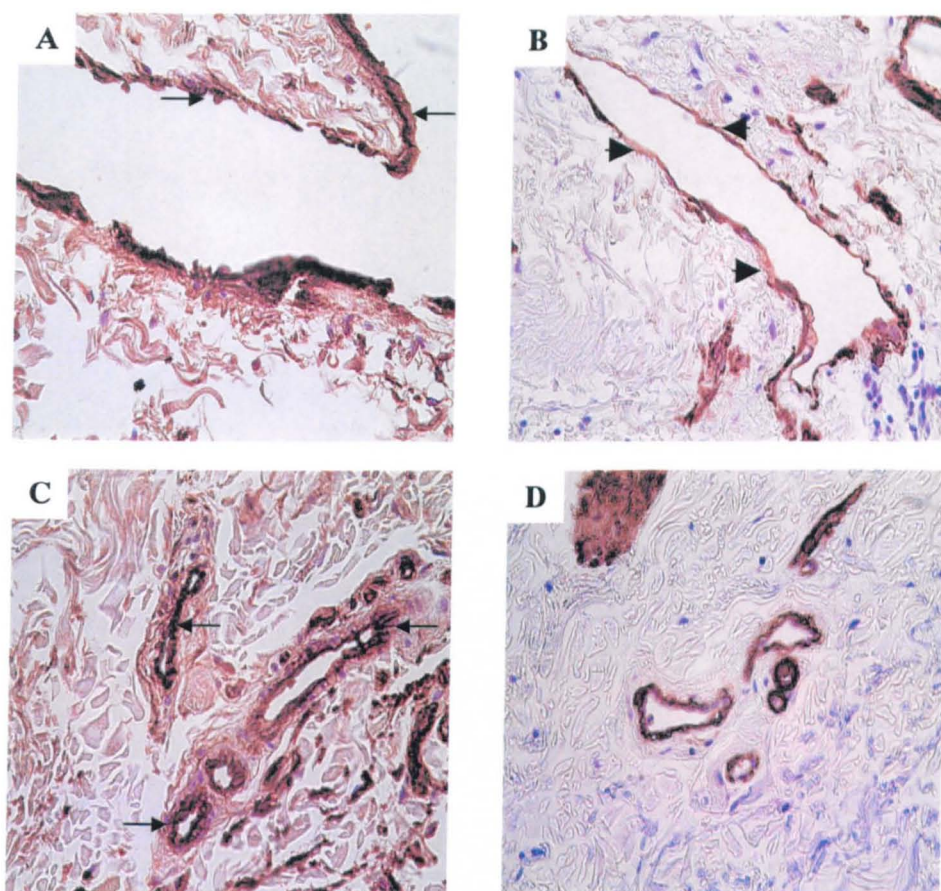
Figure 5.2 **High power view of venous malformation vessels.** Dermal venous malformation tissue was fixed in formalin and embedded in paraffin. Sections were probed with the endothelial cell marker, anti-vWF (**A** and **C**) and the smooth muscle cell marker, anti-SMA (**B** and **D**). **A**) Shows the lesional vasculature dominating the tissue section. **B**) Vessels are poorly invested with smooth muscle cells. Furthermore, smooth muscle cell association is disorganised (arrowheads). **C**) Smaller vessels within the same section are relatively normal possessing a tight a smooth muscle cell association (**D**). Original magnification x400.
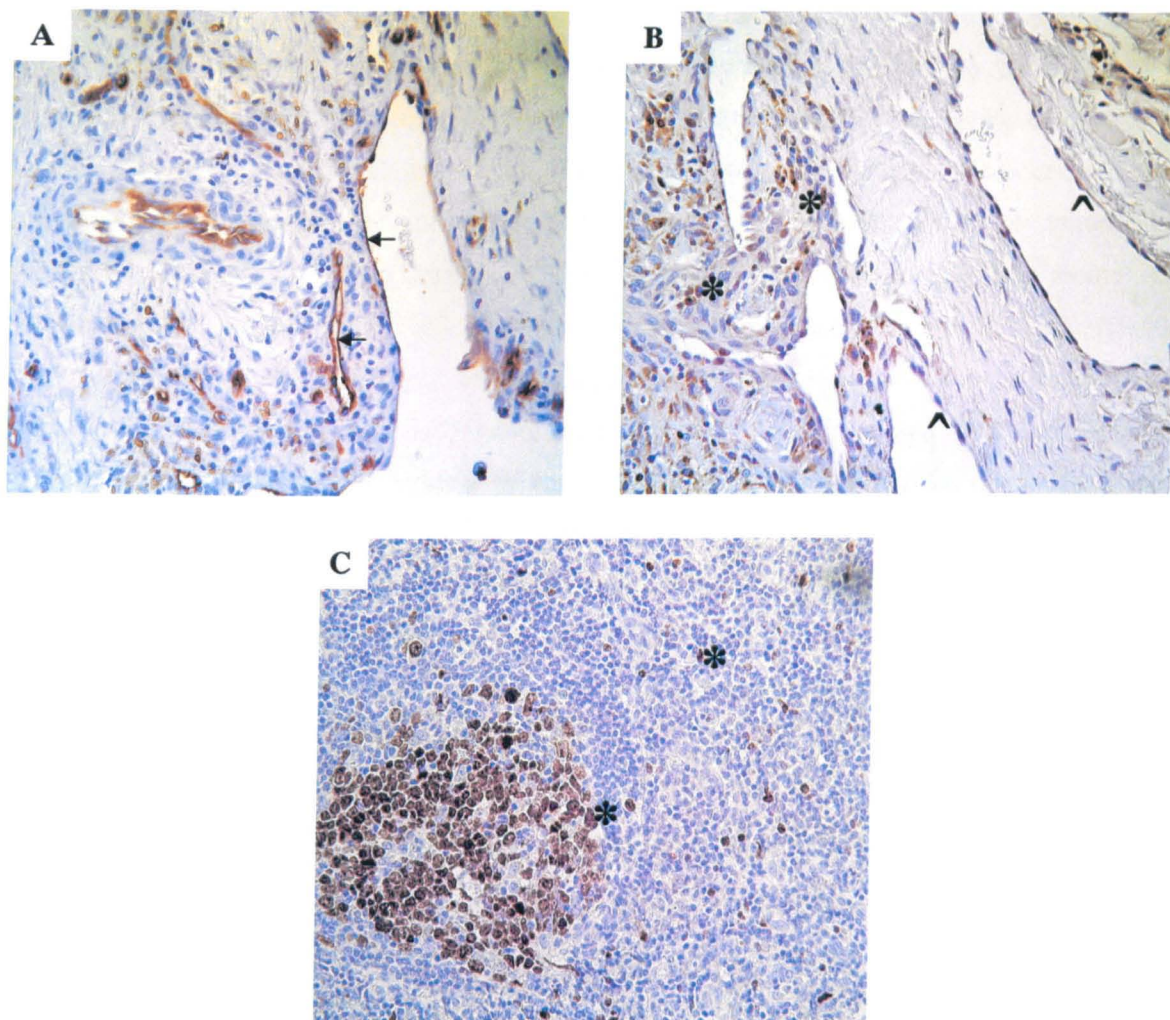
Figure 5.3 **Irregular vessels do not have high proliferative activity.** Dermal venous malformation tissue was fixed in formalin and embedded in paraffin. Sections of venous malformation tissue were probed with an endothelial cell marker, anti-CD34 (**A**), and a marker of proliferation, anti-Mib-1 (**B**). **A**) Endothelial cells are indicated by arrows. **B**) Mib-1 expressing cells were observed in the venous malformation tissue section (*) but not in the endothelial cells of the lesional vessels (^). **C**) Positive Mib-1 staining is also shown in proliferating cells in the positive control section of tonsil tissue (*). Original magnification x250.

5.2.3 Phosphorylation status of Tie2 in the venous malformation

Expression of the venous malformation mutant Tie2 in non-endothelial cells results in hyperphosphorylation of the receptor, the likely result of constitutively elevated kinase activity. This increased phosphorylation and kinase activity has been suggested to be involved in generating the lesional vessels of the venous malformation (Vikkula et al. 1996; Calvert et al. 1999). Therefore, phosphorylation of the Tie2 receptor was investigated in lesional and non-lesional vessels using an antibody specific to the synthetic Tie2 phosphopeptide (EERRTpYVNTTLpYEK(C)) (Oncogene Research Products). This corresponds to amino acid residues 1089 - 1102 of human Tie2, where the lysine in position 1092 of the native sequence has been substituted with an arginine. Surprisingly, the non-dysmorphogenic vessels exhibiting a tight mural cell association had similar staining intensity for phosphorylated Tie2 as observed in the aberrant, dysmorphogenic vessels (Figure 5.4A and B). However, the relatively normal vessels are part of the focal venous malformation and could account for the similar phosphorylation levels. The phosphorylation status of Tie2 in endothelial cells of normal skin was observed to be negative (data not shown). Both lesional and normal vessels had similar levels of Tie2 receptor expression as indicated by immunostaining with an anti-Tie2 receptor antibody (R & D Systems) (Figure 5.5C and D).

5.3 Tie2 R849W mutant expression results in receptor hyperphosphorylation

To gain insight into the cellular basis for defects in mural cell acquisition and vessel defects in venous malformations, experiments were performed with endothelial cells expressing R849W Tie2. HUVE cells were transfected with control vector or vector encoding mutant R849W Tie2. Initial experiments were aimed at determining the phosphorylation status of Tie2 in these cells. To distinguish the effects of the mutation from over-expression, parallel cultures were transfected with wild type Tie2. 80-90% confluent HUVE cells in 80cm^2 flasks were transfected with mutant R849W Tie2 DNA, full-length wild type Tie2 receptor or pcDNA 3.1 empty vector. All transfections were conducted using the Targefect F-2 reagent (Targeting Systems) (Section 2.2.4.2). After 36 hours, cells were serum starved for 4 hours at 37°C/5% CO$_2$. Prior to lysis cells were pre-treated with 10mM Na$_3$VO$_4$ for 15 minutes. Cells were then lysed as previously described in Section 2.6.3.1. The Tie2 receptor was immunoprecipitated using an anti-Tie2 antibody (R & D systems) and tyrosine-phosphorylated proteins were detected using an anti-phosphotyrosine antibody (Cell Signaling Technology).

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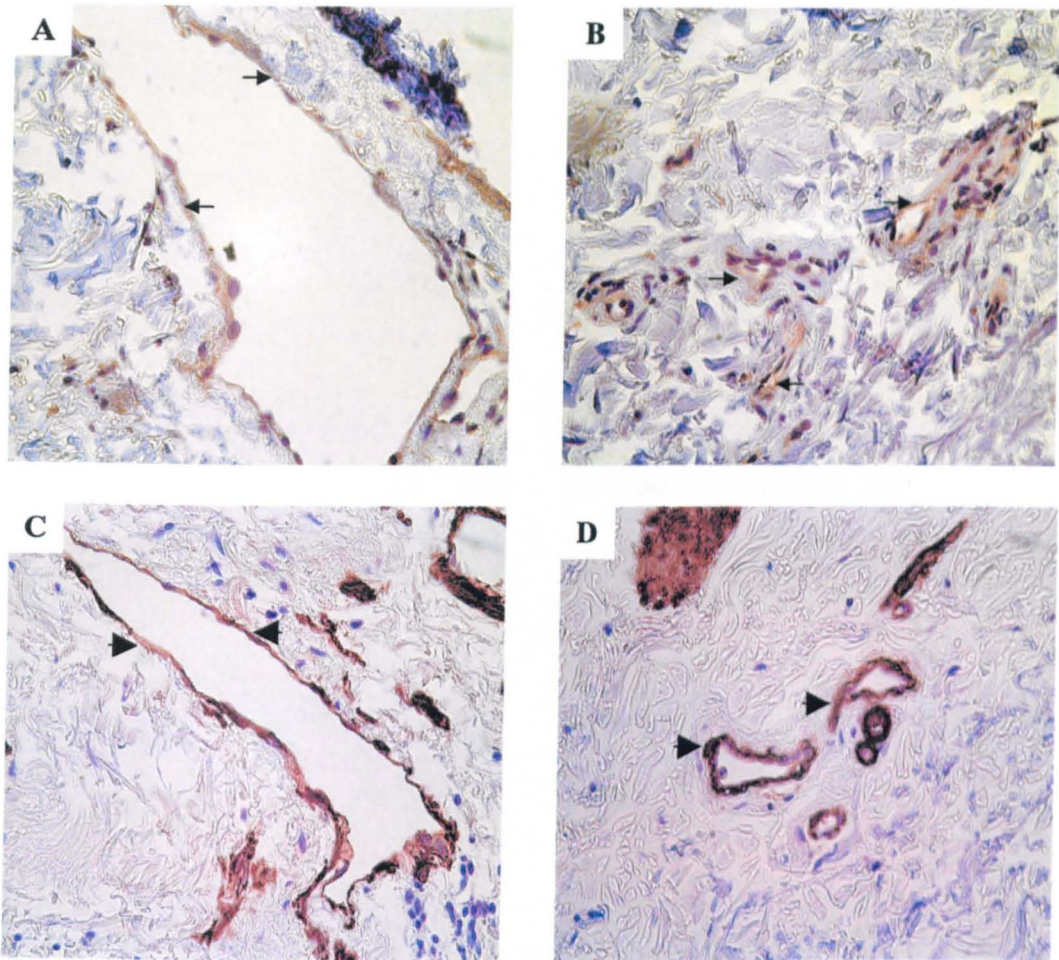
Figure 5.4 **Immunostaining for phosphorylated Tie2.** Dermal venous malformation tissue was fixed in formalin and embedded in paraffin. Sections were probed with an antibody specific to the synthetic Tie2 phosphopeptide (EERRTpYVNTTLpYEK(C)) (**A** and **B**), and anti-SMA (**C** and **D**). Positive anti-phospho-Tie2 staining was observed in normal and dysmorphogenic vessels (indicated by arrows) in (**A**) and (**B**). **C**) and **D**) Represents the smooth muscle cells association of the two subpopulations of vessels indicated by arrowheads. Original magnification x400.
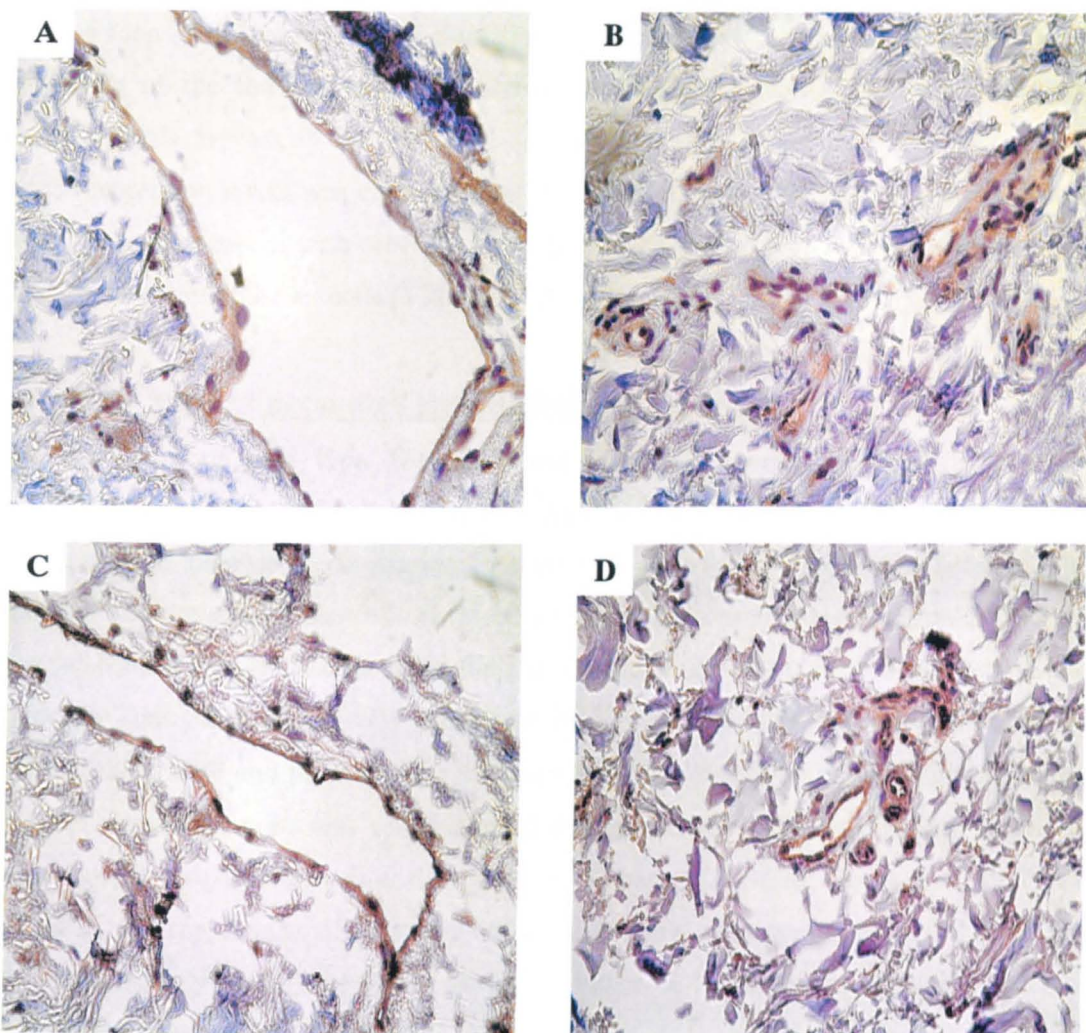
Figure 5.5 **Immunostaining for Tie2 in venous malformation.** Dermal venous malformation tissue was fixed in formalin and embedded in paraffin. Sections were probed with an antibody specific to the synthetic Tie2 phosphopeptide (EERRTpYVNTTLpYEK(C)) (**A** and **B**), and Tie2 receptor (**C** and **D**). **A**) and **B**) shows the phosphoTie2 immunoreactivity within dysmorphogenic and non-dysmorphogenic vessels. **C**) and **D**) shows Tie2 receptor expression within the vessels. Original magnification x400.

