REGULATION AND PHYSIOLOGICAL ROLE OF THE PROTEOLYTIC CLEAVAGE OF THE ENDOTHELIAL RECEPTOR TYROSINE KINASE TIE-1 IN VESSEL DESTABILISATION PRIOR TO ANGIOGENESIS

by

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The work on which this thesis is based is my own independent work except where acknowledged.

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University of Leicester

Abstract

REGULATION AND PHYSIOLOGICAL ROLE OF THE PROTEOLYTIC CLEAVAGE OF THE ENDOTHELIAL RECEPTOR TYROSINE KINASE TIE-1 IN VESSEL DESTABILISATION PRIOR TO ANGIOGENESIS

By Achilleas C Tsiamis MRCS

Angiogenesis is defined as the development of new blood vessels. It is predominantly observed during embryonic development but it plays central role in a wide variety of pathological conditions including tumour growth, invasion and metastasis, ischaemic disease and chronic inflammation. It has been recently shown that a group of growth factors including VEGF, bFGF and the angiopoietins and their relative receptors work synergistically leading to new vessel growth and maturation.

The receptor tyrosine kinase Tie-1 is a novel endothelial cell membrane receptor, with still unknown ligand, which seems to play an important role in vessel maturation.

In our study, we show that VEGF regulates the proteolytic cleavage of the Tie-1 extracellular domain resulting to the release of an extracellular fragment and the production of an intracellular fragment with potential downstream signalling functions. This is mediated by a metalloprotease, involving phosphorylation of tyrosine kinases. Nitric oxide, thrombin, angiopoietin II, Bradykinin and bFGF were not found to regulate proteolytic cleavage of the Tie-1 receptor's extracellular domain.

Cell survival and cell proliferation experiments confirmed that the Tie-1 ectodomain cleavage leads to enhanced endothelial cell survival and proliferation.

In order to examine the physiological role of Tie-1 cleavage we attempted to specifically inhibit truncation by synthesising a peptide corresponding to the truncation site and raising a monoclonal antibody against it.

The in-vivo angiogenesis model of the chick chorioallantoic membrane (CAM) model was used.

Tie-1 was identified in the developing CAM existing in both its truncated and full-length form. Treatment of the mature CAM with VEGF results to ectodomain cleavage of Tie-1. Treatment of the CAM with monoclonal

antibodies recognising the extracellular epitope of the receptor results to interstitial oedema suggesting a role for the receptor in vessel stabilisation.

Tie-1 was also identified in platelets where it is present in a different, non-cleavable isoform suggesting that proteolytic cleavage of the Tie-1 receptor's extracellular domain is endothelial cell specific phenomenon.

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ACRONYMS

Ang 1-4: Angiopoietins –1 to 4

CAM: Chorioallantoic Membrane

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl Sulphoxide

FCS: Foetal Calf Serum

EGF: Epidermal Growth Factor

FACS: Fluorescence-activated flow cytometry.

FGF: Fibroblast Growth Factor

HUVEC: Human Umbilical Vein Cell

NO: Nitric Oxide

PAGE: Polyacrylamide Gel Electrophoresis

PBS: Phosphate Buffered Saline

PMA: Phorbol Myristate Acetate

RTK: Receptor Tyrosine Kinase

SDS: Sodium Dodecyl Sulphate

TIE-1: Tyrosine kinase with Immunoglobulin-like and epidermal factor homology domains receptor

TNF: Tumour Necrosis Factor

VEGF: Vascular Endothelial Growth Factor / Vascular Permeability Factor

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CHAPTER 1: INTRODUCTION

1.1 About angiogenesis

The establishment and maintenance of a vascular supply is an absolute requirement for the growth of normal and neoplastic tissues. The cardiovascular system is the first organ system to develop and become functional during embryogenesis.

Two distinct cellular processes have been observed to mediate blood vessel formation during avian and mammalian embryogenesis. The first is *vasculogenesis*, which is defined as the primary in situ differentiation of endothelial cells from mesodermally derived precursors, and their assembly into vascular channels. The larger vascular structures of the embryo, including the heart endocardium and major blood vessels arise by this process. The second is *angiogenesis*, which describes the formation of new blood vessels by a process of sprouting from pre-existing ones or by intussusceptive vascular growth (IMG). [1] [2]

After the developing embryo has formed a primary vascular plexus by vasculogenesis, further blood vessels are generated by both sprouting and non-sprouting angiogenesis, which are progressively pruned and remodelled into a functional adult circulatory system. Recent results, particularly from the study of mice lacking some of the signalling systems involved, have greatly improved our understanding of the molecular basis underlying these events, and may suggest new approaches for treating conditions such as cancer that depend on angiogenesis. [3, 4]

The classic light-microscopic observations of Clark and Clark in transparent tails of living amphibian larvae were among the first to describe the sequence of events leading to the formation of new capillaries. These and subsequent observations have provided a detailed histological account of new blood vessel formation, which can be summarized as follows:

In response to a local angiogenic stimulus, endothelial cells of pre-existing capillaries or postcapillary venules become "activated" followed by local vasodilatation, increased vascular permeability, and the accumulation of extravascular fibrin, as well as proteolytic degradation of the basement membrane of the parent vessel. Cytoplasmic processes are then extended from the activated endothelial cells, and directed endothelial cell migration occurs into the surrounding matrix toward the angiogenic stimulus. Migrating endothelial cells elongate and align with one another to form a capillary sprout, followed by endothelial cell division, occurring proximal to the migrating tip, resulting to further increase of the sprout length. The solid sprout gradually develops a lumen proximal to the region of proliferation. The neighbouring tubular sprouts anastomose at their tips to form a functional capillary loop in which blood flow is soon established.

Intussusception causes growth of the vascular network by insertion of columns of interstitial tissue inside the vessel lumen and its subsequent partitioning. It occurs during continuous vessel perfusion and its cellular mechanisms can even be influenced by the perfusion pattern. Intussusception also contributes the leading mechanism of vascular network remodelling, which follows network formation and growth and transforms the uniform primary vascular plexus into a secondary one, which is more complex in structure.[4-6]

Vessel maturation is accomplished by reconstitution of the basement membrane and reinforcement of the vessel wall by pericytes.[7] Together, these cellular functions contribute to the process of capillary morphogenesis.

From a developmental point of view, it has been suggested that endothelial cells arise either from *angioblasts* (which differentiate exclusively into endothelial cells) or from *haemangioblasts* (which have the dual capacity to differentiate into either endothelial or haematopoietic cells). However, although the existence of the angioblast has been well established, definitive proof for the existence of the haemangioblast is still unavailable. It is currently assumed that primary endothelial cell differentiation is strictly limited to vasculogenesis. Furthermore, it seems that vasculogenesis is limited to early embryogenesis while angiogenesis occurs both during development and in postnatal life. However, the possibility that endothelial "stem cells" may persist into adult life, contributing to the formation of new blood vessels, has recently been suggested by work in a number of laboratories.[8-10]

In addition to its role during development, angiogenesis is required for the functional and structural integrity of the organism during postnatal life. It is observed during wound healing; in inflammation; in situations of ischaemia; and in female reproductive organs: in the ovary before ovulation and during the formation of the corpus luteum; in the phase or repair of the menstruating uterus and in the placenta and the mammary glands during pregnancy. [11] [12]

In all these situations, the neovascularisation is tightly regulated by the metabolic demands of the tissues concerned.

Angiogenesis also occurs in pathological conditions where excessive or abnormal growth of microvessels predominate, such as: ocular neovascularisation; psoriasis rheumatoid

arthritis as an important constituent of the inflammatory pannus that destroys articular cartilage; hemangiomas and vascular malformations; solid tumour growth, invasion and metastasis

Much of our interest in angiogenesis comes from the notion that in order for tumours to grow beyond a critical size, they must recruit endothelial cells to form their own endogenous microcirculation. This process is driven by the metabolic requirements of the rapidly growing tumour itself. Therefore, during tumour progression, two phases can be recognized: a *prevascular* and a *vascular* phase. The transition from the prevascular to the vascular phase is referred to as the *"angiogenic switch"*. The prevascular phase is characterized by an initial increase in tumour growth followed by a plateau, during which the rate of tumour cell proliferation reaches equilibrium with their rate of death. This phase may persist for many years and is clinically known as *carcinoma in situ*, characterized by no invasion and no metastases. In this phase, the tumour is rarely larger than 2 to 3 mm^3 and may contain a million or more cells. The vascular phase is characterized by exponential growth, tissue invasion, haematogenous spread of tumour cells and the development of solid metastases. During that phase, the rapid increase in tumour growth is largely due to a decrease in the rate of tumour cell apoptosis. An inverse relationship thus exists between tumour dormancy/tumour cell apoptosis and tumour angiogenesis. In a sense, tumour angiogenesis might almost be considered "appropriate," in that newly formed vessels serve to meet the metabolic demands of the rapidly growing tumour. Although angiogenesis may be beneficial to the tumour itself, it is clearly detrimental to the organism, since it is permissive for continued tumour growth allowing for the dissemination of tumour cells and the formation of metastasis. [13, 14]

1.2 Molecular regulators of angiogenesis

It is accepted that adult endothelium is normally quiescent with the exception of angiogenesis, occurring in response to tissue injury or in the female reproductive organs. The maintenance of endothelial quiescence is thought to be due to the presence of endogenous negative regulators, since positive regulators are detected in adult tissues in which no active angiogenesis is occurring. However, positive and negative regulators often coexist in tissues in which endothelial cell turnover is increased. This has led to the hypothesis that the balance between positive and negative molecular regulators determines endothelial activation status. The predominance of positive regulators results in activated (angiogenic) endothelium, whereas endothelial quiescence is achieved and maintained by the increased activity of negative regulators. [15]

The idea of the angiogenic switch, initially used to describe the transition of tumour growth from the *in situ* to the angiogenic phase, is also applicable to developmental, physiological as well as pathological angiogenesis. The switch to the angiogenic phenotype involves a change in the local equilibrium between positive and negative regulators of the growth of microvessels.