HUVE cells over-expressing the R849W Tie2 mutant exhibited a constitutive tyrosine phosphorylated receptor (Figure 5.6A). Over-expressed wild type Tie2 also exhibited increased levels of tyrosine phosphorylation compared to control. Phosphorylated Tie2 was also detected using an antibody specific for the phosphorylated tyrosine residue 992 located in the putative activation loop of the receptor (Cell Signaling Technology) (Murray et al. 2001) (Figure 5.6B). Comparison of the three transfectants showed substantially higher Tie2 phosphorylation in endothelial cells expressing R849W Tie2 compared to wild type Tie2. The amount of Tie2 receptor expression levels was confirmed by immunoblotting for Tie2 (R & D Systems) (Figure 5.6C). These data concur with previous findings that R849W Tie2 is hyperphosphorylated when expressed in non-endothelial cells (Vikkula et al. 1996).

5.4 R849W mutant promotes endothelial cell survival

Ang-1 activation of wild type Tie2 has been shown to increase endothelial cell survival (Papapetropoulos et al. 2000). It is possible therefore, that one of the effects of R849W Tie2 expression is to provide a constitutive pro-survival activity to endothelial cells. This was investigated by developing the following endothelial cell survival assay (Section 2.2.5). HUVE cells were seeded to a confluence of 80-90% in a gridded six well plate and transfected with the appropriate Tie2 construct, R849W mutant or wild type. All cells were co-transfected with a vector encoding GFP and incubated for 48 hours at 37°C/5% CO_2. Before serum starvation the number of transfected cells was counted in 10 defined fields. Cells were serum starved for 18 hours, inducing programmed cell death/apoptosis (Kovacs et al. 1996; Gupta et al. 1999; Yilmaz et al. 2003), and the number of surviving cells was counted. For comparison control transfected HUVE cells (empty vector) were also subject to either 18 hours serum starvation or no serum starvation and their survival monitored.

The maximum (20% serum) and minimum (serum free) rates of survival for the endothelial cells subjected to transfection within the survival assay were 57.67+/-9.66% and 20.67+/-9.61% of surviving cells expressing GFP, respectively (data presented as mean +/-standard deviation for three experiments). The survival of HUVE cells was significantly reduced in serum-starved conditions ($p \leq 0.05$; Figure 5.7). Endothelial cells transfected with both mutant Tie2 and wild type Tie2 had significantly increased survival compared with cells transfected with control vector (52.33+/-10.79% and 32.00+/-12.53%, data presented as mean +/-standard deviation for three experiments, $p \leq 0.05$ and 0.05, respectively; Figure 5.7).

A

B

C
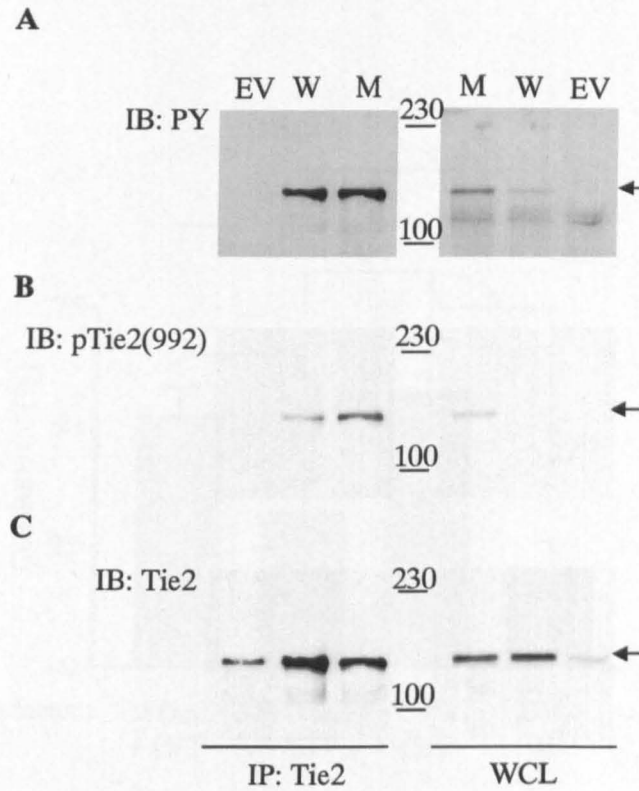
Figure 5.6 **The mutant Tie2 (R849W) receptor is constitutively phosphorylated.** $80cm^2$ flasks of HUVE cells at 80-90% confluence were transfected with mutant Tie2 (**M**), wild type Tie2 (**W**) and empty vector (**EV**). After 36 hours cells were serum starved for 4 hours at 37°C/5% CO_2. Cells were lysed as previously described (Section 2.6.3.1) and immunoprecipitated with an anti-Tie2 antibody. **A**) Tyrosine phosphorylated proteins were detected using an anti-phosphotyrosine antibody (**anti-PY**), constitutive phosphorylation was detected in both mutant Tie2 and wild type. **B**) Phosphorylated Tie2 was detected using an anti-phosphoTie2 antibody (**pTie2(992)**) encompassing the tyrosine residue at 992. Tie2 R849W was hyperphosphorylated compared to wild type. **C**) Tie2 receptor levels were detected using an anti-Tie2 antibody. **IB** – immunoblot; **IP**- immunoprecipitation; **WCL** – whole cell lysate.
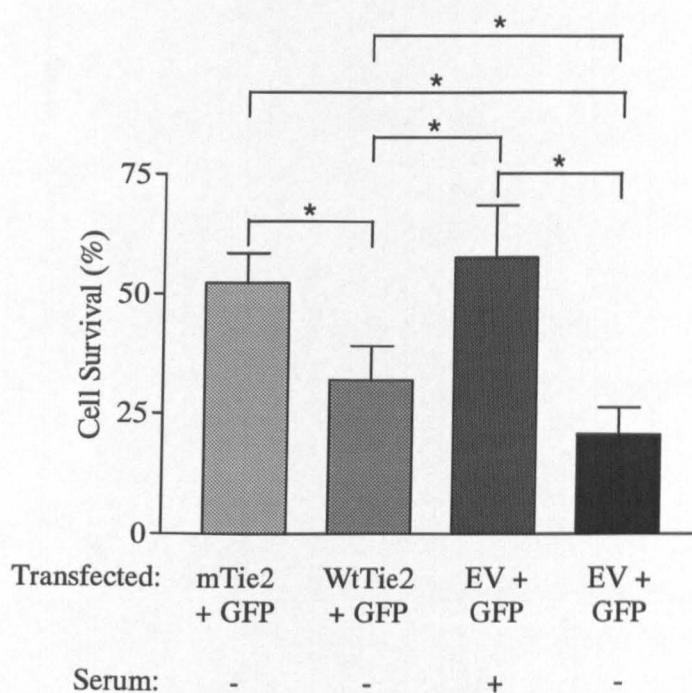
Figure 5.7 **R849W mutant Tie2 promotes endothelial cell survival after serum starvation.**
HUVE cells were co-transfected with GFP and wild type Tie2 (**WtTie2**), mutant Tie2 (**mTie2**) or
empty vector (**EV**). After 48 hours transfected cells were counted in 10 pre-defined fields. Cells
were serum starved for 18 hours and the surviving transfected cells were counted. Cells co-
transfected with GFP and empty vector were also maintained in serum or serum free conditions to
indicate maximum survival and serum free induced cell death. HUVE cells transfected with
R849W Tie2 and wild type show increased cell survival. R849W Tie2 significantly increased
endothelial cell survival compared to wild type Tie2. Data presented as mean +/-standard
deviation for three separate experiments (n=3). * p≤0.05 students 't' test.

There was no significant difference between survival of R849W Tie2 expressing cells and cells exposed to serum. HUVE cells expressing the wild type Tie2 exhibited significantly lower survival than control cells maintained in serum ($p \leq 0.05$; Figure 5.7). Furthermore, mutant Tie2 promoted endothelial cell survival better than wild type Tie2 ($p \leq 0.05$; Figure 5.7).

5.5 R849W mutant Tie2 inhibits endothelial cell apoptosis

The increased survival of endothelial cells expressing R849W suggests these cells had decreased apoptosis. This was investigated. HUVE cells were co-transfected with GFP and R849W Tie2 mutant or wild type Tie2 and incubated for 48 hours at 37°C/5% CO_2. HUVE cells were serum starved for 18 hours. Similar to the survival assay maximum and minimum levels of apoptosis were determined by transfecting HUVE cells with empty vector and either serum starving (maximum apoptosis) or maintaining in serum (minimum apoptosis). HUVE cells were fixed with 4% Paraformaldehyde solution and stained with 0.2µg/ml of the DNA specific fluorochrome 4,6-diamidino-2-phenylindole-2-HCl (DAPI) to look at the status of nuclear DNA (Section 2.2.6). HUVE cells expressing GFP and appropriate construct were analysed for evidence of apoptotic events such as nuclear blebbing, collapse and fragmentation in three separate experiments.

Figures 5.8A and B show examples of HUVE cells expressing GFP and DAPI stained nuclei, respectively. Figure 5.8B represents both apoptotic and non-apoptotic cells which coincide with GFP expression on Figure 5.8A. In three independent experiments serum starvation induced apoptosis in endothelial cells. 33.33+/-2.89% of HUVE cells in serum were apoptotic compared with 72.00+/-8.19% of those serum starved for 18 hours (data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$; Figure 5.8C). Expression of wild type Tie2 reduced apoptosis following serum starvation to 63.33+/-5.77% ($p \leq 0.05$ compared with control transfected serum starved cells, three experiments; Figure 5.8C). Expression of R849W Tie2 reduced apoptosis still further (33.33+/-2.89% of cells apoptotic compared with control transfected serum starved cells, three experiments, $p \leq 0.05$; Figure 5.8C). Significantly fewer cells over-expressing the R849W mutant were apoptotic compared to wild-type expressing cells ($p \leq 0.01$; Figure 5.8C). There was no difference between apoptosis observed in cells maintained in serum and those expressing mutant Tie2.
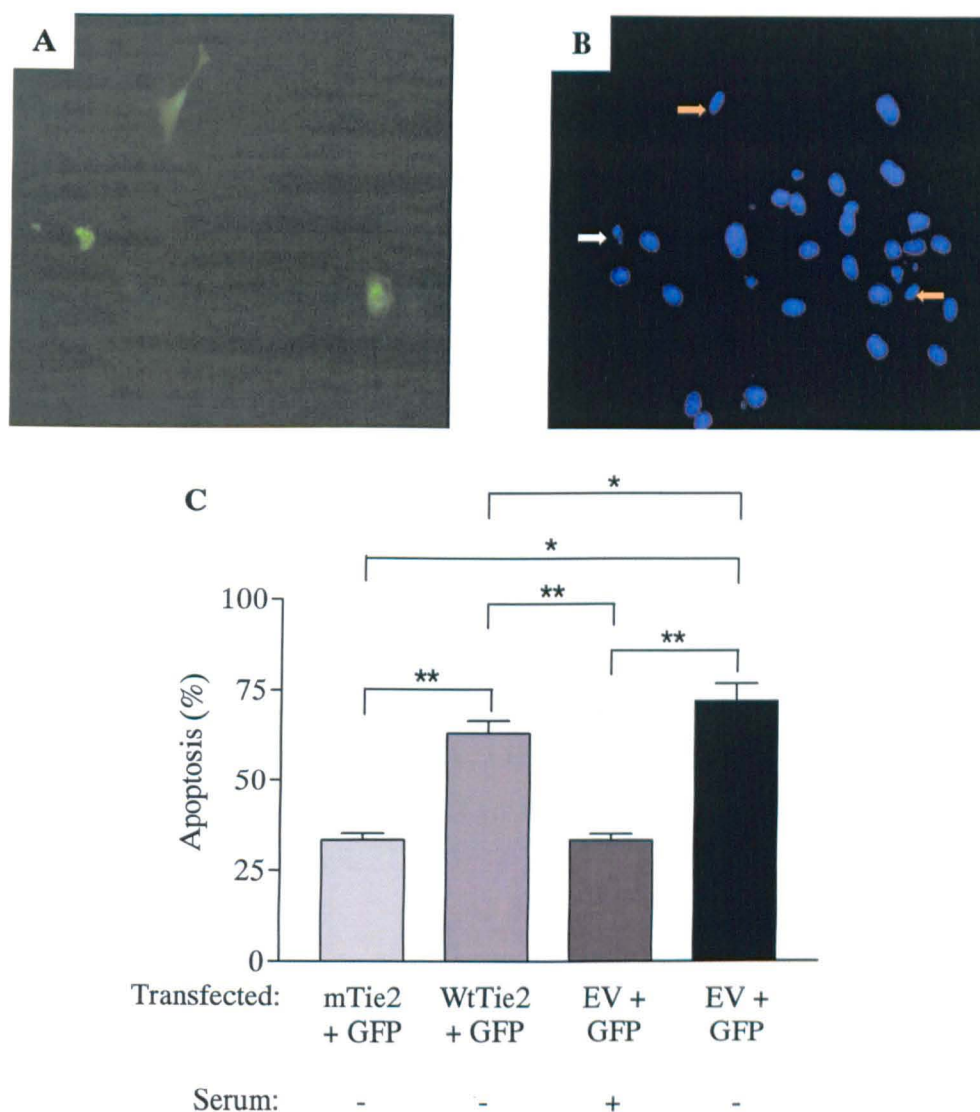
Figure 5.8 **Apoptosis in endothelial cells is decreased by R849W Tie2 over-expression.** HUVE cells were co-transfected with GFP and R849W Tie2 (**mTie2**), wild type Tie2 (**WtTie2**) or empty vector (**EV**). After 48 hours cells were subjected to serum starvation for 18 hours at $37°C/5\%$ CO_2. As in the endothelial cell survival assay, cells expressing GFP and empty vector were also maintained in serum and serum free conditions for 18 hours. Endothelial cells were fixed with 4% Paraformaldehyde solution and labelled with $0.2\mu g/ml$ of DAPI nuclear stain for 3 minutes at room temperature. Transfected cells were counted and assessed for apoptosis. **A)** HUVE cells expressing GFP. **B)** DAPI labelled HUVE cells. White arrow indicates an apoptotic GFP expressing cell. Orange arrows indicate non-apoptotic cells. **C)** R849W Tie2 and wild type Tie2 reduce HUVE cell apoptosis in serum free conditions. R849W Tie2 significantly rescues endothelial cells from an apoptotic phenotype compared to wild type Tie2. A minimum of 60 transfected cells were analysed for each transfection. Data presented as mean +/-standard deviation for three experiments (n=3). * $p \leq 0.05$; ** $p \leq 0.01$ students 't' test.

However, significantly more HUVE cells expressing wild type Tie2 were apoptotic than serum maintained cells ($p \leq 0.01$; Figure 5.8C). Therefore, the venous malformation Tie2 mutant gives rise to a substantial pro-survival effect and anti-apoptotic phenotype in cultured endothelial cells.

5.6 Mutant Tie2 reduces active caspase-3

Further evidence that the R849W mutant suppresses apoptosis was sought by assessing its effects on caspase-3 activation. The cleaved form of caspase-3 is a downstream early effector when apoptosis has been activated (Porter and Janicke 1999; Vaughan et al. 2002).

HUVE cells were seeded into a gridded six well plate at 80-90% confluence and transfected with GFP and R849W Tie2, wild type Tie2 or empty vector. Cells were incubated for 24 hours in 20% serum at 37°C/5% CO_2. Cells expressing empty vector were either serum starved or remained in 20% serum for 4 hours at 37°C/5% CO_2. HUVE cells transfected with either mutant Tie2 or wild type Tie2 were also serum starved for 4 hours. Cleaved caspase-3 was detected by immunofluorescence microscopy of cells probed with an antibody that detects the cleaved (activated) form of caspase-3 (Section 2.5.2). Cells expressing GFP (FITC fluorochrome) were analysed for cleaved caspase-3 expression (Cy3 flurochrome). Figure 5.9 shows examples of caspase-3 staining of transfected cells.

Statistical analysis of HUVE cells in 20% serum or serum free conditions showed significantly more cells exhibiting cleaved caspase-3 in serum starved conditions (17.00+/-8.19% and 64.67+/-4.51%, respectively, data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$; Figure 5.10). Following 4 hour serum starvation 25.67+/-16.01% of cells expressing R849W Tie2 were positive for cleaved caspase-3 ($p \leq 0.01$ compared with control transfected cells). 55.00+/-5.00% of cells expressing wild type Tie2 were positive for cleaved caspase-3 ($p \leq 0.05$ compared with transfected cells; Figure 5.10). Comparison of mutant Tie2 and wild type Tie2 with cells maintained in serum revealed no difference for the R849W mutant but significantly higher numbers of active caspase-3 positive cells were seen in the wild type cells ($p \leq 0.01$; Figure 5.10). Furthermore, significantly fewer cells expressing R849W Tie2 were positive for active caspase-3 than cells expressing wild type Tie2 ($p \leq 0.05$; Figure 5.10). Therefore, serum starvation induces programmed cell death reducing endothelial cell survival. This phenotype is rescued by the over-expression of both R849W Tie2 mutant and wild type Tie2, but mutant Tie2 was far more effective at inhibiting apoptosis than wild type Tie2.
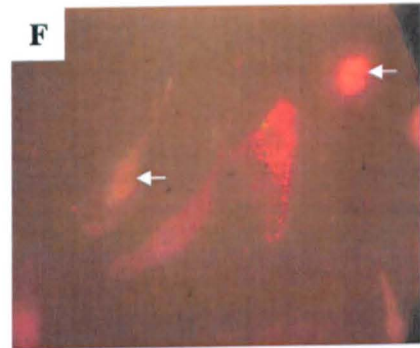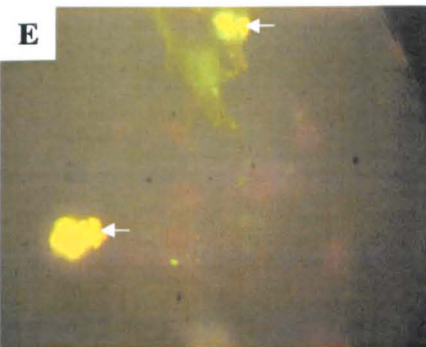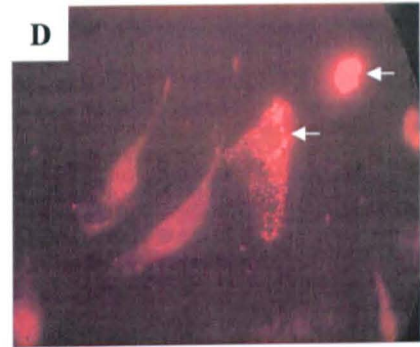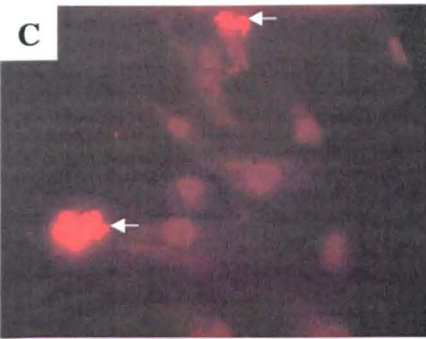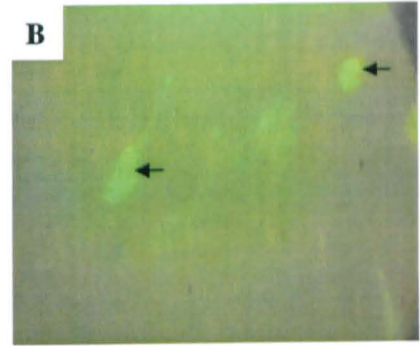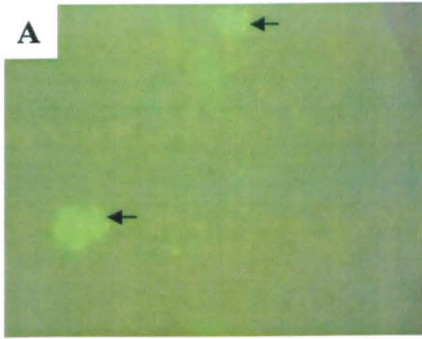
Figure 5.9 **Activated caspase-3 immunoreactivity in endothelial cells.** HUVE cells were transfected with GFP and R849W Tie2 or empty vector. After 24 hours cells were serum starved for 4 hours at 37°C/5% CO_2 and then fixed with 4% Paraformaldehyde. HUVE cells were probed with an antibody recognising the cleaved activated form of caspase-3. A) Shows GFP expression in endothelial cells transfected with GFP plus empty vector. C) Shows caspase-3 immunoreactivity of these cells. E) Shows an overlay of A) and C). B) Shows GFP expression in cells transfected with GFP plus R849W Tie2. D) Shows active caspase-3 immunoreactivity. F) Shows an overlay of B) and D). G) Shows negative control for active caspase-3 probe.

Cells positive for cleaved caspase-3 (%)

| Transfected: | mTie2 + GFP | WtTie2 + GFP | EV + GFP | EV + GFP |
| Serum: | - | - | + | - |
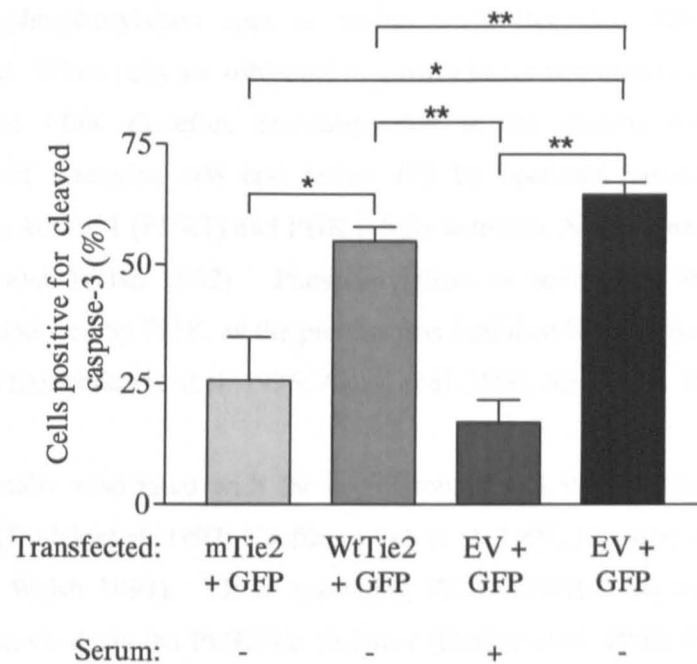
Figure 5.10 **R849W Tie2 expression reduces percentage of cells positive for cleaved caspase-3 in endothelial cells.** HUVE cells were co-transfected with GFP and R849W Tie2 (**mTie2**), wild type Tie2 (**WtTie2**) or empty vector (**EV**). After 24 hours cells were serum starved for 4 hours at 37°C/5% CO_2. A control well with endothelial cells expressing empty vector was maintained in 20% serum as an indicator of minimal caspase-3 activation. Endothelial cells were fixed in 4% Paraformaldehyde solution and immunofluorescently labelled with an antibody recognising cleaved caspase-3 (Section 2.5.2). Cells expressing GFP were analysed for caspase-3 activation under green UV light. Data presented as mean +/-standard deviation for three experiments (n=3). * $p \leq 0.05$; ** $p < 0.01$ students 't' test.

5.7 R849W Tie2 mutant induces cell survival and anti-apoptosis via Akt

Originally identified as an oncogene related to the murine AKT8 retrovirus, Akt was independently isolated as a protein kinase related to protein kinase A and C and was named as protein kinase B (PKB) (Staal 1987; Bellacosa et al. 1991; Coffer and Woodgett 1991; Jones et al. 1991). Three mammalian genes, *Akt1*, *Akt2* and *Akt3*, encode proteins containing a PH domain found in the amino terminus, plus a central kinase domain and a carboxyl terminal regulatory domain (Datta et al. 1999; Scheid and Woodgett 2001). Akt1 is the most abundant and is predominantly expressed in brain, heart and lung (Coffer and Woodgett 1991; Altomare et al. 1995 and 1998; Brodbeck et al. 1999). Akt is found in the cytoplasm in resting cells where two regulatory phosphorylation sites at amino acids threonine 308 and serine 473 are unphosphorylated. When cells are subjected to growth factor stimulation the PH domains bind to lipid products of PI3K therefore recruiting Akt to the plasma membrane. Sequential phosphorylation of threonine 308 and serine 473 by upstream kinases 3-phosphoinositide-dependent protein kinase 1 (PDK1) and PDK 2 fully activates Akt (Hemmings 1997; Downward 1998; Shiojima and Walsh 2002). Phosphorylation of serine and threonine residues was discovered to be induced by PI3K, as the process was inhibited by wortmannin, a potent inhibitor of kinase activity (Andjelkovic et al. 1996; Alessi et al. 1996; Kohn et al. 1996).

Akt/PKB is critically associated with the regulation of cell viability in a number of distinct cellular systems (Dudek et al. 1997; Kauffman-Zeh et al. 1997; Kennedy et al. 1997; Kulik et al. 1997; Fujio and Walsh 1999). VEGF activating Flk1/VEGFR-2 has been shown to enhance endothelial cell survival via the PI3K/Akt pathway (Gerber et al. 1998; Fujio and Walsh 1999). The ability to promote endothelial cell survival was not limited to VEGF receptors; the Tie system was also identified in activating the PI3K/Akt signalling pathway. Research by Kontos et al. (1998 and 2002) revealed both Tie1/2 stimulate endothelial cell survival and inhibit apoptosis via the PI3K/Akt pathway. Subsequent research has revealed that Ang-1 not only activates the Akt cell-signalling pathway to inhibit apoptosis but also inhibits neuronal cell apoptosis via the same signalling pathway (Kim et al. 2000a; Papapetropoulos et al. 2000; Valable et al. 2003). A high concentration of Ang-2 also activates a pro-survival phenotype in endothelial cells via the Akt pathway (Kim et al. 2000b). The possibility that Akt was a downstream intermediate in the survival and anti-apoptotic signalling pathway mediated by R849W Tie2 mutant over-expression was investigated using the previously described survival and apoptosis assays.

5.7.1 R849W Tie2 causes Akt phosphorylation in serum-starved conditions

The effect of R849W Tie2 on Akt activation was determined in this study by phospho-ser473 immunoblotting of transfected cells. HUVE cells were transfected with R849W Tie2, wild type Tie2 and empty vector. After 36 hours the cells were serum starved for 18 hours and cells were lysed (Section 2.6.2) and proteins separated on 10% SDS-PAGE. The activation status of Akt was tested using an anti-phospho-Akt antibody specifically recognising the phosphorylated serine 473 residue (Cell Signaling Technology). Relative expression levels of total Akt was observed using an anti-Akt antibody (Cell Signaling Technology). Over-expression of the Tie2 constructs was confirmed using an anti-Tie2 antibody (R & D Systems).

Expression of the R849W mutant elevated the phosphorylation of the serine 473 residues of the Akt molecule (Figure 5.11A). This was confirmed by comparing the relative levels of total Akt expression in the R849W mutant, wild type Tie2 and empty vector (Figure 5.11A). Densitometric analysis of three separate immunoblotting experiments was performed in which phospho-Akt/Akt ratio of R849W Tie2 expressing cells was normalised to 100% for comparison between transfectants. Cells expressing R849W Tie2 exhibited significantly higher phospho-Akt/Akt ratio than cells expressing wild type Tie2 (26.80+/-4.00% of R849W Tie2 value, data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$) or control vector (14.10+/-11.40% of R849W Tie2 value, data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$; Figure 5.11B).