The multiple cell functions that occur during angiogenesis can be classified into an early and a late stage. The early stage is that of endothelial cell activation (which encompasses initiation and progression) and the late stage phase of resolution (which encompasses termination, vessel maturation or regression). While a great deal is known about the factors that induce the activation phase, very little is known about the factors involved in the phase of resolution, in which's is mediated in part by the autocrine activity of endogenous regulators. [16-18]

Regarding activated endothelium, an important distinction must be made between physiological and pathological settings: although many of the same positive and negative regulators are operative in both, endothelial cell proliferation in the former is tightly regulated, whereas in the latter, uncontrolled angiogenesis implies continuous predominance of positive regulators, which results in uncontrolled endothelial cell growth.

Among the factors that affect endothelial cell activation status, either positively or negatively, are cytokines. On the basis of the observation that a given tissue can profoundly influence the way in which its cellular components respond to a given cytokine, it has been suggested that cytokines should be seen as "specialized symbols in a language of intercellular communication, whose meaning is controlled by context." Context is determined by (at least) three parameters:

- The concentration of other cytokines in the pericellular environment of the responding cell;
- Interactions between cells, cytokines, and the extracellular matrix; and
- The configuration of the cell cytoskeleton.

As far as angiogenesis is concerned, the ideas of both the angiogenic switch and tissue context are central to the understanding of the molecular mechanisms that govern this process.

Several potential regulators of angiogenesis have been identified, including acidic and basic fibroblast growth factors, epidermal growth factor, platelet-derived endothelial cell growth factor, transforming growth factors α and β, and tumour necrosis factor α (TNF-alpha). Vascular endothelial growth factor (VEGF) is unique among these agents by virtue of its direct and specific mitogenic effects on endothelial cells combined with the fact that it is a secreted polypeptide.[19] [20]

Vascular endothelial growth factor (VEGF) and its receptors, Flt-1 (VEGFR-1) and Flk-1 (VEGFR-2), as well as Angiopoietins-1 and 2 and their receptor, Tie-2, represent key signal transduction systems involved in the regulation of embryonic vascular development[21-23]. The expression of these molecules correlates with phases of blood vessel formation during embryogenesis. Inactivation of any of the genes encoding these molecules in mouse embryos results in defective vascular development and embryonic lethality. The cytokines that have been the most extensively studied in the context of angiogenesis are Vascular Endothelial Growth Factor, acidic Fibroblast Growth Factors (aFGF), and basic Fibroblast Growth Factor (bFGF).

Vascular Endothelial Growth Factor (VEGF) is a multifunctional cytokine expressed and secreted at high levels by many tumour cells of animal and human origin. As secreted by tumour cells, VEGF is a 34-42 kDa heparin-binding, dimeric, disulfide- bonded glycoprotein that acts directly on endothelial cells by way of specific receptors. Three high affinity VEGF receptors, all tyrosine kinases, have thus far been described. VEGF is likely to have a number of important roles in tumour biology related, but not limited to, the process of tumour angiogenesis. In addition, the VEGF signal transduction system has been implicated in the regulation of pathological blood vessel growth during certain angiogenesis-dependent diseases that are often associated with tissue ischaemia, such as proliferate retinopathy. This hypothesis is substantiated by experiments, in which the inhibition of VEGF signal transduction resulted in the inhibition of neovascularisation in these diseases. Thus, the VEGF signal transduction system represents a useful target for an anti-angiogenic therapy.[19]

As a potent permeability factor, VEGF promotes extravasation of plasma fibrinogen, leading to fibrin deposition that alters the tumour extracellular matrix. This matrix promotes the ingrowth of macrophages, fibroblasts, and endothelial cells. Moreover,

VEGF is a selective endothelial cell growth factor in vitro, and it stimulates endothelial cell proliferation in vivo. Furthermore, VEGF has been found in animal and human tumour effusions by immunoassay and by functional assays and very likely accounts for the induction of malignant ascites. In addition to its role in tumours, VEGF has recently been found to have a role in wound healing and its expression by activated macrophages suggests that it probably also participates in certain types of chronic inflammation. VEGF is expressed in normal development and in certain normal adult organs, notably kidney, heart, adrenal gland and lung. Its functions in normal adult tissues are under investigation.[24]

The finding that in vitro VEGF and FGF positively regulate many endothelial cell functions, including proliferation, migration, extracellular proteolytic activity, and tube formation, has led to the characterisation of these factors as direct-acting positive regulators of angiogenesis. However, although the role for VEGF in developmental, pathological and tumour angiogenesis has been clearly established, the role of other growth factors such as the FGFs alone or in combination with VEGF remains to be clearly defined.

The ultimate target for both positive and negative regulators is the endothelial cell. This has led to the hypothesis that angiogenesis regulators may act either directly on endothelial cells or indirectly by regulating the production of direct-acting regulators by non-endothelial cells. Thus, in contrast to VEGF and FGF (which are direct endothelial cell mitogens), Transforming Growth Factor -ß and Tumour Necrosis Factor-α inhibit endothelial cell growth in vitro and have therefore been considered as direct-acting negative regulators. Nevertheless, both TGF-ß and TNF are shown to be angiogenic *in vivo*. It has been demonstrated that these cytokines induce angiogenesis indirectly by stimulating the production of direct-acting positive regulators from stromal and inflammatory cells. In this

context, then, TGF-ß and TNF are considered indirect positive regulators. In view of its capacity to directly inhibit endothelial cell proliferation and migration, reduce extracellular proteolysis, and promote matrix deposition in vitro, TGF-ß has also been proposed to be a potential mediator of the later stage of angiogenesis leading to vessel maturation. In vitro, TGF-ß also promotes the organization of single endothelial cells embedded in three-dimensional collagen gels into tubelike structures, a phenomenon that is likely to be representative of the latter stages of angiogenesis.

Other cytokines that have been reported to regulate angiogenesis in vivo include: HGF, EGF/TGF, PDGF-BB, Interleukins (IL-1, IL-6, and IL-12), Interferons, GM-CSF, PlGF, Proliferin and proliferin-related protein.

Chemokines that regulate angiogenesis in vivo have to date only been identified in the -C-X-C- family such as IL-8 and platelet factor IV.

Angiogenesis can also be regulated by a variety of noncytokine or nonchemokine factors, including: Enzymes (angiogenin and PD-ECGF/TP), Inhibitors of matrix-degrading proteolytic enzymes (TIMPs) and of PAs (PAIs), extracellular matrix components/coagulation factors or fragments of (thrombospondin, angiostatin, hyaluronan, and its oligosaccharides), Soluble cytokine receptors, Prostaglandins, Adipocyte lipids, and Copper ions.

1.3 The role of endothelial cell receptor tyrosine kinases in angiogenesis

Tyrosine kinases, first described as oncogenes, have been shown to play a role in normal cellular processes. Signal transduction by receptor and cytoplasmic tyrosine kinases is a process of critical importance in cellular proliferation and differentiation. Phosphorylation on tyrosine of cellular proteins has been detected in vivo using anti-phosphotyrosine antibodies. Genetic and biochemical studies in this large family of proteins have shown that different receptor tyrosine kinases (RTKs) are responsible for transducing important developmental, proliferative, cell survival and migratory signals from the outside to the inside of the cell using a complex combination of protein systems. (Table 1) [25-27].

Categories	Examples
Enzymatic proteins	Phosphatydil inositol 3-kinase (PI3K)[25]8] Phospholipase Cγ (PLCγ) Src family kinases Tyrosine phosphatase SHP-2[26]9]
Non enzymatic adaptor proteins	Grb-2 [25]8] Shc Nck
Cytoskeletal proteins	Tensin
Transcription factors	STATs

Table 1: Examples of Proteins participating in intracellular signalling of RTKs

Distinct receptors for vascular endothelial growth factor, and other receptor tyrosine kinases, appear to regulate very different aspects of early vessel formation including endothelial cell differentiation, tube formation and differentiation of blood vessels into microvasculature versus large vessels. [3, 30-35] In later development and in the adult circulation, remodelling adapts arteries to chronic changes in haemodynamic function.[28, 29]

Receptor	Ligands	Signal transduction proteins	Physiological role
VEGFR-1 (Flt-1)	VEGFA VEGFB PIGF	PLCγ PI3K SHP2 SAPK	Ligand control molecule Monocyte migration PIGF stimulated trophoblast survival
VEGFR-2 (Flk-1/KDR)	VEGFA VEGFC VEGFD	Grb2 Shc, Nck, SHP-2 FAK, Pyk2 PI3K Ras/Raf/Mek/ p42/44 MAPK pathway P38 MAPK PLCγ/PKC pathway	Haematopoietic cell differentiation Endothelial cell differentiation Endothelial cell proliferation Endothelial cell survival

Table 2: Receptor Tyrosine kinases in vascular development

Receptor	Ligand	Physiological roles
VEGFR-3 (Flt-4)	VEGFC VEGFD	Lymphatic endothelium growth
PDGFRα	PDGF AA, AB, AB, BB	Vessel stabilisation?
PBGFRβ	PDGFB	VSMC recruitment and migration? Vessel formation and maintenance
Tie-1[22, 36, 37]		Capillary sprouting [36, 38] Haemodynamic stress resistance Fluid exchange across capillaries[39]
Tie-2/Tek[22]	Ang1, 2,3,4 [40-43]	Recruitment and interaction with per endothelial support cells[39, 44, 45]

Table 2 : Receptor Tyrosine kinases in vascular development

1.4 Cell–Extracellular Matrix Interactions: Integrins and Proteases

The coordinate induction of protease activities and cell migration is a principal feature of endothelial cells invading the interstitial space in the initial step of angiogenesis. However, the molecular mechanisms of these events are not fully characterized.[46]

Alterations in at least four endothelial cell functions occur during angiogenesis:

1. Increased proliferation, which provides new cells for the growing and elongating vessel, with a subsequent return to the quiescent state once the new vessel is formed;
2. Initial increase and subsequent decrease in migration, which allows the cells to migrate toward the angiogenic stimulus and stop when they reach their destination;
3. Endothelial cell-to-cell interactions; and
4. Interactions of endothelial cells with the extracellular matrix.

The extracellular matrix is an intricate and complex network of protein fibres and other macromolecules that influences cellular function and tissue architecture. During capillary formation, endothelial cell migration and organization are critically dependent on surrounding basement membrane proteins. These proteins serve as a physical support and are likely to provide signals, which regulate migration and organization of the cells. Reconstituted basement membrane (Madrigel) activates tyrosine phosphorylation of several proteins including focal adhesion kinase. Inhibition of this pathway with tyrosine kinase inhibitors impairs localization of paxillin to focal adhesions and organization of actin filaments, decreases motility and elongation of endothelial cells, and prevents their organization into cords or tubes on basement membrane. These data demonstrate that basement membrane-induced modulation of endothelial cell motility, shape, and organization is critically dependent on tyrosine kinase signalling pathways involving cytoskeletal proteins.[47]

Among the molecules that are relevant to cell–extracellular matrix interactions are integral membrane proteins, including integrins, which provide a link between the extracellular matrix and the cytoskeleton, and extracellular proteases and their inhibitors, which mediate focal degradation of the extracellular matrix during cellular invasion.

Integrins are heterodimeric cell-surface receptors composed of two associated transmembrane glycoproteins that connect adhesive proteins in the extracellular matrix to the cytoskeleton. Integrins mediate attachment of cells to their substratum. They are also involved in intracellular signalling. At present, 15 different α and 8 different ß subunits have been identified, which associate to form more than 20 receptors recognizing one or more extracellular legends. Endothelial cells express a number of different integrins, one of which, namely αvβ3 has been shown to be particularly important during angiogenesis. αvβ3 is a receptor for a number of proteins with an exposed Arg-Gly-Asp (RGD) tripeptide, including Vitronectin, Fibronectin, Fibrinogen, Laminin, Thrombospondin, osteopontin and von Willebrand factor.

In vivo, this receptor is not widely expressed. It appears to be most prominent on activated endothelial cells during angiogenesis in a wide variety of settings including blood vessels in human wound granulation tissue but not in the normal skin and the chick chorioallantoic membrane. Smooth muscle cells in also express it: Post angioplasty restenosis, atherosclerotic plaques and healing arterial wounds.

It has been shown that αvβ3 antagonists (antibodies and cyclic RGD peptides) inhibit angiogenesis during development, wound healing, retinal neovascularisation and in growing tumours (in which they induce tumour regression). A number of angiogenic cytokines, including bFGF, VEGF, and TGF-ß1, have been shown to increase expression of the αv and ß3 subunits in endothelial cells, and vß3 antagonists have been shown to markedly inhibit angiogenesis induced by bFGF and TNF-β in the chicken chorioallantoic membrane and rabbit corneal micropocket assays. In addition, αvβ3 has been identified as an important mediator of survival of blood vessels as it is highly expressed in angiogenic

endothelial cell where it suppresses apoptosis promoted by p53 and the cell cycle inhibitor p21WAF1/CIP1 [48]

Moreover, several lines of evidence suggest that VEGFR-2 does not work alone to mediate endothelial cell function, but instead works in conjunction with adhesion receptors including vascular endothelial –cadherin and integrins αvβ3 and αvβ5 during angiogenesis [49-51]. αvβ3 may enhance the signalling capacity of VEGFR-2 directly by binding to VEGFR-2 in response to VEGF stimulation. Evidence suggesting that αvβ3 function affects VEGFR-2 signalling comes from the observation the plating of cultured endothelial cells on the αvβ3 ligand vitronectin enhancesVEGFR-2 tyrosine phosphorylation, cell proliferation and migration in response to VEGF. Furthermore, an anti-αvβ3 antibody substantially decreases VEGF-induced proliferation, cell shape changes, and migration without affecting cell adhesion or binding of VEGFR-2 to VEGF. [51]

The αvβ5 integrin also affects VEGFR-2 function but apparently via a mechanism distinct from that of αvβ3. In corneal or chorioallantoic membrane models VEGF-stimulated angiogenesis is blocked by antibody antagonists to αvβ5 integrin. Activation of αvβ5 integrin contributes to VEGF-induced activation of PKC family members. Calphostin C (a protein kinase C inhibitor) blocked angiogenesis potentiated by αvβ5 but not αvβ3 [50] . While the exact nature of the connection between VEGFR-2 and integrin signalling remains to be elucidated, it is feasible that integrins act to enhance clustering and dimerization of VEGFR-2 subunits in a manner similar to integrin enhanced activation of the PDGFR- β. Ligand-independent tyrosine phosphorylation of the PDGFR- β has been shown in cells plated on collagen type I and fibronectin. This event appears to be due to increased receptor clustering in response to increased mechanical tension or stress exerted on cells upon engagement of integrins by underlying matrix proteins.[52] Indeed, it has

recently been shown that subjecting endothelial cells to shear stress results in clustering and tyrosine phosphorylation of VEGFR-2 [53]. It is also possible that the signal transduction mechanisms of VEGFR-2 and integrins converge at a common signalling protein such as MAPK resulting in synergistic activation of this molecule. Enhanced and sustained MAPK activation mediated by multiple receptor tyrosine kinases has been shown to require collaboration with integrins. [54]

Strong evidence demonstrating a role for VE-cadherin in VEGF-mediated endothelial cell function in vivo comes from a recent paper describing targeted mutations in the gene encoding VE-cadherin mice. [49]Targeted mutations that completely disrupted VE-cadherin, or expressed a truncated protein that no longer binds β–cadherin, a potential downstream effector, resulted in identical vascular defects, which led to lethality by E9.5. The defects were observed at the remodelling/maturation phase of angiogenesis highlighted by impaired sprouting, vessel regression, and reduced endothelial cell survival. In vitro analysis suggests that impaired intracellular signalling downstream of VEGFR-2 leading to decreased endothelial cell survival may also contribute to the VE-cadherin mutant phenotypes. Previously it had been shown that VEGFA, results in tyrosine phosphorylation of VE-cadherin[55]. Consistent with the idea that VE-cadherin is required to coordinate an anti-apoptotic signal transmitted downstream of VEGFR-2 activation, VEGFA, but not other growth factors tested, promoted survival of wild-type cultured in serum-free medium. Further analysis revealed that VE-cadherin promotes VEGFR-2 signalling by acting as part of a VEGF-induced multicomponent complex with activated VEGFR-2, β–cathenin, and PI3K which leads to Akt activation, increased Bcl2 expression and endothelial cell survival [49].The relevance of $\alpha v\beta 3$ to angiogenesis and its potential as an important therapeutic target has therefore been clearly established.

Basement membrane degradation, extracellular matrix invasion, and capillary lumen formation are essential components of the early angiogenic phase. These cellular phenomena are dependent on a variety of proteases and protease inhibitors produced by endothelial and nonendothelial cells. Extracellular proteolysis has also been implicated in the regulation of cytokine activity. In addition, matrix degradation results to the generation of a variety of degradation products, many of which themselves have biological activity. Although a number of enzymatic systems have been implicated in extracellular proteolytic events, many of the relevant enzymes belong to one of two families: the serine proteases, in particular the PA/plasmin system and the MMPs.

Although many mechanisms, including transcriptional and translational controls and secretion and activation of proenzymes, are involved in the regulation of extracellular proteolysis, one mechanism that appears to be particularly relevant to cell migration and morphogenesis is spatial localization. Spatial localization, which appears to have evolved to concentrate proteolysis near the cell surface as well as to restrict its activity to the immediate pericellular environment, can be achieved by two mechanisms: Binding to cell-surface receptors and matrix-binding sites and the production of protease inhibitors.

The MMP family of enzymes contributes to both normal and pathological tissue remodeling. MMPs play a key role in the migration of normal and malignant cells through the body. They also act as regulatory molecules, both by functioning in enzyme cascades and by processing matrix proteins, cytokines, growth factors and adhesion molecules to generate fragments with enhanced or reduced biological effects.
The matrix metalloproteinases (MMPs) are members of a family of at least 15 Zn-dependent endopeptidases that function extracellularly The MMPs each contain a protease domain that has a conserved HExGHxxGxxHS/T sequence in which the three Histidine

residues form a complex with a catalytic Zn atom. In addition, all MMPs contain a regulatory domain (pro-piece) with a conserved PRCGxPD motif that is responsible for maintaining latency in MMPs via binding of the cysteine residue to the active site Zn. The simplest MMP is MMP-7 (matrilysin), which consists of a pro-piece and catalytic domain only. The other MMPs maintain this basic unit but have a variable number of structural domains added. Although most MMPs are secreted proteins, the recently described membrane-type MMPs (MT-MMPs) are anchored to the cell membrane by a transmembrane and intracytoplasmic domain. X-ray crystallography has shown that the catalytic domains of the different MMPs have similar structure, but the topology of the active site clefts differs, accounting for some of the differences in substrate specifictties. Differences in the other domains confers further substrate specificity, regulates binding to matrix proteins, and determines interactions with the Tissue Inhibitors of Metalloproteinases (TIMPs), the natural inhibitors of MMP activity.[56, 57]

Much of the early literature suggested that each MMP had its own particular substrate. This concept led to the use of substrate-focused nomenclature for MMPs such that the collagenases broke down intact fibrillar collagens, gelatinases degraded denatured collagen, and metalloelastase attacked elastin. It is now recognized that MMPs usually degrade multiple substrates, with considerable substrate overlap between individual MMPs. For example, interstitial collagenase (MMP-1) is capable of degrading casein, gelatin, a-1 antitrypsin, myelin basic protein, L-Selectin, pro-TNF and IL-1a and pro-MMP-2 and -9. 72-kDa gelatinase (MMP-2) can degrade fibrillar collagen, elastin, IGF-binding proteins, FGF receptor and can activate MMP-1, -9 and -13. MMP-12 is highly active against type IV collagen, gelatin, fibronectin, vitronectin and plasminogen, but it is not very effective at degrading elastin.