5.7.2 R849W mutant Tie2 receptor activates endothelial cell survival via Akt

Akt mediation of endothelial cell survival by the R849W mutant was tested using a mutant construct kindly donated by Dr. Brian Hemmings, Friedrich Miescher-Institut, Basel, Switzerland. A triple mutant dominant negative form (referred to as Akt3A) in which residues lysine (179), threonine (308) and serine (473) were substituted with alanine residues (K179A/T308A/S473A) was used (Andjelkovic et al. 1997). Expression of the dominant negative Akt construct was investigated. HUVE cells (80-90% confluence) were transfected with Akt3A or empty vector. Whole cell lysates were prepared as indicated in Section 2.6.2 and samples were fractionated using 10% SDS-PAGE. Akt expression was determined using an anti-Akt antibody (Cell Signaling Technology) and endothelial cells transfected with dominant negative Akt exhibited over-expression (Figure 5.12).

A
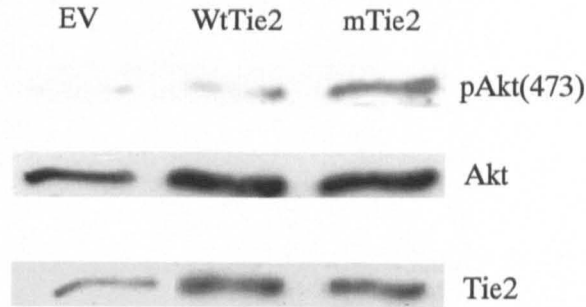
B
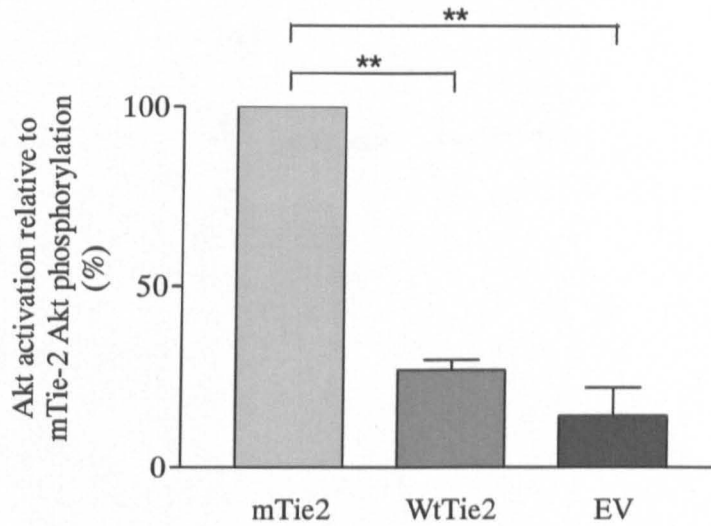
Figure 5.11 **Expression of R849W Tie2 increases Akt activation.** HUVE cells were transfected with R849W Tie2 (**mTie2**), wild type Tie2 (**WtTie2**) and empty vector (**EV**) (Section 2.2.4.2). After 48 hours cells were serum starved for 18 hours and lysed as previously described (Section 2.6.2). **A)** Akt phosphorylation was determined using an anti-phospho-Akt (ser473) (**pAkt473**) antibody. Relative expression of total Akt was determined using an anti-Akt antibody. Transfection of R849W Tie2 and wild type Tie2 constructs was determined by an anti-Tie2 antibody. **B)** Densitometric analysis was undertaken on the phospho-Akt and total Akt blots. Phosphorylated Akt levels relative to total Akt in the R849W Tie2 were taken as 100%. Data presented as mean +/-standard deviation of three experiments (n=3). ** $p \leq 0.01$ students 't' test.

Figure 5.12 **Expression of Akt constructs in endothelial cells.** HUVE cells were plated to 80-90% confluence in six well plates and transfected with Akt3A or empty vector (**EV**) (Section 2.2.4.2). Cells were lysed as previously described (Section 2.6.2) and samples were fractionated using 10% SDS-PAGE. Levels of total Akt expression were determined using an anti-Akt antibody. Increased levels of Akt indicate the transfection of the dominant negative Akt compared to empty vector (arrow).

The survival assay was conducted using HUVE cells seeded to 80-90% confluence in a gridded six well plate and co-transfected with R849W Tie2, either Akt3A or empty vector and GFP. Cells were incubated for 48 hours and GFP expressing cells were counted in 10 pre-defined fields. HUVE cells were serum starved for 18 hours and surviving cells counted. Again, HUVE cells co-transfected with empty vector and GFP were either maintained in serum as an indicator of maximum endothelial cell survival or subjected to serum starvation as an indicator of minimum endothelial cell survival.

Maximum (serum) and minimum (serum starvation) survival was observed at 98.50+/-6.28% and 29.98+/-7.41%, respectively (data presented as mean +/-standard deviation for three experiments, $p \leq 0.05$; Figure 5.13). Again, R849W Tie2 mediated an increase in cell survival (61.12+/-2.63%, data presented as mean +/-standard deviation for three experiments) compared to the control (serum starvation) (p ≤ 0.05; Figure 5.13). However, the introduction of the dominant negative mutant Akt 3A abolished R849W Tie2 mediated endothelial cell survival (37.66+/-2.63%, data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$; Figure 5.13). Therefore, survival of endothelial cells expressing the R879W mutant appears to be mediated by Akt.

5.7.3 The anti-apoptotic effects of R849W Tie2 is mediated by Akt

The R849W mutant was shown to have an anti-apoptotic effect in Sections 5.5 and 5.6. The role of Akt in mediating this effect was investigated.

HUVE cells were transfected as described in Section 5.7.2 and the apoptosis assay was conducted as previously described (Section 5.5). As expected endothelial cells maintained in serum were 21.67+/-5.77% apoptotic compared to 70.00+/-5.00% of serum starved cells (data presented as mean +/-standard deviation for three experiments, $p \leq 0.05$; Figure 5.14). Cells expressing R849W Tie2 mutant rescued the apoptotic phenotype in serum starvation compared to cells transfected with empty vector (41.67+/-2.89%, data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$; Figure 5.14). Expression of the Akt 3A dominant negative mutant negated the anti-apoptotic properties of the R849W Tie2 mutant. 70.00+/-5.00% of cells expressing R849W Tie2 plus Akt3A were apoptotic ($p \leq 0.05$; Figure 5.14).

These data demonstrate that the survival and anti-apoptotic signalling pathway used by the constitutively active R849W Tie2 mutant appears to be via the Akt molecule.

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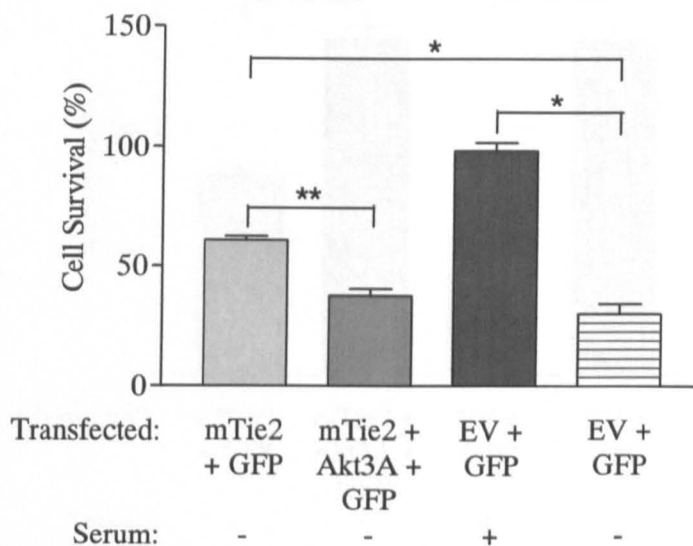
Figure 5.13 **Endothelial cell survival induced by the R849W Tie2 mutant is mediated by the Akt signalling pathway.** HUVE cells were co-transfected with GFP and R849W Tie2 (**mTie2**) or empty vector (**EV**) with Akt3A or vector. After 48 hours cells emitting green light in UV/fluorescence conditions were counted in 10 predefined fields. Cells were serum starved for 18 hours and the surviving cells were counted. Cells co-transfected with GFP and empty vector were also maintained in serum throughout the duration of the experiment to act as an indicator of maximum survival. Survival of endothelial cells expressing R849W Tie2 was abrogated by the expression of dominant negative Akt. Data presented as mean +/-standard deviation for three experiments (n=3). * p≤0.05; ** p≤0.01 students 't' test.
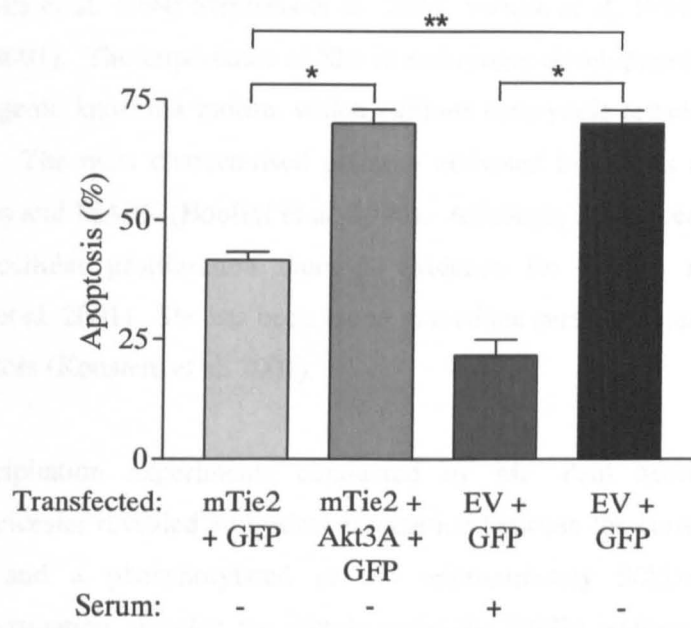
Figure 5.14 **The anti-apoptotic effects of R849W Tie2 in endothelial cells are mediated through the Akt signalling pathway.** HUVE cells were co-transfected with GFP and R849W Tie2 (**mTie2**) or empty vector (**EV**) with Akt3A or empty vector. After 48 hours cells were subjected to serum starvation for 18 hours at 37°C/5% CO_2. As in the endothelial cell survival assay, cells expressing GFP and empty vector were also maintained in serum conditions. Endothelial cells were fixed with 4% Paraformaldehyde solution and labelled with 0.2µg/ml of DAPI nuclear stain for 3 minutes at room temperature and assessed for apoptosis. R849W Tie2 reduction of HUVE cell apoptosis is abrogated by the expression of an Akt dominant negative construct. Data presented as mean +/-standard deviation for three experiments (n=3). * $p \leq 0.05$; ** $p \leq 0.01$ students 't' test.

5.8 Potential involvement of Shc in R849W Tie2 mediated endothelial cell survival and anti-apoptotic activity

Another, possible downstream signal candidate for the modulation of endothelial cell survival is the adapter protein, Shc, which is found ubiquitously expressed in mammalian cells in its ShcA homologue. ShcA is expressed in three isoforms of 46, 52 and 66kDa, which are generated by alternative splicing of mRNA or alternate translational initiation (Pelicci et al. 1992; Migliaccio et al. 1997). All Shc isoforms possess the ability to bind to phosphotyrosine sequences such as PTB and SH2 domains (Pelicci et al. 1996). Several growth factors including EGF, insulin, PDGF and VEGF signal through Shc (Pelicci et al. 1992; Rozakis-Adcock et al. 1992; Pronk et al. 1994; Sasaoka et al. 1994; Stephens et al. 1994; Yokote et al. 1994; Gelderloos et al. 1998; Ravichandran 2001). The importance of Shc in embryonic development has been highlighted in the ShcA transgenic knockout mouse, which exhibits embryonic lethality at day 11.5 (Lai and Pawson 2000). The most characterised pathway activated by Shc is the mitogenic signalling pathway via Ras and MAPK (Bonfini et al. 1996). Although, Shc appears to have a central role in regulating cellular proliferation there is evidence for it also mediating cell survival (Ravichandran et al. 2001). Shc has been found to mediate survival signals via the oestrogen and androgen receptors (Kousteni et al. 2001).

Co-immunoprecipitation experiments conducted by Mr. Paul Morris, Dept. of Surgery, University of Leicester revealed a physical association between the constitutively active R849W Tie2 receptor and a phosphorylated protein approximately 50kDa in endothelial cells. Subsequent investigation revealed the protein to be the 52kDa isoform of ShcA. Shc did not associate with wild type Tie2 receptor or control vector in these experiments.

The role of Shc in the R849W Tie2 mediated endothelial cell survival and anti-apoptotic effects was investigated using a Shc dominant negative construct. The dominant negative form of Shc used was obtained from Dr. A. Raymond Frackelton Jr., Department of Pathology and Laboratory Medicine, Brown University, Providence, Rhode Island, USA. This dominant-negative mutant Shc was constructed by replacing the tyrosine residue at position 317 with a phenylalanine. Y317F Shc retains its SH2 and PTB domains, but cannot be tyrosine phosphorylated at residue 317. It is therefore defective in its binding of Grb2-SOS and subsequent Ras signalling (Salcini et al. 1994; Ravichandran et al. 1995). Although the mutant Shc is defective in activating Ras, it still retains its ability to compete with normal cellular Shc for docking to activated receptor

tyrosine kinases, thereby inhibiting the ability of the endogenous normal Shc to link activated receptor to Ras (Salcini et al. 1994; Pelicci et al. 1995; Gotoh et al. 1996; Sasaoka et al. 1996; Gotoh et al. 1997; Ishihara et al. 1997; Thomas and Bradshaw. 1997; Stevenson et al. 1999). This construct also includes Glutathione S-transferase (GST) fused to the amino terminus.

Expression of the dominant negative Shc construct was confirmed by immunoblotting. HUVE cells were seeded to 80-90% confluence in six well plates and transfected with Shc dominant negative or PEBG, empty vector. Cells were incubated for 48 hours at 37°C/5% CO_2 and then serum starved for 18 hours. Cells were lysed (Section 2.6.2) and fractionated by 10% SDS-PAGE. Shc expression was detected using an anti-Shc antibody (Cell Signaling Technology) (Figure 5.15A). The 80kDa dominant negative Shc-GST fusion protein is clearly expressed in HUVE cells compared to control cells.

5.8.1 Dominant negative Shc suppresses mutant Tie2 receptor induced endothelial cell survival

HUVE cells plated to 80-90% confluence in six well plates were co-transfected with GFP and R849W Tie2 or wild type Tie2 with either the dominant negative Shc or empty vector, PEBG. Cells were incubated for 48 hours at 37°C/5% CO_2 and GFP expressing cells were counted in 10 pre-defined fields. HUVE cells were serum starved for 18 hours and surviving cells counted. Maximum and minimum values of endothelial cell survival were determined by maintaining or serum starving endothelial cells co-transfected with GFP, empty vector and PEBG empty vector.