Although the link between single MMPs and individual substrates is not as direct as once thought, it is clear that as a family, the MMPs are capable of breaking down any extracellular matrix component. In normal physiology, MMPs produced by connective tissue are thought to contribute to tissue remodeling in development, in the menstrual cycle, and as part of repair processes following tissue damage. The obvious destructive capability of MMPs initially focused most research onto diseases that involve breakdown of the connective tissues. Leukocytes, particularly macrophages, are major sources of MMP production. MMPs released by leukocytes play vital roles in allowing leukocytes to extravasate and penetrate tissues, a key event in inflammatory disease. In an analogous way, metastatic cancer cells also use MMPs to get in and out of tissues and to establish a blood supply.[58] Low molecular weight MMP inhibitors are now available that have shown efficacy in models of these diseases, reinforcing their central role in pathology.

The MMP axis is highly regulated to avoid excessive tissue damage. Most MMPs , are not constitutively expressed in normal tissues. Inflammatory cytokines such as IL-1 and TNF, growth factors such as TGF-b and noxious stimuli are required to initiate transcription. MMPs are also expressed as inactive zymogens (the pro-piece must be dissociated from the catalytic domain before the enzyme is activated). This dissociation can be achieved by autocatalysis or by the action of enzymes such as furin, plasmin or even other MMPs. For example, the activation of pro-MMP-2 occurs at the surface of many cells and is mediated by MT-MMPs. Once activated, MMPs are subject to inactivation by TIMPs and by binding to plasma proteins such as alpha-2 macroglobulin. It is thought that the local balance of MMP expression and activation versus the level of TIMP governs the level of destruction mediated by MMPsleading to significant pathologies such abdominal aortic aneurysm formation.[59] This is of great significance when studying MMP involvement in disease processes.

By preserving matrix integrity and thereby ensuring normal tissue architecture, protease inhibitors play an important permissive role during angiogenesis. These findings have led to the notion that a precise protease-antiprotease equilibrium allows for localized pericellular matrix degradation during cell migration, while at the same time protecting the extracellular matrix against inappropriate destruction. However, since the net balance of proteolysis required for invasion is always likely to be positive, it has been suggested that antiproteolysis could be effective in inhibiting angiogenesis. In this respect, the requirement for MMP46 47 48 49 and PA50 51 activity during experimentally induced angiogenesis in vivo has been clearly demonstrated. These studies provide evidence for a causal role for the MMP and PA/plasmin systems during angiogenesis in vivo. [60-64]

Accumulation of extravascular fibrin is one of the hallmarks of angiogenesis. Fibrin accumulates in the extracellular environment after injury and because of vascular hyperpermeability seen during the inflammation processes and in solid tumour growth. Fibrin constitutes a provisional matrix that is progressively removed and replaced by other matrix components, including collagen. Although the mechanisms by which fibrin induces mature matrix formation are poorly understood, fibrin itself is chemotactic for inflammatory cells and regulates endothelial cell and fibroblast migration. Recent observations strongly suggest that the fundamental and possibly only physiological role of the PA/plasmin system is to mediate fibrinolysis. With respect to angiogenesis, it has been demonstrated that endothelial cells express uPA, uPA receptor, and PAI-1 during angiogenesis in vivo and that in vitro, and all of these components are induced by VEGF and bFGF. It should be noted that from a quantitative point of view, alterations in expression of the PA/plasmin system are usually far more dramatic than those seen with the MMP system. However, developmental and physiological angiogenesis appears to occur normally in PA-, uPA receptor–, PAI-1–, and plasminogen-deficient mice. Possible

explanations for these apparent discrepancies include redundancy (in which different proteins share the same function) and compensation (in which removal of one protein results in upregulation of another protein with a related function). However, it has also been suggested that in certain settings, for example, during development, in which fibrin is not a major component of the extracellular matrix uPA, tPA, PAI-1, and plasminogen may have no functional role. However, all of these components are expressed during cellular invasion and tissue remodelling in a precisely controlled temporospatial manner [28, 29]. One explanation might be that the mechanisms that regulate angiogenesis are unable to distinguish situations that require fibrinolysis from those that do not. Thus, irrespective of the context, a consistent pattern of changes in expression of a cohort of genes will occur in endothelial cells in response to VEGF and bFGF. This might include proteases and protease inhibitors as well as alterations in synthesis of matrix components and integrins. This hypothesis suggests that it should be possible to inhibit angiogenesis by interfering with the PA/plasmin system in settings such as wound healing, inflammation, and tumour growth, in which fibrin is a major component of the extracellular matrix.

In summary, angiogenesis is dependent on precisely controlled sequential alterations in a number of endothelial cell functions, including proliferation, migration, and cell–extracellular matrix interactions, all of which are potential targets for antiangiogenic strategies. Positive (therapeutic angiogenesis) or negative (antiangiogenic) manipulation of the angiogenic process could provide therapies for pathologies ranging from cancer to ischaemic disease.

1.5 Antiangiogenesis in tumours

The vascularisation of tumours forms a central part in the process of carcinogenesis. The ability of tumours for further growth from the in situ situation and subsequently to invade and metastasise appears to be directly related to their ability to induce new blood vessel growth. [65]

The vascularity of tumours and the capacity of the growing tumour to elicit the production of new capillary endothelium from the host have been noted for many years. [66] Tannock showed that the rate of division of tumour cells decreased in proportion to their distance from the supplying blood vessel and related this to diminishing oxygen supply. Moreover, he showed that the overall rate of growth was dictated not by proliferation of tumour cells but by the lower rate of proliferation of endothelial cells, concluding that the supply of oxygen and nutrients to the tumour limited its growth.[67, 68]

Anti-angiogenesis as a therapeutic concept was developed three decades ago .It was based on original observations that tumours that failed to vascularise did to grow beyond a few millimetres in diameter. [65]In a series of classic experiments, J Folkmann compared the growth of transplanted tumours in the avascular aqueous humour of a rabbit eye with those in the vascular iris and showed that tumour growth had distinct avascular and vascular phases. The start of the vascular phase of growth coincided with tumours growing beyond 2-3 mm3. This coincided with a 20-fold increase in the rate of tumour growth. Tumours in the aqueous humour were unable to reach the vascular phase and remained dormant.[69] The conclusion was that new blood vessel development was essential to tumour growth and suggested that disruption of this process was a potential novel therapeutic strategy.[70]

Foremost among the settings in which the notion of antiangiogenesis has been promoted is the inhibition of solid tumour growth and the spread of metastasis. In his classic article on tumour angiogenesis in 1974, Folkmann highlighted a number of points in the angiogenic process that might be targets for antiangiogenesis therapy. [71]These included

1. interruption of angiogenesis-angiogenesis factor (TAF) synthesis;
2. blockade of transmission of TAF through tissues;
3. prevention of endothelial cell mitosis;
4. prevention of vessels from penetrating the tumour.

Based on the above points, the following strategies for antiangiogenic treatment are currently under extensive investigation:

- Neutralising angiogenic promoters
    - Viral delivery of dominant negative receptors to vascular endothelial growth factor
    - Prevent release and activation of fibroblast growth factor 2
- Endogenous angiogenic inhibitors
    - Supply angiogenic inhibitors directly-such as angiostatin, endostatin
    - Gene transfer of DNA of angiogenesis inhibitors-angiostatin
- Endothelial cell targets
- Synthetic angiogenic inhibitors[72]

A large amount of descriptive data has clearly pointed to the importance of VEGF and its receptors in tumour angiogenesis. These observations have led to the development of strategies aimed at inhibiting tumour growth by interfering with cytokine-receptor interactions, including:

- anti-VEGF antibodies,
- soluble VEGF receptors,
- antisense VEGF,

- a VEGF-toxin conjugate,
- and a dominant negative approach using a truncated form of VEGF receptor

Since VEGF is an endothelium-specific mitogen, these findings unambiguously demonstrate the essential requirement for angiogenesis in tumour growth. They also point to the importance of establishing an angiogenic profile in patients with cancer and other chronic angiogenesis-associated diseases, for by knowing which angiogenic cytokine is involved, it will be possible in the near future to specifically tailor antiangiogenic therapy to the individual needs of the patient. Another concept that has recently received convincing experimental support is that of the inverse relationship between tumour dormancy/tumour cell apoptosis and tumour angiogenesis (see above). This follows from Folkman's earlier concept of '"antiangiogenesis' as a means of causing tumours to remain avascular and dormant. Thus, it has been demonstrated that administration of the potent angiogenesis inhibitor angiostatin, an internal-cleavage product of plasminogen that inhibits the growth of a number of tumours in immunoincompetent mice, achieves its effect by inducing a high rate of tumour cell apoptosis. When angiostatin administration was terminated, angiogenesis proceeded unabated, the rate of tumour cell apoptosis was dramatically decreased (in the face of an unaltered rate of tumour cell proliferation), and tumours entered an exponential phase of growth. These findings demonstrate that one of the consequences of antiangiogenesis therapy is the induction and/or maintenance of tumour dormancy; this phenomenon may be explained by either the lack of nutrient supply or the removal of endothelial cell–derived tumour cell survival and growth factors. These observations highlight the importance of reciprocal trophic interactions between tumour cells and endothelial cells. They also serve to illustrate that long-term (possibly even lifelong) antiangiogenesis therapy will be required if one is to achieve a beneficial therapeutic effect. [73]

In addition to VEGF/VEGF receptor antagonists and angiostatin, a large number of polypeptide and nonpolypeptide inhibitors have been successfully used for the inhibition of tumour angiogenesis. Some of these are potential endogenous inhibitors (i.e., they may be involved in the physiological regulation of angiogenesis) and include:

- inhibitors of matrix-degrading proteases (TIMPs and PAIs),
- interferons,
- thrombospondin,
- platelet factor 4,
- a 16-kD fragment of prolactin,
- C-X-C chemokines,
- IL-12,
- and certain steroids and their metabolites.