Maximum (serum) and minimum (serum starvation) percentage endothelial cell survival was 67.50+/-5.65% and 19.90+/-2.55%, respectively (data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$; Figure 5.16). Endothelial cell survival was increased in serum starvation conditions by the R849W Tie2 mutant (50.00+/-3.60%, data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$) and partially wild type Tie2 (29.93+/-1.45%, data presented as mean +/-standard deviation for three experiments, $p \leq 0.05$; Figure 5.16). Comparison of the mutant and wild type Tie2 revealed that the R849W Tie2 increased cell survival significantly more than the wild type Tie2 consistent with previous observations ($p \leq 0.01$; Figure 5.16). In the presence of dominant negative Shc, R849W Tie2 mediated endothelial cell survival was abrogated (26.13+/-1.12%, data presented as mean +/-standard deviation for three experiments, $p \leq 0.05$; Figure 5.16).
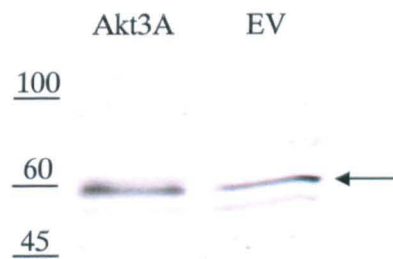
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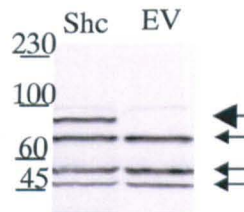
Figure 5.15 **Expression of Shc constructs in endothelial cells.** HUVE cells were plated to 80-90% confluence in six well plates and transfected with a dominant negative Shc-Y317F-GST fusion or PEBG empty vector (**EV**) donated by Dr. A. Raymond Frackleton (Section 2.2.4.2). Cells were lysed as previously described (Section 2.6.2) and samples were fractionated using 10% SDS-PAGE. An anti-Shc antibody confirmed the presence of the endogenous Shc isoforms (arrows). Expression of Shc was observed as the GST fusion protein is seen at 80kDa (arrowhead).
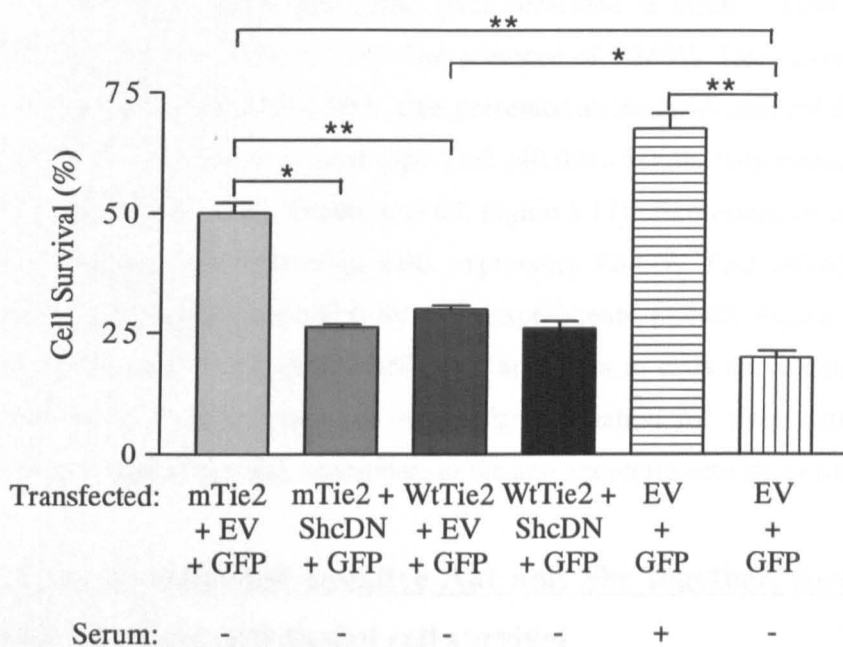
Figure 5.16 **Shc modulates endothelial cell survival in R849W Tie2 expressing cells.** HUVE cells were co-transfected with GFP, R849W Tie2 (**mTie2**) or wild type Tie2 (**WtTie2**) with either ShcDN or empty vector (**EV**), PEBG. After 48 hours cells emitting green light in UV/fluorescence conditions were counted in 10 predefined fields. Cells were serum starved for 18 hours and the surviving GFP-expressing cells were counted. Cells co-transfected with GFP and empty vector were also maintained in serum throughout the duration of the experiment to act as an indicator of maximum survival. R849W Tie2 and wild type Tie2 increased endothelial cell survival. Survival of endothelial cells expressing R849W Tie2 was abrogated by the expression of the dominant negative Shc, no effect was seen on wild type Tie2 endothelial cells. Data presented as mean +/-standard deviation for three experiments (n=3). * p$\leq$0.05; ** p$\leq$0.01 students 't' test.

Wild type Tie2 mediated endothelial cell survival was unaffected by dominant negative Shc (25.97+/-2.58%, data presented as mean +/-standard deviation for three experiments; Figure 5.16).

5.8.2 Dominant negative Shc suppresses R849W Tie2 mediated anti-apoptotic effects

HUVE cells were co-transfected as described in Section 5.8.1 and serum starved for 18 hours at 37°C/5% CO_2. Cells were labelled with DAPI at a concentration of 0.2μg/ml.

In the presence of serum apoptosis was observed in 20.00+/-5.00% of cells compared with 56.67+/-5.77% of cells in serum free media (data presented as mean +/-standard deviation for three experiments, p≤0.05) (Figure 5.17). The presence of R849W Tie2 decreased the level of endothelial cell apoptosis (28.33+/-2.89%, data presented as mean +/-standard deviation for three experiments, p≤0.05) compared to wild type Tie2 (40.00+/-5.00%, data presented as mean +/-standard deviation for three experiments, p≤0.05; Figure 5.17). Expression of dominant negative Shc increased apoptosis in endothelial cells expressing R849W Tie2 (46.67+/- 2.89%, data presented as mean +/-standard deviation for three experiments, p≤0.05; Figure 5.17). Dominant negative Shc did not have a significant effect on apoptosis in cells expressing wild type Tie2 (45.00+/-5.00%, data presented as mean +/-standard deviation for three experiments; Figure 5.17). These data suggest that Shc contributes to the anti apoptotic activity of R849W Tie2.

5.9 Expression of dominant negative Akt and Shc together, further abrogate R849W Tie2 mediated endothelial cell survival

In previous sections of this Chapter endothelial cell survival and a reduction in programmed cell death was found to occur upon expression of the mutant Tie2 (R849W) receptor in endothelial cells. Expression of dominant negative Akt inhibited the effects of R849W Tie2 on cell survival and apoptosis. Similarly, the cell survival and anti-apoptotic effects of the mutant receptor were also inhibited by dominant negative Shc. These data suggest both Akt and Shc and involved in the anti apoptotic effects of mutant Tie2. To investigate this, additional experiments were performed to determine the effect of expressing dominant negative constructs Akt and Shc together.

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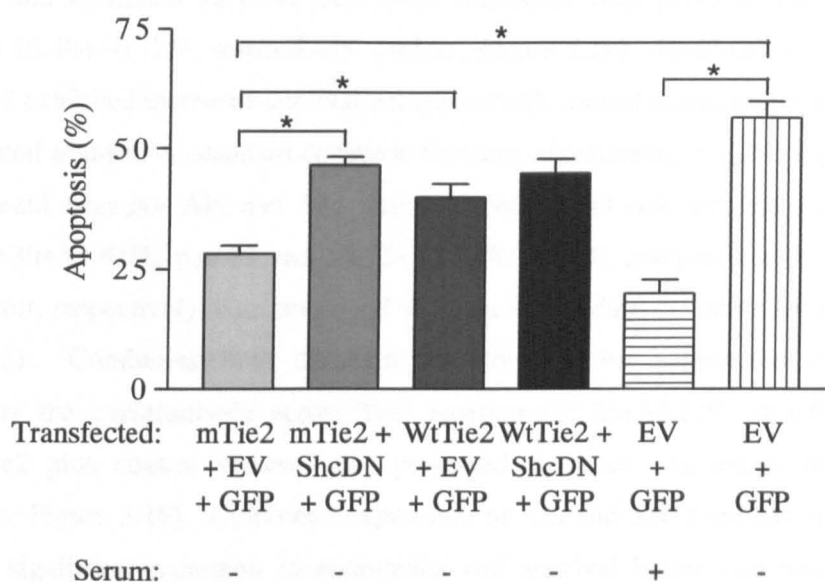
Figure 5.17 **Anti-apoptotic effects of R849W Tie2 are abrogated by dominant negative expression of Shc.** HUVE cells were co-transfected with GFP and R849W Tie2 (**mTie2**) or wild type Tie2 (**mTie2**) and ShcDN or PEBG, empty vector (**EV**) (Section 2.2.4.2). After 48 hours cells were subjected to serum starvation for 18 hours at 37°C/5% CO_2. Cells expressing GFP and empty vector were also maintained in serum conditions. Endothelial cells were fixed with 4% Paraformaldehyde solution and labelled with 0.2μg/ml of DAPI nuclear stain for 3 minutes at room temperature and assessed for apoptosis. Mutant Tie2 reduction of HUVE cell apoptosis is abrogated by the expression of a Shc dominant negative construct. The Shc dominant negative has no effect upon endothelial cells expressing wild type Tie2. Data presented as mean +/- standard deviation for three experiments (n=3). * $p \leq 0.05$ students 't' test.

Using the previously defined survival assay HUVE cells were seeded to 80-90% confluence in gridded six well plates and co-transfected with GFP and either dominant negative Akt or Shc in combination with the R849W Tie2 mutant. Furthermore, dominant negative Akt and Shc were co-transfected together along with GFP and mutant Tie2 to investigate the ability of the two proteins in abrogating endothelial cell survival. Cells were incubated for 48 hours at 37°C/5% CO_2 and GFP expressing cells were counted in 10 pre-defined fields. HUVE cells were serum starved for 18 hours and surviving cells counted. Maximum and minimum levels of endothelial cell survival were determined as before using serum maintenance and starvation over the period of 18 hours.

Maximum and minimum survival rates were consistent with previous experiments (64.93+/-6.56% and 15.40+/-0.72%, respectively, $p \leq 0.01$; Figure 5.18). Endothelial cells expressing the mutant Tie2 exhibited increased survival compared with control serum free cells (41.90+/-3.00%, data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$; Figure 5.18). Again, both dominant negative Akt and Shc inhibited endothelial cell survival induced by R849W mutant (20.30+/-1.81%, $p \leq 0.05$ and 18.73+/-1.85%, $p \leq 0.01$ compared with R849W Tie2 plus control vector, respectively, data presented as mean +/-standard deviation for three experiments; Figure 5.18). Combining both dominant negatives further suppressed the survival effect exhibited by the constitutively active Tie2 receptor (13.23+/-0.81%, $p \leq 0.01$ compared with R849W Tie2 plus control vectors, data presented as mean +/-standard deviation for three experiments; Figure 5.18). Combined expression of Akt and Shc dominant negative constructs revealed a significant reduction in endothelial cell survival below that seen with Akt alone ($p \leq 0.05$; Figure 5.18) but not Shc alone.

5.10 The expression of dominant negative Akt and Shc further abrogates R849W Tie2 anti-apoptotic effects

The anti-apoptotic effect of R849W Tie2 in the presence of the dominant negative mutants was investigated as described above. HUVE cells were treated in exactly same manner as in Section 5.9. Again, the relative levels of endothelial cell apoptosis with and without serum were consistent with previous experiments (28.33+/-7.64% and 66.67+/-2.89%, respectively, data presented as mean +/-standard deviation for three experiments $p \leq 0.01$; Figure 5.19). Mutant Tie2 rescued the apoptotic phenotype of the serum starved endothelial cells (40.00+/-5.00%, data presented as mean +/-standard deviation for three experiments $p \leq 0.05$; Figure 5.19).
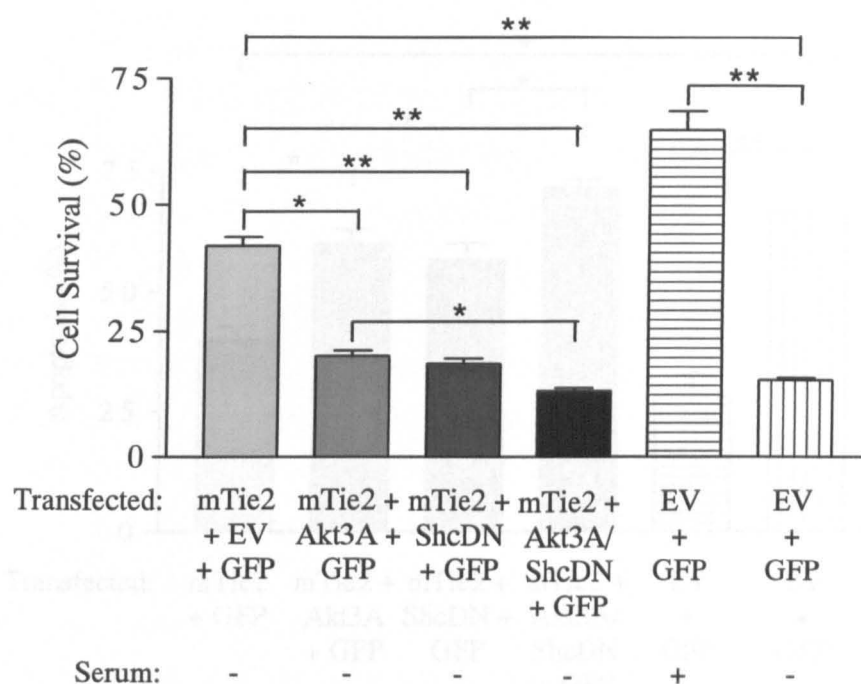
Figure 5.18 **Over-expression of both Akt and Shc dominant negative mutants further abrogates endothelial cell survival mediated by the expression of the R849W Tie2 receptor.** HUVE cells were co-transfected with GFP and R849W Tie2 (**mTie2**) or empty vector (**EV**) with Akt3A and/or ShcDN, where indicated (Section 2.2.4.2). After 48 hours cells emitting green light in UV/fluorescence conditions were counted in 10 predefined fields. Cells were serum starved for 18 hours and the surviving cells were counted. Cells co-transfected with GFP and empty vector were also maintained in serum throughout the duration of the experiment to act as an indicator of maximum survival and proof that serum free conditions mediated cell death. R849W Tie2 increases endothelial cell survival which was abrogated by expression of Akt and Shc dominant negative constructs. Expression of both Akt and Shc further reduces R849W mediated endothelial cell survival. Data presented as mean +/-standard deviation for three experiments (n=3). * $p \leq 0.05$; ** $p \leq 0.01$ students 't' test.
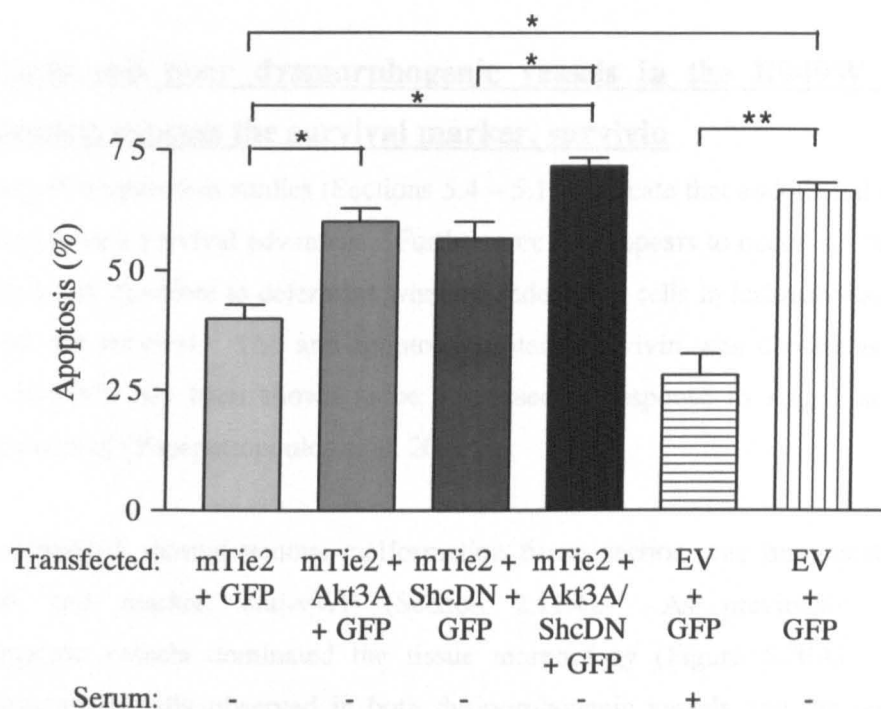
Figure 5.19 **R849W Tie2 ability to abrogate apoptosis is significantly reduced by the dual expression of Akt and Shc dominant negative mutants.** HUVE cells were co-transfected with GFP and R849W Tie2 or empty vector with Akt3A and/or ShcDN, where indicated (Section 2.2.4.2). After 48 hours cells were subjected to serum starvation for 18 hours at 37°C/5% CO$_2$. As in the endothelial cell survival assay, cells expressing GFP and empty vector were also maintained in serum. Endothelial cells were fixed with 4% Paraformaldehyde and labelled with 0.1μg/ml of DAPI nuclear stain for 3 minutes at room temperature and assessed for apoptosis. R849W Tie2 reduction of HUVE cell apoptosis is abrogated by the expression of an Akt dominant negative construct and Shc dominant negative, although not significantly. Expression of both dominant negative mutants further abrogates the effect of R849W Tie2. Data presented as mean +/-standard deviation for three experiments (n=3). * p≤0.05; ** p≤0.01 students 't' test.