Other inhibitors include:

- AGM-1470/TNP-470 (a fumagillin derivative) and other angiostatic antibiotics,
- synthetic metalloproteinase inhibitors,
- angiostatic polysaccharides,
- suramin analogues,
- integrin (particularly vß3) antibodies and other antagonists,
- genistein (a tyrosine kinase inhibitor),
- and thalidomide

A list of the anti angiogenic treatments currently under various clinical trials as well as the proposed mechanism of action follows:[74-89]

Drug	*Trial*	*Mechanism*
Marimastat	Phase III small cell lung cancers	Synthetic inhibitor of matrix metalloproteinases (MMPs)
COL-3	Phase I/II brain, Kaposi's Sarcoma	Synthetic MMP inihibitor. Tetracycline® derivative
Neovastat	Phase II Multiple Myeloma, Phase III renal cell (kidney) cancer, Phase III non-small cell lung cancer	Naturally occurring MMP inhibitor
BMS-275291	Phase I/II Kaposi's; Phase II/III Advanced or Metastatic Non-Small Cell Lung	Synthetic MMP inhibitor

Table 3: Drugs that block matrix breakdown:

Table 4: Drugs that inhibit endothelial cells directly:

Drug	*Trial*	*Mechanism*
Thalidomide	Phase I Malignant Glioma, Phase I/II for advanced Melanoma, Phase II ovarian, metastatic prostate, Phase II with chemotherapy against solid tumors; adjuvant study in recurrent or metastatic colorectal cancer; Myelofibrosis with myeloid metaplasia, follicular lymphoma, myelodysplastic syndrome, refractory ovarian, Phase II gynecologic sarcomas, liver cancer; metastatic melanoma, CLL, Multiple Myeloma; Phase III non-small cell lung, nonmetastatic prostate, refractory multiple myeloma, renal cancer.	Unknown
Squalamine	Phase II non small cell lung cancer; Phase II Ovarian; Brain; Phase I Advanced Cancers	Extract from dogfish shark liver; inhibits sodium-hydrogen exchanger, NHE3
2-ME	Phase I solid tumour studies	Inhibition of endothelial cells

Table 5 : Drugs that block activators of angiogenesis:

Drug	*Trial*	*Mechanism*
SU5416	Phase I recurrent head & neck, Phase I advanced malignancies, recurrent or progressive brain(pediatric), Phase I/II AML; advanced malignancies, advanced colorectal, Recurrent Brain, Soft tissue sarcoma; Phase II von-Hippel Lindau disease, advanced soft tissue; Phase II prostate cancer, metastatic melanoma, multiple myeloma, malignant mesothelioma, metastatic renal, advanced or recurrent head and neck	Blocks VEGF receptor signaling
SU6668	Phase I against advanced tumors	Blocks VEGF, FGF, and PDGF receptor signaling
Interferon-alpha	Phase II/III	Inhibition of bFGF and VEGF production
Anti-VEGF Antibody	Advanced head and neck. Phase II metastatic renal cell cancer, Phase II with chemotherapy in untreated advanced colorectal, metastatic breast; Phase II non-hodkin's lymphoma, hematologic malignancies, metastatic prostate, previously untreated advanced colorectal, inflammatory breast cancer, Advanced or recurrent cervical, non-small cell lung; Phase II/III Advanced non-small cell lung; Phase III with chemotherapy in untreated metastatic colorectal, Phase III metastatic breast	Monoclonal antibody to vascular endothelial growth factor (VEGF)

Table 6: Drugs that inhibit endothelial-specific integrin/survival signalling:

Drug	*Trial*	*Mechanism*
Medi-522 (Vitaxin II)	Phase I/II trial in CPT-11 (irinotecan) refractory advanced colorectal cancer	Antibody that blocks the integrin present on endothelial cell surface
EMD121974	Phase I in patients with HIV related Kaposi's Sarcoma, Phase I/II progressive or recurrent Anaplastic Glioma	Small molecule blocker of integrin present on endothelial cell surface

Table 7: Drugs with non-specific mechanism of action:

Drug	*Trial*	*Mechanism*
CAI	Phase I studies in combination against solid tumors, Phase II ovarian cancer, metastatic renal cell cancer	Inhibitor of calcium influx
Celecoxib	Phase I Prostate; Phase I/II Cervical; Phase II Basel Cell, Metastatic Breast	Enzyme cyclo-oxygenase 2 (COX-2)
Interleukin-12	Phase I/II Kaposi's sarcoma	Up-regulation of interferon gamma and IP-10
IM862	Phase II for untreated metastatic cancers of the colon and rectum; Ovarian	Unknown mechanism

Table 8: Antiangiogenic drugs in current clinical trials

Other situations in which antiangiogenesis therapy has been successful include interferon alfa-2a in the treatment of life or sight threatening hemangiomas, thalidomide for ocular neovascularisation due to macular degeneration, minocycline for arthritis, TNP-470 as a vascular pannus inhibitor in experimental inflammatory arthritis, and proliferative retinopathy (in which IFN- and thalidomide are currently being tested in clinical trials). The proven efficacy of IFN2a in inducing early resolution of juvenile haemangiomas is likely to be related to its capacity to inhibit angiogenesis in vivo. Although inhibitors of angiogenesis have not been studied in clinical trials in patients with rheumatoid arthritis, it is likely that TIMPs (possibly via a gene therapy approach) as well as other MMP inhibitors will prove to be efficacious due to their combined antiangiogenic and chondroprotective effects.

Preclinical research into tumour angiogenesis has led to the identification of several antivascular treatments with impressive efficacy in animal models of human cancer. Currently, at least 17 antivascular agents are being assessed in clinical trials, mostly still phase I and II trials that involve treating patients with advanced metastatic disease that is resistant to other treatments. There are occasional reports of striking clinical remissions, but the real efficacy of these agents will only become apparent over the next decade as they are fully evaluated in extensive clinical studies either as stand alone or as combination treatments with known chemotherapeutic agents. As inhibition of angiogenesis may induce dormancy of a tumour rather than killing it, there is

growing appreciation that the administration of these agents, and their assessment in clinical trials, may need to be different from that currently used for cytotoxic drugs. Indeed, it is possible that these agents could be effective in maintaining long-term remission, an approach not currently used for solid tumours.

1.6 Angiogenic neovascularisation in ischaemic disease

The age-adjusted prevalence of peripheral vascular disease (PVD) in the U.S. population has been estimated to approach 12%. The clinical consequences of occlusive PVD include pain on walking (claudication), pain at rest, and loss of tissue integrity in the distal limbs; the latter may ultimately lead to amputation of a portion of the lower extremity. Surgical bypass techniques and percutaneous catheter-based interventions may be used to successfully revascularise the limbs of certain patients with PVD. In many patients, however, the anatomic extent and distribution of arterial occlusion is too severe to permit relief of pain and/or healing of ischaemic ulcers. No effective medical therapy is available for the treatment of such patients

Therapeutic angiogenesis is defined as the clinical use of growth factors to enhance or promote the development of blood vessels in ischaemic tissue.

There are obvious advantages in the possibility of inducing neovascularisation in peripheral vascular disease without the need for surgical intervention. Progress in understanding the cellular and molecular steps of angiogenesis, the isolation of angiogenic growth factors, successful in vivo studies, and promising early clinical results have created great excitement about the potential of therapeutic angiogenesis. Although many questions remain, therapeutic angiogenesis may be the next major advance in the treatment of ischaemic heart and peripheral vascular disease.

Previous animal research has shown that it is possible to induce therapeutic angiogenesis in models of coronary or peripheral ischaemia with VEGF or bFGF. The growth factors were injected intracoronary, intramyocardial, intrapericardial, or intravenously in the case of myocardial ischaemia and intra-arterial and intramuscular in models of peripheral ischaemia. [22-24] Similar results were obtained by the use of gene transfer techniques encoding for the growth factors instead of the proteins themselves. These results led to a dramatic increase in the interest of using of therapeutic angiogenesis in humans.

The first successful trial of angiogenic treatment for ischaemic heart disease by Schumacher and colleagues used direct injection of bFGF into the myocardium of patients undergoing coronary arterial by-pass graft surgery distal to the anastomosis in order to by-pass non-graftable stenoses. Twelve weeks later, dense neovascularisation, bypassing the stenosed areas, was observed angiographically in the treated group with no response in the control group. [26] In another uncontrolled trial, eight patients undergoing coronary artery bypass graft surgery received intramyocardial injections of bFGF in slow release beads in an area of the myocardium not amenable to revascularisation. Three patients had improved perfusion in the non-revascularised region on follow up nuclear perfusion tests. In a preliminary dose finding study, 15 patients with viable but underperfused myocardium who were not suitable for coronary revascularisation received increasing doses of intracoronary VEGF165. Clinical results included improved myocardial

perfusion in seven patients, angiographic evidence of increased collateral density in seven patients, and symptomatic improvement in13 patients. [90] Finally, five patients who were given intramyocardial injection of naked DNA plasmid coding for VEGF165 during minimally invasive surgery showed improvements in angina, nuclear perfusion scans, and angiography. [91] Although these results are very promising, firm conclusions from these studies are limited by the small patient numbers and lack of placebo controls.