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The dominant negative forms of Akt and Shc increased apoptosis in the R849W mutant expressing endothelial cells although dominant negative Shc did not reach statistical significance in this experiment series (60.00+/-5.00%, $p \leq 0.05$ and 56.67+/-5.77%, respectively, data presented as mean +/-standard deviation for three experiments; Figure 5.19). In the presence of both Akt and Shc mutants apoptosis in R849W Tie2 expressing cells increased to 71.67+/-2.89% of apoptotic cells (data presented as mean +/-standard deviation for three experiments $p \leq 0.01$; Figure 5.19). In contrast to the survival assay, the combined expression of Akt and Shc dominant negatives elevated the number of apoptotic endothelial cells compared to the endothelial cells expressing Shc ($p \leq 0.05$) but not Akt (Figure 5.19).

5.11 Mural cell poor dysmorphogenic vessels in the R849W Tie2 venous malformation express the survival marker, survivin

Data from cell transfection studies (Sections 5.4 – 5.10) indicate that endothelial cells expressing mutant Tie2 have a survival advantage. Furthermore this appears to occur via the Akt pathway. It was of interest therefore to determine whether endothelial cells in lesional vessels express any markers of cell survival. The anti-apoptotic protein, survivin was chosen as a pro-survival marker. Survivin has been shown to be expressed in response to Ang-1 activation of the PI3K/Akt pathway (Papapetropoulos et al. 2000).

Paraffin embedded, dermal venous malformation tissue section was immunostained with the endothelial cell marker, anti-vWF (Section 2.11.4). As previously described, the dysmorphogenic vessels dominated the tissue morphology (Figure 5.20A). Expression of survivin was specifically observed in both dysmorphogenic vessels and the relatively normal vessels (Figure 5.20B).

5.12 Expression of R849W mutant Tie2 does not effect mural cell migration

Vikkula et al. (1996) postulated that the generation of the dysmorphogenic vessels in the venous malformation was due to an inability of the vessels to recruit and interact with mural cells. The recruitment and adhesion of smooth muscle cells by endothelial cells expressing the mutant Tie2 was therefore investigated. To obtain meaningful data from these experiments it was necessary to use a population of endothelial cells all expressing mutant Tie2. Transient expression in HUVE cells produces a variable and relatively small subpopulation of transfected cells.
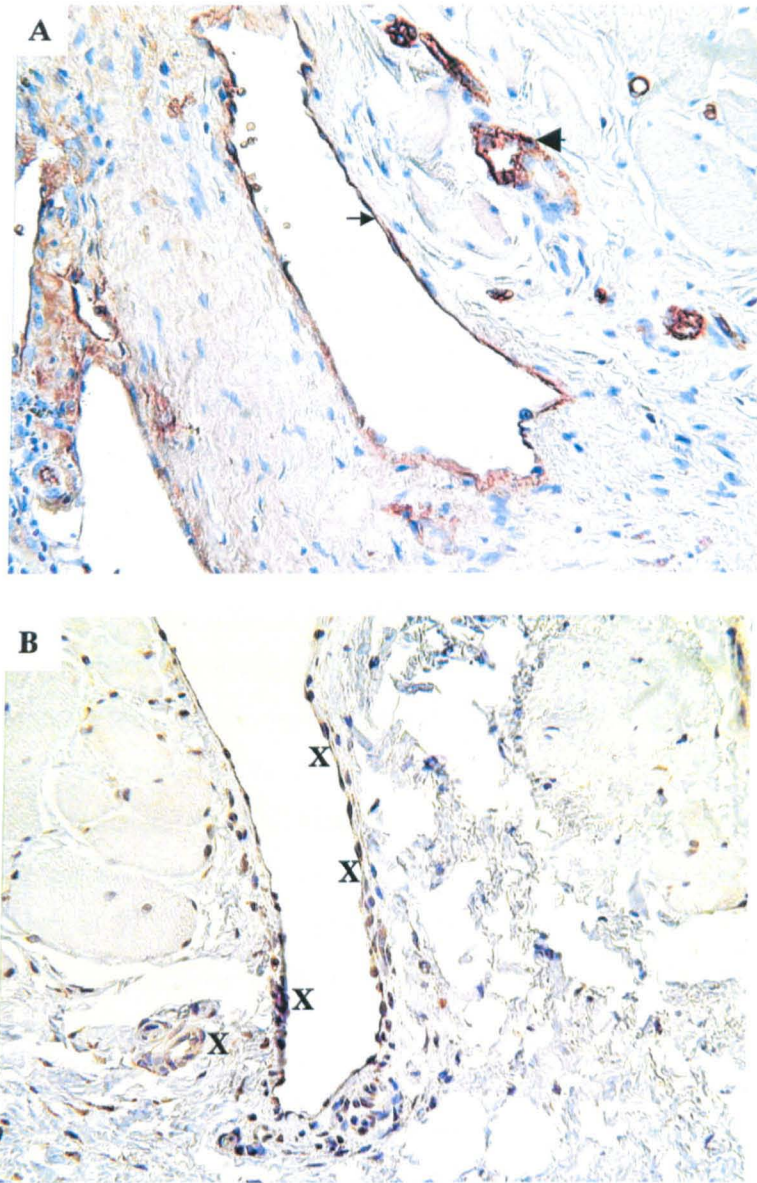
Figure 5.20 **Endothelial cells in the lesional vessels express survivin.** Dermal venous malformation tissue was fixed in formalin and embedded in paraffin. Sections were probed for endothelial cells with anti-vWF (**A**) and an antibody specific to survivin (**B**). **A**) Normal vessels (arrowheads) and dysmorphogenic vessels (arrows) both exhibit survivin expression indicated by **X** (**B**). Negative control for survivin staining can be found in Figure 5.1A inset. Original magnification x250.

In experiments on migration and adhesion, therefore, a stably transfected mixed population of BAE cells expressing mutant Tie2, wild type Tie2 or control vector were used.

Hyperphosphorylation of R849W Tie2 in these cell lines was confirmed by immunoprecipitation. 80cm^2 flasks of BAE cells expressing each construct were grown to confluence, serum starved for 2 hours and lysed as previously described (Section 2.6.3.1). Phosphorylated Tie2 proteins were immunoprecipitated using an anti-phosphotyrosine antibody (Section 2.6.3.2). Proteins were fractionated using 10% SDS-PAGE and tyrosine phosphorylated proteins were detected by immunoblotting with an anti-phosphotyrosine antibody (New England Biolabs). Levels of Tie2 receptor expression were confirmed using an anti-Tie2 antibody (R & D Systems). Figure 5.21 reveals an increase in tyrosine phosphorylation of both mutant and wild type Tie2. Mutant R849W Tie2 phosphorylation showed consistently higher phosphorylation levels as previously observed.

The ability of conditioned media from endothelial cells expressing wild type Tie2 or the R849W mutant form to attract vascular smooth muscle cells was investigated. Conditioned media from BAE cells expressing the mutant Tie2, full length wild type Tie2 as well as normal cells (control) was collected after 48 hours and plated into the lower chamber of a 24 well plate. Transwells were inserted into the wells and vascular smooth muscle cells were added to the insert (10^5 cells per well). Vascular smooth muscle cells were allowed to migrate towards the conditioned media for a period of 6 hours at 37°C/5% CO_2. Migration was stopped and inserts were fixed and the presence of vascular smooth muscle cells on the lower surface of the insert counted following staining with haematoxylin and eosin.

There was no significant difference in migration of VSM cells towards medium conditioned by control endothelial cells and endothelial cells over-expressing wild type or mutant R849W Tie2 (Figure 5.22).
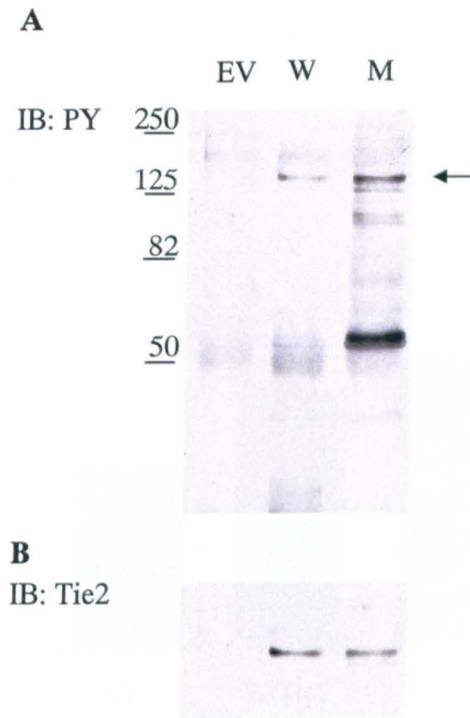
Figure 5.21 **Phosphorylation status of R849W Tie2 and wild type Tie2 in BAE cells.** BAE cells stably expressing R849W Tie2 (**M**), wild type Tie2 (**W**) or empty vector (**EV**) were grown to 100% confluence in 80cm^2 flasks and then serum starved for 2 hours. Cells were lysed and phosphorylated tyrosine proteins were immunoprecipitated using an anti-phosphotyrosine antibody (Section 2.6.3.2). Samples were fractionated by 10% SDS-PAGE. Tyrosine phosphorylated proteins (**A**) and the Tie2 receptor (**B**) were identified using specific human antibodies. R849W Tie2 and wild type Tie2 are phosphorylated. Endogenous levels of Tie2 in BAE cells are not recognised by the antibodies due to differing species. **IB** – immunoblot.

The migration assay was conducted to determine the effect of expressing R849W Tie2 on a vascular smooth muscle cell chemoattractants. Conditioned media was obtained from BAE cells expressing wild type Tie2, R849W Tie2 or empty vector.
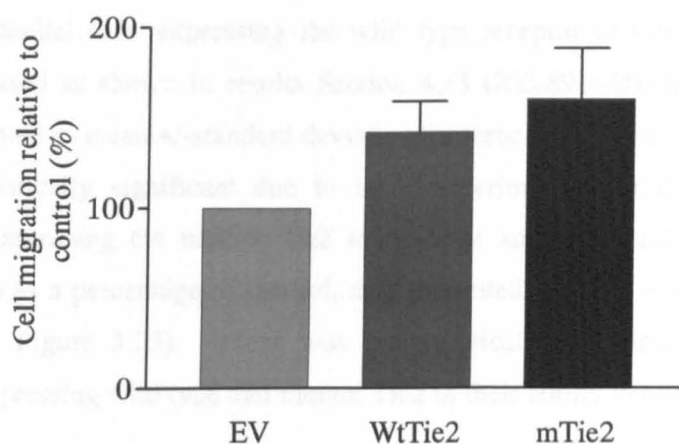
Figure 5.22 **Expression of R849W Tie2 does not affect production of smooth muscle cell chemoattractants compared with expression of wild type Tie2.** Conditioned media was obtained from BAE cells expressing wild type Tie2 (**WtTie2**), R849W Tie2 (**mTie2**) or empty vector (**EV**) over a 48 hour period. The media was plated into the lower chamber of a 24 well plate. Transwells were inserted into the 24 well plate and VSM cells were added (10^5 cells) and incubated at $37°C/5\%$ CO_2. After 6 hours the assay was stopped and inserts were fixed. The presence of vascular smooth muscle cells on the lower surface of the insert were counted following staining with haematoxylin and eosin. Both conditioned media from the wild type and mutant Tie2 expressing BAE cells showed no significant effect upon the migration of vascular smooth muscle cells compared to control cells. Data presented as mean +/-standard deviation for four experiments (n=4).

5.13 Expression of R849W mutant Tie2 does not effect endothelial cell mediated mural cell adhesion

An adhesion assay was conducted to determine the effects of expressing R849W Tie2 on adhesion of mural cells. A monolayer of VSM cells were grown to confluence and maintained overnight in complete media in a 96 well plate. BAE cells expressing wild type full length Tie2, mutant Tie2 or empty vector (control) were pre-labelled for 15 minutes with a fluorescent dye, 5µg/ml of Calcein AM, washed and brought into suspension prior to addition onto the monolayer of smooth muscle cells and incubated for 30 minutes 37°C/5% CO_2. Adherence was expressed as percentage of cells adhered of the total used in each well and then normalised to adhesion of control endothelial cells.

Adhesion of endothelial cells expressing the wild type receptor to smooth muscle cells was significantly increased as shown in results Section 4.13 (233.89+/-20.08% as a percentage of control, data presented as mean +/-standard deviation for three experiments, $p \leq 0.01$; Figure 5.23). Although not statistically significant due to large experimental variability the adhesion of endothelial cells expressing the mutant Tie2 to vascular smooth muscle cells was increased (312.83+/-107.28% as a percentage of control, data presented as mean +/-standard deviation for three experiments; Figure 5.23). There was no statistically significant difference between endothelial cells expressing wild type and mutant Tie2 in their ability to adhere to smooth muscle cells (Figure 5.23).

5.14 Effect of expression level on phosphorylation

Vikkula et al. (1996) suggest the expression level of R849W Tie2 in endothelial cells determines its activation state. They further hypothesise that the reason why dysmorphogenic vessels are focal in nature is that it is in these vessels mutant Tie2 has reached or exceeded a level of expression necessary for its constitutive activation (Vikkula et al. 1996). It was of interest therefore to determine whether Tie2 activation state depends on expression level. As a preliminary test of this HUVE cells were transfected with three different amounts of vector encoding R849W Tie2, 1, 0.5 and 0.25µg per well, and the phosphorylation status of expressed Tie2 determined.