As far as peripheral ischaemic disease is concerned, promising clinical results have also been reported. In the first published human study of therapeutic angiogenesis, plasmid encoding for VEGF165 was introduced in the popliteal artery of a single patient with critical lower limb ischaemia by a balloon angioplasty catheter. This resulted in improvements of resting and maximal blood flow as well as increased collateralisation shown by subtraction angiography.[92] In a following study, nine patients with critical limb ischaemia received two intramuscular injections of naked plasmid DNA encoding VEGF165. This resulted in increased serum concentrations of VEGF, angiographic evidence of improved collateral blood flow, and clinical improvement (healing of ischaemic ulcers, limb salvage, and resolution of rest pain). [93] Preliminary results of a double blind, placebo controlled trial of bFGF in 19 patients with claudication indicated that the13 treated patients had improved blood flow in the calf and a decrease in claudication at the highest dose.[94] A 85-year-old woman with chronic critical leg ischemia was enrolled in an experimental protocol to induce therapeutic angiogenesis which

consisted of six consecutive, weekly intravenous infusions of bFGF. A beneficial clinical response was detectable by week four of therapy. The clinical improvement was sustained throughout the remaining weeks of therapy and at follow-up. [95]

Phase I trials with intravenous VEGF165 and intracoronary bFGF have been completed, but the results are not yet published. Currently under way are phase I trials with intramyocardial injections of naked plasmid coding for VEGF using minimally invasive surgical approach, intramyocardial injections of adenovirus coding for VEGF121, and intracoronary infusions of adenovirus coding for bFGF. *(Table 9)[96-102]*

Current applications of therapeutic angiogenesis have been confined to patients with severe ischaemic heart disease or peripheral vascular disease. However, the indications for angiogenic treatment may be extended to include ischaemic cardiomyopathies, accelerated atherosclerosis in cardiac transplant patients, restenosis following angioplasty or surgery, microvascular disease, left ventricular hypertrophy, pulmonary hypertension, cerebrovascular and renal vascular disease. Non-cardiovascular indications include peptic ulcer disease, wound healing and increased survival of free or pedicled grafts.

Despite these promising initial results, several questions remain unanswered regarding:

- the optimal angiogenic factor or cocktail of angiogenic factors,

- the best method and route of delivery,
- the dilemma between protein or gene therapy,
- doubt if those early results will be confirmed in placebo controlled trials,
- if the benefits will be sustained,
- if treatment with angiogenic growth factors will be sufficient, or will it be used as adjunctive therapy to other forms of intervention such as bypass graft surgery, percutaneous angioplasty, or the more recent techniques of transmyocardial laser revascularisation or enhanced external counterpulsation, which are also thought to stimulate angiogenesis[103],
- the problem of pathological angiogenesis: The theoretical possibility that therapeutic angiogenesis could exacerbate otherwise dormant coexisting angiogenic diseases such as diabetic retinopathy, rheumatoid arthritis or growth and dissemination of solid tumours [73]

The basis of many of the previous questions can be rephrased as the quality and stability of the produced neovessels. It is now apparent that the initiation of angiogenesis and stabilisation of the produced neovessels require distinct molecular pathways. As discussed earlier, the paracrine actions of a variety of polypeptide growth factors, such as PDGF, VEGF, TGFb and the angiopoietins, appear to be orchestrated in a complex sequence of steps that lead to the development of the mature vascular system.[15]. Moreover, angiogenic treatments with VEGF are associated with complications associated with vessel instability and leakage such as spider angiomas developing after VEGF gene transfer and limb oedema. [104]It is therefore

reasonable to suggest that the optimal strategy for angiogenesis would involve administering an angiogenic cocktail aiming to both new blood vessel formation and stabilization. The Tie-1 class of molecules that is involved in controlling stability and vessel integrity seems like a likely candidate for improving vessel quality in therapeutic angiogenesis.

Year	Author	Type of study	No of pts	Disease	Growth Factor	Method of administration	Results
1996	Isner	Case Report	1	Critical ischaemia (Peripheral vascular disease)	VEGF	Intraarterial plasmide application	↑Resting Flow, ↑Maximal Flow, ↑collateralisation
1998	Sellke Fw	Uncontrolled Trial	8	Ischaemic heart disease	BFGF	Direct intramyocardial injections	Improved perfusion in the non-revascularised region on follow up nuclear perfusion tests
1998	Henry TD	Uncontrolled dose finding study	15	Ischaemic heart disease	VEGF	Direct intramyocardial injections	Improved myocardial perfusion ,Increased collateral density, Symptomatic improvement
1998	Lazarous DF	Preliminary results of a controlled trial	19	Claudication	BFGF	Direct intraartelial infusion	Improved blood flow in the calf and a decrease in claudication at the highest dose
1998	Baumgartner I	Uncontrolled Trial	9	Critical ischaemia (Peripheral vascular disease)	VEGF	Plasmid Injections (intramuscular)	Increased serum concentrations of VEGF improved collateral blood flow.Clinical improvement
1998	Schumacher	Controlled Trial	20	Ischaemic heart disease	BFGF	Direct intramyocardial injections	↑Neovascularisation

1999	Losordo DW	Uncontrolled Trial	5	Ischaemic heart disease	VEGF	Plasmid Injections (intramyocardial)	Improvements in angina, nuclear perfusion scans, and angiography
1999	Cooke Jp	Case Report	1	Critical Ischaemia	BFGF	Intravenous infusions	Clinical improvement
2001	Simovic	Open-label, dose-escalating trial	29	Ischaemic neuropathy	phVEGF	Plasmid Injections (intramuscular)	Clinical improvement
2002	Lederman	Radomised trial	190	Claudication	BFGF	Direct intraartelial infusion	Clinical improvement
2003	Henry	Radomised trial	178	Ischaemic heart disease	VEGF	Intracoronary & intravenous infusions	Clinical improvement, QoL improvement
2003	Grines	Randomised trial	52	Ischaemic heart disease	BFGF	Intracoronary administration of adenovirus	Improved perfusion
2004	Morishita	Non-randomised prospective sudy	6	Critical ischaemia (Peripheral vascular disease)	HGF	Plasmid Injections (intramuscular	Clinical improvement
2005	Kastrup	Randomised Trial	80	Ischaemic heart disease	phVEGF	Intramyocardial gene transfer	Improved regional wall motion

Table 9: Therapeutic angiogenesis in humans

1.7 Therapeutic Angiogenesis and Antiangiogenesis: Methods and routes of delivery

The therapeutic modulation of angiogenesis can be achieved by two means:

- direct intraarterial or intravenous use of an angiogenic factor (recombinant protein or a nonpolypeptide factor) or
- gene therapy .

A central issue in future clinically applied angiogenesis research will be to determine which of these delivery options will be the most cost effective, and devoid of serious side effects.

In favour of the use of polypeptide and nonpolypeptide regulators are:

- the ability to accurately regulate their dose and thus be able to define the therapeutic window between efficacy and toxicity,
- the ability to clearly define a toxicity profile with immediate withdrawal of treatment when necessary.

Factors against the use of this approach are:

(1) the considerable cost involved in producing sufficient quantities of purified antigen free factor;

(2) the requirement for repeated or prolonged administration of protein via an indwelling venous or arterial catheter;

(3) the appearance of factor specific significant side effects such as seen during prolonged administration of bFGF (hypotension, thrombocytopenia, anaemia) or VEGF (oedema, spider hemangiomas).

Gene therapy denotes the introduction of exogenous genetic material into somatic cells of an organism. The aim is to achieve high levels of sustained gene expression without provoking adverse host reactions. Many gene therapy approaches to date have concentrated on transferring genes into patients with single-gene inherited disorders. They usually involve the transfer of a functionally normal copy into a cell with a defective or abnormal gene followed by the expression of the relative phenotype. These are usually single-gene recessive disorders but may also include dominant disorders in which a wild-type gene will override the abnormal dominant phenotype. Other approaches have been used to stimulate the immune system, treat chronic diseases, and provide molecular markers. Human trials are presently under way for genetic diseases (e.g., adenosine deaminase deficiency, cystic fibrosis, and familial hypercholesterolemia), as well as AIDS, cancer, rheumatoid arthritis, and cardiovascular diseases (ischaemia and restenosis). Although traditionally favoured target tissues include lymphocytes (adenosine deaminase deficiency), airway epithelium (cystic fibrosis), skeletal myocytes, hepatocytes, and in the case of cancer, tumour cells themselves.

With respect to angiogenesis, gene therapy can be used for both aspects: stimulation of angiogenesis for ischaemic disease and antiangiogenesis in cancer, inflammatory arthritis, and proliferative retinopathy. At the present time, gene transfer has been used successfully for therapeutic angiogenesis in animal models and humans. Moreover, successful use of this approach for the inhibition of angiogenesis has been reported in mice using adenoviral vector

to deliver a recombinant, soluble Tie-2 receptor (AdExTie-2) capable of blocking Tie-2 activation. This resulted to inhibition of primary tumour growth and significant reduction in metastasis development. [105]

Factors that favour this approach would include:

- the ability to induce regulating angiogenesis locally in the desired lesion or anatomical area, if local integration of the vector could be achieved without promoting extralesional angiogenesis;
- decreased cost compared to protein treatment;
- single shot injection with transient or permanent results without the need of long-term intraarterial catheters;

Factors against the use of gene therapy include:

- the general problems encountered in current forms of gene transfer i.e.
  - retroviruses require dividing cells,
  - adenoviruses frequently induce non-specific inflammation,
  - use of liposomes and naked DNA is generally inefficient, etc
- limited control in growth factor administration and expression;
- the presence of predictable as well as unpredictable secondary effects, which may be related to either the vector or the gene product that it encodes;

It is important to recall that adenovirus-mediated gene transfer does not result in stable incorporation of the transgene into the host genome; transgene expression is therefore transient. This would certainly be desirable for

therapeutic angiogenesis, since a relatively brief period of transgene expression is likely to be all that is required to induce collateral vessels that, once formed, are likely to be maintained by local hemodynamic factors.[106-108]

A number of endothelial cell properties make them very attractive as vehicles for the delivery of therapeutic recombinant molecules in vivo: Endothelial cells synthesise and secrete many different proteins, they can sustain a high level of transgene expression, they exist in the interface of blood and tissues within the different vascular environments of the body and they are ideally positioned for efficient delivery of therapeutic gene products to achieve local or systemic effects. It is possible to deliver recombinant genes directly into the vasculature at specific sites either by local application of the vector, by surgical or radiological insertion of vascular grafts seeded with genetically engineered endothelial cells or by direct injection of genetically engineered endothelial cells aimed to induce new vessel growth. Despite the fact that the duration of gene expression after transfer appears at present to be a limiting factor, as a drug delivery system for the treatment of local or systemic vascular and nonvascular diseases, gene therapy employing endothelial cells may prove to be an important strategy aided by techniques to stabilise the newly formed vessels or destabilise the undesirable ones.[107]

1.8 Diagnostic and prognostic applications of angiogenesis.

The question of measurement of angiogenesis has been the subject of extensive research. The main targets are either direct visualisation of neovessls in histological specimens or, indirectly, the detection of angiogenic factors or their receptors in biological fluids or tissue. Assessment of these parameters has potentially important clinical applications, including their use as diagnostic markers, prognostic indicators and, and for monitoring the response to angiogenic and other treatments.

Quantitation of microvessel density in breast carcinoma and a variety of other tumour types, including lung, prostate, head and neck, rectal, testicular and bladder carcinoma, malignant melanoma, soft tissue tumours, and multiple myeloma, has shown significant between tumour angiogenesis and a negative clinical outcome including increased risk of metastasis, tumour recurrence, or death.

It has been suggested that high microvessel density is a successful predictor of metastatic risk because high density increases the area of vascular surface, facilitating the escape of tumour cells into the circulation. An angiogenic cell shed from the primary tumour is more likely than a nonangiogenic one to develop into a detectable metastasis. However, the inability to demonstrate this association in a number of other studies has led some authors to question the usefulness of a static measure of microvessel density as a prognostic tool in cancer. The main limitation of direct assessment is the degree of

subjectivity involved in the selection of the appropriate visual fields for counting as well as variations in the type and specificity of various monoclonal antibodies used in the immunocytochemical detection of neovessels

As far as indirect assessment of angiogenesis is concerned , the majority of the available studies report the concentrations of the mainly studied angiogenic factors, namely bFGF and VEGF. Thus, although bFGF is detectable in trace amounts in the serum and plasma of normal adults, elevated levels of have been detected in the serum of patients with prostate and ovarian carcinoma[109, 110]. Moreover, bFGF is present in the urine of normal individuals and in slightly higher concentration in females than males.[111] However, elevated levels of bFGF have been detected in the urine of patients with a wide spectrum of tumours, in haematological malignancies, and in the cerebrospinal fluid of children with brain tumours[111, 112]. The concentration of bFGF in cerebrospinal fluid correlated with the density of microvessels in histological sections.[113]

Regarding VEGF, high concentrations have been detected in the serum of patients with pulmonary[114], ovarian, and uterine tumours[115]; in malignant ascites[116]; in cyst fluid and tissue extracts of glioblastomas [117]; in the ocular fluid of patients with diabetic retinopathy and other retinal disorders[118] ; in the serum of patients with inflammatory bowel disease [119]; and in the urine of women undergoing gonadotrophin treatment[120].

Other angiogenic cytokines that have been detected at elevated levels in cancer patients include the following: (1) aFGF in the urine of patients with bladder cancer[121]; (2) TGF-ß in the plasma of patients with prostatic and hepatocellular carcinoma and in the serum of patients with ovarian carcinoma[122, 123]; (3) HGF in urine and tissue extracts of individuals with bladder carcinoma and in the sera of patients with hepatocellular carcinoma [124, 125]; (4) EGF/TGF-α in the urine of patients with glial tumours and metastatic breast cancer [126]; and (5) angiogenin, TNF-α , GM-CSF, and interleukins-2, -6, -7, -8, and -10 in patients with ovarian carcinoma.[127] In one study, plasma TGF-ß1 levels were found to correlate positively with the extent of tumour vascularity but not with tumour size or underlying liver disease in hepatocellular carcinoma[122].

Although studies published to date have measured only positive regulators of angiogenesis, it will be important in the future to also consider negative regulators, since their loss may be permissive for allowing endothelial cells to enter the activation phase of angiogenesis. Finally, measurement of positive and negative regulators and the establishment of an angiogenic profile may also help to determine their importance in settings such as juvenile hemangioma, rheumatoid arthritis, and possibly infertility.

Furthermore, advances in molecular biology allow now the measurement of mRNA encoding for those receptors either in situ in tissue samples or in

biological fluid increasing the diagnostic accuracy of the detection of the angiogenic factors or their receptors.

Identification of mutations in endothelial cell receptor tyrosine kinases (VEGF receptors-1, -2, and -3; Tie-1; and Tie-2 has provided significant insight in the pathogenesis of various vascular malformation syndromes such as of venous malformation and for the Klippel-Trénaunay-Weber syndrome. Venous malformations, the most common errors of vascular morphogenesis in humans, are composed of dilated, serpiginous vascular channels. The walls of the channels have variable thickness of smooth muscle cover; some mural regions lack smooth muscle altogether. A missense mutation resulting in an arginine-to-tryptophan substitution at position 849 in the kinase domain of the receptor tyrosine kinase Tie-2 is associated with dominantly inherited venous malformation in two unrelated families. Using proteins expressed in insect cells, it was shown that the mutation results in increased activity of Tie-2 causing inherited venous malformations suggesting that the Tie-2 signalling pathway is critical for endothelial cell to smooth muscle cell communication in venous morphogenesis. [128]

1.9 The potential role of the endothelial cell receptor Tie-1 in angiogenesis

Tie-1 and Tie-2 define a new class of receptor tyrosine kinases that are specifically expressed in developing vascular endothelial cells. In a series of experiments to study the functions of Tie-1 and Tie-2 during vascular endothelial cell growth and differentiation in vivo, targeted mutations of the genes in mice were introduced by homologous recombination. Embryos deficient in Tie-1 failed to establish structural integrity of vascular endothelial cells resulting in oedema and subsequently localized haemorrhage. However, analyses of embryos deficient in Tie-2 showed that it is important in angiogenesis, particularly for vascular network formation in endothelial cells. These in vivo analyses indicate that the structurally related receptor tyrosine kinases Tie-1 and Tie-2 have important but distinct roles in the formation of blood vessels. [39]

Angiogenesis is thought to depend on a precise balance of positive and negative regulation. Angiopoietin-1 (Ang1) is an angiogenic agonist that signals via Tie-2. Like VEGF, Ang1 is essential for normal vascular development in the mouse. An Ang1 relative, termed angiopoietin-2 (Ang2), was identified by homology screening and shown to be a naturally occurring antagonist for Ang1 and Tie-2.[42, 43] Transgenic overexpression of Ang2 disrupts blood vessel formation in the mouse embryo. In adult mice and humans, Ang2 is expressed only at sites of vascular remodelling[129]. The

discovery of a negative regulator acting on Tie-2 emphasizes the need for exquisite regulation of this angiogenic receptor system.[42]

The receptor tyrosine kinase Tie-1 (Tyrosine kinase with Immunoglobulin and EGF homology domains) is essential for angiogenesis where it appears to have a role in vessel maturation[38]. It possesses two extracellular Immunoglobulin –like loop domains separated by three tandem Epidermal Growth Factor domains. These are followed by three FNIII domains located closest to the transmembrane domain. The intracellular domain contains two tyrosine kinase domains interrupted by short kinase insert sequence and a carboxyl terminal tail [37, 130]. These structural properties are shared by Tie-2 and these two receptors constitute a distinct subfamily of RTKs[37, 131]Tie-1 is expressed in cells as a doublet of 135 and 125 kD; the 135-kD band represents mature cell surface receptor containing sialic acid and N-linked oligosaccharide residues, whereas the 125-kD band represents an intracellular pool of immature receptor.[132] Tie-1 is expressed in vascular endothelia during development, in human and murine hematopoietic stem cells[133-135] and in some megacaryoblastic and erythroleukemia cell lines[136, 137]. By in situ hybridisation, TIE-1 mRNA was localised to endothelial cells of 9.5 to 18 day mouse embryos[36, 138], in proliferating ovarian capillaries, in the granulation tissue during wound healing[36], in the endothelia of brain neoplasms[139] and the vasculature of arteriovenous malformations[140]. However, Tie-1 is expressed in most of the non-proliferating adult endothelium[21, 36]. Although no specific ligand has yet been identified for

the receptor, targeted disruption of the Tie-1 gene by mutagenesis in mice results to a lethal phenotype. Mice die over a variable period, ranging from E15 to birth exhibiting extensive haemorrhage and oedema resulting from a lack of endothelial cell integrity and hyperpermeability of blood vessels.[39]

The unique structure of Tie-1 extracellular domains may indicate that it has evolved for multiple protein-protein interactions[31]. Moreover, both hypoxia and VEGF were found to increase Tie-1 in a time-dependent manner. The effects of hypoxia and VEGF were not additive. Experiments with actinomycin D indicate that these activators regulate Tie-1 at the transcriptional level.[141]

The currently available data suggest a potentially significant role of Tie-1 in regulating later stage of angiogenesis, namely vessel maturation and regression[142]. However, no known ligand or modes of intracellular signalling have been identified. Therefore the scope of the present thesis is to identify the physiological regulators of activation and expression of the Tie-1 receptor as well as further clarify the *in vitro* and *iv vivo* physiological significance of Tie-1. Apart for the obvious biological importance, manipulation of the Tie-1 signalling pathway could also have great importance in therapeutic angiogenesis contributing to vessel stabilisation following angiogenic treatment.

CHAPTER 2: REGULATION OF SURFACE EXPRESSION OF THE ENDOTHELIAL CELL RECEPTOR TYROSINE KINASE TIE-1

2.1 Introduction

Signal transduction by receptor and cytoplasmic tyrosine kinases is a process of critical importance in cellular proliferation and differentiation. Genetic and biochemical studies in this large family of proteins have shown that different receptor tyrosine kinases (RTKs) are responsible for transducing important developmental, proliferative, cell survival and migratory signals from the outside to the inside of the cell.

Recent studies of endothelial cell specific RTKs have shown that their expression can vary significantly depending on cell origin, activation state, and whether cells are transformed. Several different pathways, including the proteolytic release of the extracellular domain as a soluble receptor, can modulate the expression and activity of RTKs at the cell surface.