BAE cells were transfected with a total of 1μg of DNA where WtTie2 DNA was transfected at concentrations of 0.25, 0.5 and 1μg. The concentration of DNA was made up to 1μg using empty vector. Cells were incubated for 36 hours prior to lysis (Section 2.6.2) as described in Section 2.6.2. Cells were lysed (Section 2.6.2) and samples were immunoblotted for pTie2.

Immunoblots were probed with an antibody specific to the phosphorylated tyrosine residue in the putative activation loop of the Tie2 receptor (Cell Signalling Technologies). Protein expression levels were determined by immunoblotting with an antiTie2 antibody (Santa Cruz).

Receptor phosphorylation are shown in Figure 5.24. Densitisation of the phospho-Tie2 relative at 3 different levels of expression revealed no significant trend in the increase in the phosphorylated Tie2 bands (Figure 5.25). To test more conclusively the importance of the phosphorylation, another WtTie2 expression level under the same conditions will be tested.

5.15 Effects of Ang-2 on R849W Tie2 phosphorylation

Therefore, it appears that an increase in R849W Tie2 receptor expression does not result in an increase in R849W Tie2 phosphorylation and activation, possibly because the formation of dysmorphogenic vessels was tested using the classic morphogenesis assay previously described. As previously indicated, Ang-2 the antagonistic ligand to Tie2 inhibits Ang-1 signalling, however, high concentrations of Ang-2 has been shown to induce the activation and weak activation of the PI3K/Akt pathway (Kim et al. 2000b). Preliminary experiments were undertaken to determine the role of Ang-2 as a possible mediator in the development and maintenance of the R849W Tie2 venous malformation.

Figure 5.23 **Endothelial cells expressing R849W Tie2 adhere to smooth muscle cells similarly to endothelial cells expressing wild type Tie2.** BAE cells over-expressing wild type Tie2 (**WtTie2**), R849W Tie2 (**mTie2**) or empty vector (**EV**) were fluorescently labelled with Calcein AM (5μg/ml), washed and brought to suspension prior to seeding onto a confluent monolayer of vascular smooth muscle cells and incubation for 15 minutes at 37°C/5% CO_2. Adhesion of the BAE cells are expressed as a percentage of the total amount of cells and normalised to control BAE cells. BAE cells over-expressing the Tie2 receptor adhered preferentially to vascular smooth muscle cells compared to normal BAE cells. Data presented as mean +/- standard deviation for three experiments (n=3). * p≤0.05 students 't' test.

HUVE cells were transfected with a total of 1μg of DNA where R849W Tie2 or wild type Tie2 concentrations consisted of 0.25, 0.5 and 1μg. The concentration of DNA was made up to 1μg using empty vector. Cells were incubated for 36 hours prior to serum starvation for 4 hours at 37°C/5% CO_2. Cells were lysed (Section 2.6.2) and samples were fractionated on 10% SDS-PAGE.

Immunoblots were probed with an antibody specific to the phosphorylated tyrosine residue at 992 in the putative activation loop of the Tie2 receptor (Cell Signaling Technology). Tie2 receptor expression levels were determined by immunoblotting with an anti-Tie2 antibody (R & D Systems).

Examples of immunoblots are shown in Figure 5.24. Quantitation of the phospho-Tie2/Tie2 ratio at 3 different levels of expression revealed no significant effect of expression level on phosphorylated Tie2 signal (Figure 5.25). To test more completely the dependence of tyrosine phosphorylation and R849W Tie2 expression level a wider range of expression will need to be tested.

5.15 Effects of Ang-2 on R849W Tie2 phosphorylation

Although, it appears that an increase in R849W Tie2 concentration does not modulate an increase in R849W Tie2 phosphorylation and activation. Another, possible mechanism for the mediation of dysmorphogenic vessels was tested using the chronic over-expression of an angiogenic growth factor. As previously indicated, Ang-2 the antagonistic ligand of Tie2 inhibits Ang-1 activation, however, high concentrations of Ang-2 has been shown to enhance endothelial cell survival by activation of the PI3K/Akt pathway (Kim et al. 2000b). Preliminary experiments were undertaken to determine the role of Ang-2 as a possible mediator of dysmorphogenic vessels in the R849W Tie2 venous malformation.

HUVE cells were transfected and treated in the same manner as Section 5.14 except prior to lysis the HUVE cells were pre-treated with Ang-2 (200ng/ml) or control (PBS) for 15 minutes at 37°C/5% CO_2. Tie2 phosphorylation was detected by immunoblotting with an anti-phospho-Tie2 antibody (Cell Signaling Technology). Relative levels of receptor was analysed using an anti-Tie2 antibody (R & D Systems).
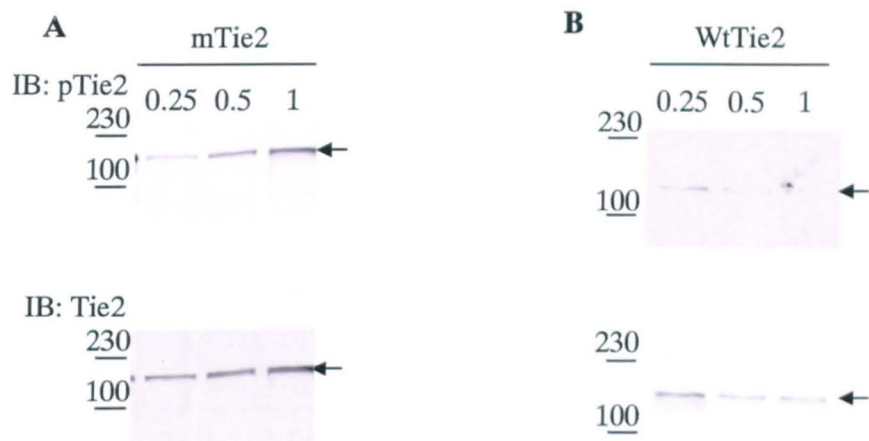
Figure 5.24 **Hyperphosphorylation of the R849W Tie2 mutant maybe concentration dependent.** HUVE cells were transfected with a total of 1μg of DNA where mutant Tie2 or wild type Tie2 consisted of 0.25, 0.5 and 1μg. The concentration of DNA was made up to 1μg using empty vector. Cells were incubated for 36 hours prior to serum starvation for 4 hours at 37°C/5% CO_2. Cells were lysed (Section 2.6.2) and samples were fractionated on 10% SDS-PAGE. **A)** After probing with phospho-Tie2 (992) and Tie2 it was noted that increased expression of R849W Tie2 mutant increased hyperphosphorylation. **B)** HUVE cells expressing wild type Tie2 probed with the phospho-Tie2 antibody revealed less receptor phosphorylation and phosphorylation did not correspond to increased Tie2 receptor levels. **IB-** immunoblot.
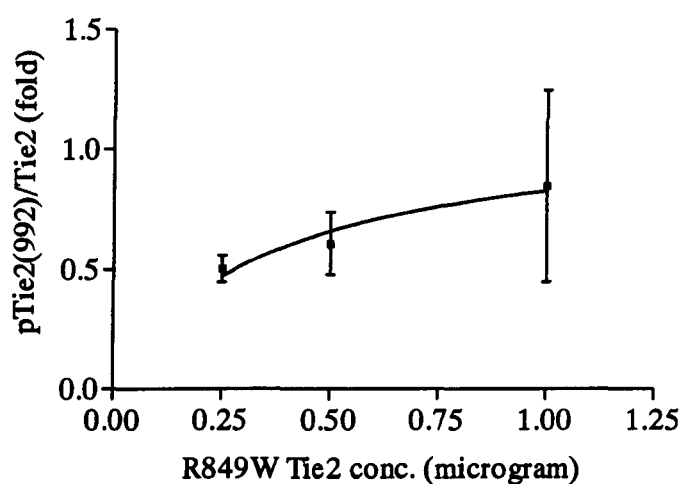
Figure 5.25 **Increased expression of R849W Tie2 does not effect receptor tyrosine phosphorylation.** Densitometry was used to determine the level of tyrosine phosphorylation in relation to increased mutant receptor expression. The phospho-Tie2/Tie2 ratio at 0.25, 0.5 and 1μg of receptor revealed no significant effect of expression level on phosphorylated Tie2 signal. Data presented as mean +/-standard deviation for three experiments (n=3).

Increased phosphorylation of tyrosine protein at position 992 of the Tie2 receptor was observed in the presence of Ang-2 for the mutant R849W Tie2 receptor (Figure 5.26A). A small increase was noted in endothelial cells expressing wild type Tie2 (Figure 5.26B). In order to determine the precise effect of Ang-2 upon Tie2 receptor activation further experiments would need to be performed.

5.16 Discussion

The aim of the studies described in this Chapter was to determine how the venous malformation mutant form of Tie2 could contribute to development of mural cell poor microvessels. Examination of microvessels within lesions taken from a patient with the R849W Tie2 mutant version of Tie2 confirmed the existence of mural cell poor vessels. These vessels were dysmorphogenic, again suggesting an association between lack of perivascular support cells and vessel dysmorphogenesis.

To gain insight into how the venous malformation mutant form of Tie2 could lead to formation of mural cell poor vessels the mutant receptor was expressed in endothelial cells consistent with reports in insect cells and non-endothelial cells (Vikkula et al. 1996) the mutant form of Tie2 exhibited increased levels of tyrosine phosphorylation compared with wild type receptor. The mechanism for this elevated constitutive activity is not known. It is possible that the substitution of an arginine 849 by tryptophan could increase hydrophobic interactions between receptor monomers leading to transphosphorylation and receptor hyperphosphorylation in the absence of the ligand. Indeed arginine 849 has been reported to lie at a dimer interface in the Tie2 structure published by Shewchuk et al. (2000).

Examination of the possible affect of R849W Tie2 on endothelial:mural cell adhesion revealed no significant suppression of adhesion between the cell types resulting from expression of mutant receptor. Indeed, endothelial cells expressing R849W Tie2 had increased adhesion. This is consistent with data reported in Chapter Four that indicated increased signalling by Tie2 can increase endothelial:smooth muscle cell adhesion. Endothelial cells expressing mutant Tie2 also did not appear to be compromised in their ability to secrete smooth muscle cell chemoattractants.
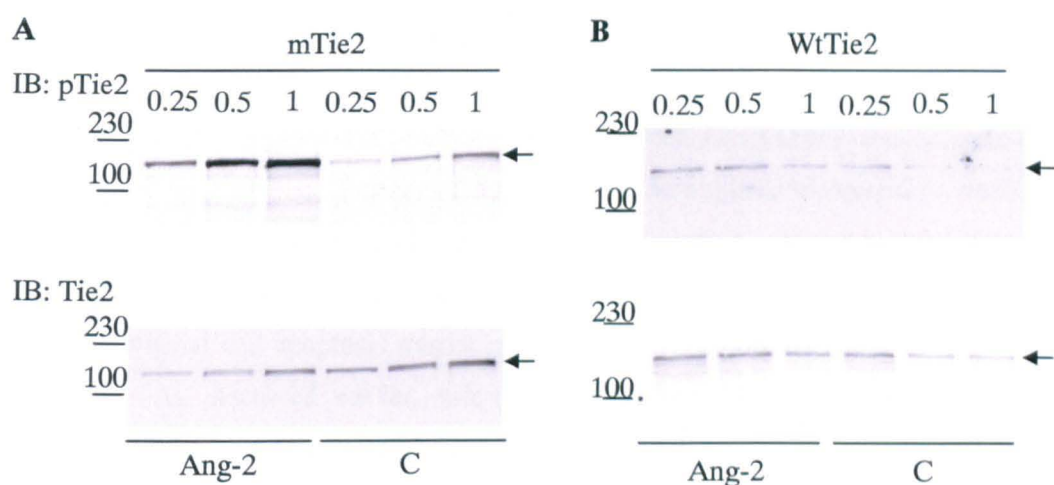
Figure 5.26 **The presence of Ang-2 increases activation of mutant R849W Tie2.** HUVE cells were transfected with a total of 1μg of DNA where mutant Tie2 or wild type Tie2 consisted of 0.25, 0.5 and 1μg. The concentration of DNA was made up to 1μg using empty vector. Cells were incubated for 36 hours prior to serum starvation for 4 hours at 37°C/5% CO_2. Prior to lysis cells were treated with Ang-2 (200ng/ml) or PBS for 15 minutes at 37°C/5% CO_2. Cells were lysed (Section 2.6.2) and samples were fractionated on 10% SDS-PAGE. **A)** Ang-2 substantially increased phosphorylation after immunoblot detection with phospho-Tie2 antibody. **B)** HUVE cells expressing wild type Tie2 showed a slight increase in tyrosine phosphorylation in the presence of Ang-2.

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Lack of suppression of endothelial:smooth muscle cell adhesion and chemoattractant production by expression of R849W Tie2 in endothelial cells suggests the mural cell poor vessels seen in venous malformations are not the result of defects in recruitment or adhesion between endothelial cells and mural cells. It should be noted, however, that the *in vivo* situation differs markedly from the cell culture assays performed in this study. It is possible that R849W Tie2 could inhibit recruitment and/or adhesion between endothelial cells and mural cells in the complex *in vivo* environment.

An important finding in this part of the study was that R849W Tie2 expression has a marked effect on endothelial cell survival. Cells expressing the mutant receptor were significantly protected from apoptosis and showed increased survival compared with control and wild type Tie2 over-expressing cells subjected to serum deprivation. This protective effect was mediated, at least partially by Akt. R849W Tie2-induced endothelial cell survival was also suppressed by a dominant negative form of Shc. Further experiments will be required to determine whether Shc or Akt act in the same pathway or parallel pathways, both of which are required for suppressing apoptosis in the conditions of these experiments. The finding that R849W Tie2 is a potent inhibitor of endothelial cell apoptosis maybe relevant to the presence of mural cell poor vessels found *in vivo*. As discussed earlier, microvessels lacking mural cells normally undergo regression unless maintained by survival factors. It is possible that in the venous malformation some of the mural cells poor vessels that arise during normal angiogenic responses escape vessel regression on withdrawal of initiating growth factors by virtue of the constitutive anti-apoptotic activity of R849W Tie2. Such vessels may expand and become dysmorphogenic due to the lack of regulatory influences normally secreted by mural cells. Indeed, immunohistochemical staining did show mural cell poor vessels in the venous malformation lesion to be expressing the pro-survival marker, survivin.

Chapter Six

Discussion

Chapter Six

Discussion

6.1 Conclusions and Discussion

Pericyte recruitment and interaction with the endothelium is important for the development of a patent vasculature. In this study three pathological situations, atherosclerotic plaque from the carotid artery, PMR treated myocardium and Tie2 mutant venous malformation, were examined for mural cell poor vessels. In each case the mural cell poor vessels were dysmorphogenic.

In the case of the atherosclerotic plaque a possible association between mural cell paucity, dysmorphogenesis and plaque instability was observed. These observations raise two important questions: (i) how is mural cell recruitment normally regulated? and (ii) how do mural cell poor vessels arise in the pathologies examined?

The Ang/Tie2 system has been implicated in mural cell recruitment but there is very little known about how this signalling pathway may regulate mural cell acquisition. To gain insight into mural cell recruitment a direct assay was established to examine adhesion between endothelial cells and smooth muscle cells/pericyte precursors. Migration of mural cells/pericyte precursors was also examined to study the involvement of the Ang/Tie2 system. It was discovered that Ang-1 stimulation increased mural cell adhesion to endothelial cells. Furthermore, integrins and N-cadherin were found to be involved in this intercellular adhesion. Ang-1 was also found to be an adhesive substrate for both endothelial cells and smooth muscle cells via the integrins. In addition, evidence is presented suggesting a positive signalling role for Tie2 in endothelial:mural cell adhesion. Over-expression of Tie2 in several different conditions resulted in increased adhesion to mural cells. Mechanistically Tie2 could increase the affinity of adhesion via N-cadherin and/or integrins associated with the intercellular interaction. However, further work would be required to determine the mechanism of Tie2 signalling and definitively test the hypothesis that Ang-1 is an intercellular adhesive substrate.

In the presence of endothelial cells, migration of mural cell precursors was greatly increased. Furthermore, Ang-1 increased endothelial cell mediated recruitment. These data correlates directly with the very recent report that Ang-1 enhances endothelial cell stimulated smooth muscle cell migration via up-regulation of endothelial cell-derived heparin binding EGF-like

growth factor (HB-EGF) (Iivanainen et al. 2003). It is possible that Ang-1 enhances migration of mural cell precursors in a similar manner.

Based on the lack of mural cell support in venous malformation vessels it was anticipated that the venous malformation mutant of Tie2 would interfere with mural cell adhesion and/or migration. This would allow a more in depth dissection of the functional role of Tie2 within mural cell recruitment and adhesion. Therefore, the functional effects of the venous malformation mutant Tie2 were examined using the adhesion and migration assays. Surprisingly, no inhibition of either adhesion or migration was found. However, over-expression of venous malformation mutant Tie2 in endothelial cells resulted in an anti-apoptotic phenotype.

Examination of the mechanism governing endothelial cell survival stimulated by venous malformation Tie2 was conducted focussing upon two signalling proteins, Akt and Shc. The mechanism of mutant Tie2 venous malformation mediated by endothelial cell survival appears to occur through the PI3K/Akt signalling pathway. Over-expression of dominant negative Akt abrogated the anti-apoptotic effects of mutant Tie2 in endothelial cells. Akt is associated with endothelial cell survival upon activation of the Tie2 receptor by Ang-1 (Kontos et al. 1998).

Abrogation of mutant Tie2 mediated endothelial cell survival was also achieved by the over-expression of dominant negative Shc. Recent research suggested an association between Shc-A and the cytoplasmic domain of Tie2 (Audero et al. 2003). The association and phosphorylation of Shc-A is increased by the presence of Ang-1. However, in the study expression of dominant negative Shc revealed no effect on endothelial survival (Audero et al. 2003). These data support my finding that mutant Shc had no effect on the survival of endothelial cells overexpressing wild type Tie2.

From the observations reported in this thesis a mechanism for the survival of mural cell poor vessels in venous malformation can be formulated. It is possible that constitutive phosphorylation of the mutant Tie2 receptor enhances activation of PI3K thus increasing the survival signal mediated by Akt. The survival signal pathway provided by Akt and Shc would require further investigation.

A common feature of atherosclerotic plaques, PMR treated myocardium and venous malformation vessels was the presence of anti-apoptotic stimuli for endothelial cells. In the

plaque and PMR this was found to be VEGF, whereas, in the venous malformation constitutively active Tie2 could provide an anti-apoptotic signal. These findings suggest in the three situations examined, mural cell poor vessels may be present because they escape regression mechanisms that normally remove these vessels. The reason why mural cell poor vessels initially arise was not determined; though it is thought that in an angiogenic response many mural cell poor neovessels arise initially and subsequently regress (Benjamin et al. 1998).

The association between lack of mural cells and vessel dysmorphogenesis seen in the three examples in this study suggests that acquisition of mural cells may be important in controlling normal vessel morphology. Supporting evidence includes the observations made by Abramsson et al. (2003) in the mouse fibrosarcoma model. In this model, tumours transplanted into mice deficient in the PDGF-B retention motif resulted in a reduction in pericytes correlating with an increase in vessel diameter and haemorrhage (Abramsson et al. 2003). These vessels showed similar phenotypes to the three pathological situations in this study. Furthermore, the tortuous tumour vessel with absent or loosely associated pericytes or vascular smooth muscle cells is often abnormal (Jain 2003). The requirement of pericytes is also evident in studies concerning diabetic retinopathy (Klinghoffer et al. 2001; Enge et al. 2002). An experimental model in murine neonates generated a vascular network where mural cell recruitment was blocked by antagonism of PDGFR-β. The mural cell poor vessels were dysmorphogenic, poorly remodelled and leaky consistent with previous observations. Therefore, mural cell acquisition would appear to be crucial for normal vessel development and morphology.

Mural cells could influence vessel morphology by (i) physical restriction on vessel expansion by mural cells, or (ii) restriction of vessel expansion by signals from mural cells such as TGF-β. As previously described, mice deficient in TGF-β resulted in mural cell poor vessels. Vessels in the pathological situation hereditary haemorrhagic telangiectasia are dysmorphogenic due to failed mural cell acquisition. This disorder is associated with mutations in the TGF-β receptor, Alk-1 (Oh et al. 2000).

Another possibility, is mural cell acquisition increases Tie2 signalling enhancing N-cadherin mediated endothelial:mural cell interaction. In fact, smooth muscle cell proliferation is increased by the dismantling of cadherin-mediated cell:cell contacts (Uglow et al. 2003). This research and

observations in this study suggests a role for N-cadherin in maintaining the mature vascular network.

6.2 Future Work

To determine the validity of Ang-1 as an intercellular adhesive substrate further work is required. Ang-1 was found to adhere to endothelial cells and mural cells in the present study, but its adhesiveness to other cell types was not tested. Integrins are expressed on a wide range of cell types and were implicated in adhesion to endothelial cells as shown in this study and other research (Carlson et al. 2001). The cell specificity of Ang-1 intercellular adhesion could be tested using CHO cells, a cell line previously found to be unaffected by Ang-1 in the smooth muscle cell adhesion assay. This experiment would confirm whether Ang-1 is able to bind cells or requires endothelial and mural cell specific characteristics.

The ability of Ang-1 to bind directly to immobilised extracellular domains of integrins and N-cadherin would elucidate the adhesion molecules involved in Ang-1 adherence. Recognition of Ang-1 binding to these adhesion molecules could be determined by ELISA using the FC domain or myc/His epitopes of Ang-1* and Ang-1, respectively.

Even if Ang-1 were confirmed to be an adhesive substrate in the interaction of endothelial cells and mural cells it could still activate Tie2 mediated signalling pathways and this may be also important in enhancing adhesion. To investigate this possibility the endothelial:mural cell adhesion assay including Ang-1 could be conducted with Tie2 kinase inhibitors. If adhesion were abrogated by blockade of Tie2 signalling it would support the possibility that part of the pro-adhesive effect of Tie2 involves activation of signalling pathways.

Furthermore, if Tie2-mediated signalling was found to contribute to intercellular pro-adhesive effects of Ang-1 further studies should be conducted with dominant negative signalling intermediates to define the precise signalling mechanisms involved.

It will be important to test the hypothesis, suggested by the present data, that mural cell poor vessels found in pathologies such as atherosclerotic plaque and venous malformation are sustained by survival signals. In the case of the atherosclerotic plaque it would be predicted that blockade of VEGF action may involve regression of mural cell poor vessels, failure to do so would indicate the possible involvement of additional pro-survival signals. In the case of venous

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malformation, specific inhibition of Tie2 pro-survival activity would be expected to lead to regression of venous malformation mural cell poor vessels. These experiments would require establishment of *in vivo* models, in the case of venous malformation possibly transgenic models.

Chapter Seven

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—lacZα ATG

M13 Reverse priming site

Hind III Kpn I Sac I BamH I Spe I BstX I EcoR I

CAGGAAACAG CTATGACCAT GATTACGCCA AGCTTGGTAC CGAGCTCGGA TCCACTAGTA ACGGCCGCCA GTGTGCTGGA
GTCCTTTGTC GATACTGGTA CTAATGCGGT TCGAACCATG GCTCGAGCCT AGGTGATCAT TGCCGGCGGT CACACGACCT

EcoR I EcoR V BstX I Not I Xho I Nsi I Xba I

ATTCGCCCTT [PCR product] AAGGGCGAAT TCTGCAGATA TCCATCACAC TGGCGGCCGC TCGAGCATGC ATCTAGAGGG
TAAGCGGGAA [PCR product] TTCCCGCTTA AGACGTCTAT AGGTAGTGTG ACCGCCGGCG AGCTCGTACG TAGATCTCCC

Apa I

T7 promoter/ priming site   M13 Forward (-20) priming site   M13 Forward (-40) priming site

CCCAATTCGC CCTATAGTGA GTCGTATTAC AATTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA AC
GGGTTAAGCG GGATATCACT CAGCATAATG TTAAGTGACC GGCAGCAAAA TGTTGCAGCA CTGACCCTTT TG

Appendix 1 **Multiple cloning region of the pCR2.1 vector from Invitrogen.**

P_{CMV} →

T7 promoter/priming site

                                     Hind III   Kpn I   BamH I

ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGT TAA GCT TGG TAC CGA GCT CGG ATC CAC TAG TCC
                                                               *** Ala Trp Tyr Arg Ala Arg Ile His *** Ser

BstX I EcoR I   EcoR V   BstX I Not I   Xho I Xba I   Apa I Sac I

AGT GTG GTG GAA TTC TGC AGA TAT CCA GCA CAG TGG CGG CCG CTC GAG TCT AGA GGG CCC TTC GAA
Ser Val Val Glu Phe Cys Arg Tyr Pro Ala Gln Trp Arg Pro Leu Glu Ser Arg Gly Pro Phe Glu

c-myc epitope   Age I   Polyhistidine (6xHis) region

CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT ATG CAT ACC GGT CAT CAT CAC CAT CAC CAT TGA
Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Met His Thr Gly His His His His His His ***

Pme I   pcDNA3.1/BGH Reverse priming site
GTTTAAACCC GCTGATCAGC CTCGACTGTG CCTTCTAG

Appendix 2 **Multiple cloning region of the pcDNA3.1 myc/His A vector from Invitrogen.**

Translational Initiation

Hind III   Not I   EcoR I   Cla I

A CC ATG GAC TAC AAA GAC GAT GAC GAC AAG C TTG CGG CCG CGA ATT CAT
    Met Asp Tyr Lys Asp Asp Asp Asp Lys>

Multiple Cloning Region

Bgl I   EcoR V Kpn I   Sal I   Xba I   BamH I   Sma I

CGA TAG ATC TGA TAT CGG TAC CAG TCG ACT CTA GAG GAT CCC GG G

Multiple Cloning Region
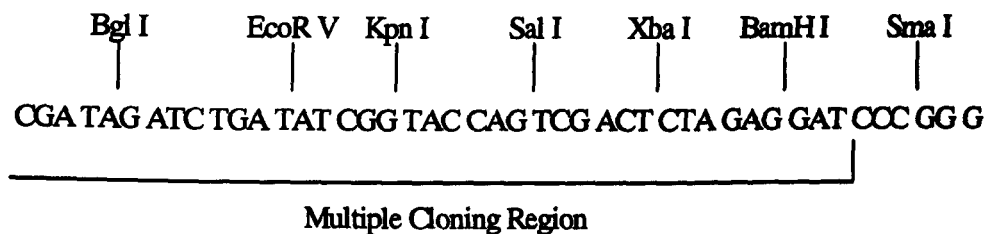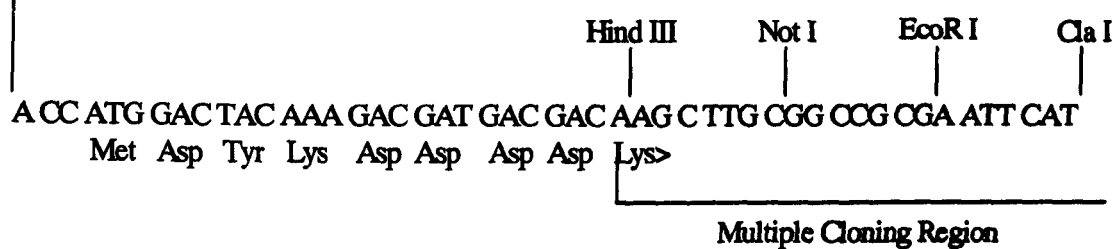
Appendix 3 **Multiple cloning region of the pFlag vector from Sigma.**

```
GTGTCCTTTC      AGAAAGCAAG      CAAACTGGAN      CTGATGACNC
AGTCCACACC      CTTGTCAATC      TTGCAACTAA      GAAGGTGTTN
TACTAAAGGA      GGAAAAGAGA      GGAAGAGAAA      CCATTTAGTG
ACTGTGCAGA      TGTATATCAA      GCTGGTTTTA      ATAAAAGTGG
AATCTACACA      TTTTATATTA      ATAATATGCC      AGAACCCAAA
AAGGTGTTTT      GCAATATGGA      TGTCAATGGG      GGAGGTTGGA
CTGTAATACA      ACATCGTGAA      GATGGAAGTC      TAGATTTCCA
AAGAGGCTGG      AAGGAATATA      AAATGGGTTT      TGGAAATCCC
TCCGGTGAAT      ATTGGCTGGG      GAATGAGTTT      ATTTTTGCCA
TTACCAGTCA      GAGGCAGTAC      ATGCTAAGAA      TTGAGTTAAT
GGACTGGGAA      GGGAACCGAG      CCTATTCACA      GTATGACAGA
TTCCACATAG      GAAATGAAAA      GCAAAACTAT      AGGTTGTATT
TAAAAGGTCA      CACTGGGACA      GCAGGAAAAC      AGAGCAGCCT
GATCTTACAC      GGTGCTGATT      TCAGCAACTA      AAGATGCTGA
TAATGACAAC      TGTATGTGCA      AATGTGCCCT      CATGTTAACA
GGAGGATGGT      GGTTTGATGC      TTGTGGCCCC      TCCAATCTAA
ATGGAATGTT      CTATACTGCG      GGACAAAACC      ATGGAAAACT
GAATGGGATA      AAGTGGCACT      ACTTCAAAGG      GCCCAGTTAC
TCCTTACGTT      CCACAACTAT      GATGATTCGA      CCTCTCGAGTC
TAGAGGGCCC      TTCGAACAAA      AACTCATCTC      AGCCGAGGAT
CTGAATATGC      ATACCGGTCA      TCATCACCAT      CACCATTGAG
TTTAAACCCG      CTGATCAG
```

Appendix 4 **Confirmation of introduction of XhoI restriction site in Angiopietin-1 sequence.** Wizard prep DNA (refer to section 2.7.3) of the mutated Ang-1 sequence, where a *XhoI* was inserted into the Ang-1 during subcloning experiments (refer to section 2.8), was confirmed by sequencing from the pcDNA3.1/BGH Reverse priming site in pcDNA3.1 myc/HisA vector by Cambridge BioScience Limited, Cambridge.

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Last but not least my fiancée Becky, words cannot describe the love, support, laughter and comfort you have brought me. I just hope I can be as brilliant throughout your PhD as you have been throughout mine. I love you very much.