Several cell surface growth factor receptors, including those with tyrosine kinase activity generate a soluble form of the extracellular domain and a truncated membrane receptor by proteolytic cleavage[143]. In a few cases,

such as Erb-4, L selectin, cadherin, heparin binding EGF, Transforming Growth Factor a, amphiregulin and Tumour Necrosis Factor, metalloproteases are involved in proteolytic cleavage as [144],[145],. In some cases, the physiological significance of these cleavage effects has been demonstrated to be physiologically significant: release of diffusible growth factors, reduced cell adhesion or increased migration.

The endothelial receptor Tie-1 is subject to regulated proteolytic cleavage. Following treatment with Phorbol 12-myristate 13-acetate (PMA) the 135-kD band, representing mature Tie-1, disappears from the cell surface. This is accompanied by the appearance of a 100-kD band in cell supernatants, shown to be a soluble form of the Tie-1 receptor containing the extracellular domain. The 100-kD band continues to accumulate in the media throughout the duration of PMA treatment during which mature Tie-1 receptor is undetectable. The release of soluble Tie-1 has been shown to be mediated through the activation of protein kinase C (PKC). These results indicate that Tie-1 receptor expression on endothelial cells is regulated by the release of a soluble extracellular fragment following activation of PKC.[132]

Here, the hypothesis that the proteolytic cleavage of Tie-1 is modulated by angiogenic growth factors leading to the production of a soluble extracellular fragment and an intracellular fragment with possible downstream signalling capabilities is examined. In addition, the signalling activity mediated by growth factor –activated cleavage is examined.

2.2 Methods and Materials

2.2.1 Materials

2.2.1.1 Reagents and Solutions

Phosphate buffered saline (PBS) pH7.4, containing disodium hydrogen orthophosphate anhydrous 80mM, sodium dihydrogen orthophosphate 20mM, and Sodium Chloride 100mM in distilled water.

Endothelial Cell Complete Medium, containing Medium 199, 20% (v/v), dialysed foetal calf serum, 100 IU/ml penicillin, 100µg/ml streptomycin, 5 IU/ml heparin and 100µg/ml endothelial growth supplement (ECGS).

Recombinant Human Vascular Endothelial Growth Factor (VEGF) was purchased from Genzyme Diagnostics.

Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma and diluted in DMSO to a final concentration of 10ng/ml (16nmol/L).

Bovine Serum Albumin, Thrombin, Bradykinin acetate, Human Angiotensin II, Brefeldin-A, Basic Fibroblast Growth Factor (bFGF), PKC Inhibitors: (HA100 and H7) were purchased from Sigma

Batimastat (BB94) was kindly provided by at SmithKline Beecham Pharmaceuticals (Harlow, Essex, United Kingdom)

2.2.1.2 Antibodies

Affinity purified goat antibodies recognizing the Tie-1 and Tie-2 extracellular domains were obtained from R& D Systems (Abingdon, Oxon, UK).This antibody has previously been characterised and shown to react specifically with Tie-1 and non-specifically ith unidentified proteins of aapproximately 55kDa

Rabbit antibody against the intracellular domain of Tie-1 was obtained from Santa Cruz Biotechnology Inc (supplied by Autogen Bioclear, Wilts, UK).

Monoclonal antiphosphotyrosine antibody (clone 4G10) was obtained from Upstate Biotechnology, Inc (Lake Placid, New York).

2.2 Methods

2.2.1 HUVEC isolation

Having obtained Ethical Committee aproval and patient informed consent fresh umbilical cords were collected in sterile PBS with 1% (w/v) streptomycin and penicillin. The cord was washed with PBS and parts of the cord with any clamp marks were removed. A syringe containing PBS was inserted into the umbilical vein and PBS flushed through to remove debris. The open end of the vein was then clamped and the vein was distended with DMEM collagenase and incubated at 37° C for 15 min. Following incubation the cord was gently kneaded throughout its length, the clamp was removed and the solution was drained into a container. The vein was then flushed with further 20mls of 20% DMEM/FCS. The cell suspension

obtained was centrifuged at 500 g for 5 min, the cell pellet was resuspended in complete medium, placed into 2% gelatine coated 25cm^2 flask with a final volume of 10mls medium and incubated at 37° overnight. The medium was then removed and the cells were washed with PBS. Once confluent the cells were passaged 1:3.

2.2.2 Cell culture

Human Umbilical Vein Cells (HUVECs) were cultured in flasks precoated with 2 % (w/v) gelatine in Endothelial Cell Complete Medium. Prior to treatment with agonists or PMA the cells were washed once with PBS and incubated for 30 minutes in serum free Medium199.

2.2.3 Cell treatment

Following serum free incubation, cells were treated with PMA (10 ng/ml) for 60 min, VEGF at various concentrations and time, bFGF (10ng/ml), Thrombin (0.1 units/ml), Bradykinin (100 nM) and Angiotensin II (1μm) for 60 min. Batimastat (BB94) was used as a matrix metalloprotease inhibitor in concentrations 10μm to 10nM. Cells were treated with BB94 for 60 min prior to activation with VEGF or PMA.

Following the indicated period of activation time the cells were washed with PBS and lysed by direct addition of Laemmli sample buffer containing 100 mM DTT (10X Laemmli Buffer: for 10.41 liters1500 g Glycine, 314 g Tris base, 104 g SDS) [146]

2.2.4 SDS/Polyacrylamide Gel Electrophoresis and Western Blotting.

The cell lysates were boiled for 10 minutes at 95^{O} C, sonicated for 30 sec and centrifuged for 10 min at 12000g.

Proteins in whole cell lysates or were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% stacking gel (0.125 M Tris, pH 6.8, distilled H_2O 3.075 ml, 0.5 M Tris-HCl, pH 6.8 1.25 ml, 20% (w/v) SDS 0.025 ml Acrylamide/Bis-acrylamide (30%/0.8% w/v) 0.67 ml, 10% (w/v) ammonium persulfate 0.025 ml, TEMED 0.005 ml) and a 12% running gel (0.375 M Tris, pH 8.8,distilled H_2O 3.4 ml , 1.5 M Tris-HCl, pH 8.8 2.5 ml, 20% (w/v) SDS 0.05 ml, Acrylamide/Bis-acrylamide (30%/0.8% w/v) 4.0 ml, 10% (w/v) ammonium persulfate 0.05 ml, TEMED 0.005 ml) at constant 150V for 2hs in running buffer (5X Running Buffer, pH 8.3 Tris Base15 g, Glycine 72 g, SDS 5 g, distilled water to 1 liter.)

Proteins were electrotransferred from the gel to nitrocellulose membrane at constant 110mA overnight (Transfer Buffer (500 ml, pH 8.3) glycine 1.450 g (39 mM), Tris base 2.900 g (48 mM), SDS 0.185 g (0.037%), methanol 100.00 ml (20%)).

Following electrotransfer the blots were incubated in a protein blocking solution of 5% BSA in TBS for 1 hour at 37°C or in 0.5% non-fat milk in PBS at 37°C for 1 hour. After incubation with the protein blocking solution, the blots were washed for 5 minutes in a wash solution of 0.1% (w/v) BSA

in TBS. The wash cycle was repeated 3 times followed by incubation with the primary monoclonal antibody, which has been diluted 1:1000 with an antibody incubation solution of 1% BSA, 0.05% Tween-20 in TBS, for 2 hours at room temperature with gentle agitation. The wash step was repeated, followed by incubation with one of the following enzyme-conjugated second antibody working solutions for 2 hours at room temperature.

Horseradish Peroxidase (HRP) conjugated rabbit anti-mouse IgG (1:500 dilution in 1% BSA, 0.05% Tween-20 in TBS).

Alkaline Phosphatase conjugated goat anti-mouse IgG (1:500 dilution in 1% BSA, 0.05% Tween-20 in TBS).

Finally, the immunoreactive bands were detected using the ECL chemiluminescent blotting detection reagents (Amersham Life Sciences). The immunoblots were scanned and the optical density of each band was determined. Statistical analysis involving calculation of means +/- SDwas performed with the use of Statistica software (StatSoft, Inc. 1995).

The author performed all the following experiments personally. Extraction of HUVECs usually performed by technical staff at the department of surgery, University of Leicester.

2.3 Results

2.3.1 Activation of endothelial cells with PMA and VEGF results to Tie-1 endodomain production.

Here we examined the results of endothelial cell activation with PMA or VEGF in the Tie-1 receptor structure.

The HUVECs were stimulated with PMA (10ng/ml) for 30 min and VEGF (100ng/ml) for 5, 15, 30 and 60 min and then they were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting as described in materials and methods.

Immunoblotting of cell lysates with an antibody directed against the carboxy terminus of the intracellular domain of Tie-1, characterised before[141] revealed the presence of a double band of 125 and 135 kDa. This band does not appear when a Tie-1 blocking peptide is used during immunoblotting with anti-Tie-1 antibody. The 55kDa band, however, still appeared in the presence of the blocking peptide suggesting it was a non-specific reaction of the antibody. The double band of 125 and 135 kDa corresponds to the previously described partially glycosylated Tie-1 and surface expressed fully glycosylated Tie-1. Moreover the non-specific staining of the 55kDa band made it possible to use it for normalisation purposes for gel loading.

Stimulation of cells with PMA (10 ng/ml) resulted in the loss of the cell membrane associated 135kDa band of the Tie-1 125/135 kDa doublet and the corresponding appearance of a band of approximately 46 kDa. Similar phenomena are observed following activation with VEGF (100 ng/ml) from 5 min to 60min. The non-specific 55kDa band remained unchanged.

Results of five independent experiments using HUVECs from different cords demonstrate increased production of a 46 kDa protein following activation of the endothelial cells with PMA (10ng/ml) or VEGF (100 ng/ml). This protein is structurally related to Tie-1 and recognised by the anti-carboxy terminus anti-Tie-1 antibody. It is also related with decreased Tie-1 cell surface expression.

One possible explanation for the above protein production is the proteolytic cleavage of the cell surface expressed full-length receptor causing release of the extracellular domain and generation of a fragment consisting of the transmembrane and intracellular domains.

Nevertheless, relatively small amount of TIE-1 endodomain is present in the control specimen indicating that constitutional truncation occurs in the quiescent endothelial cells. **(Fig1).**

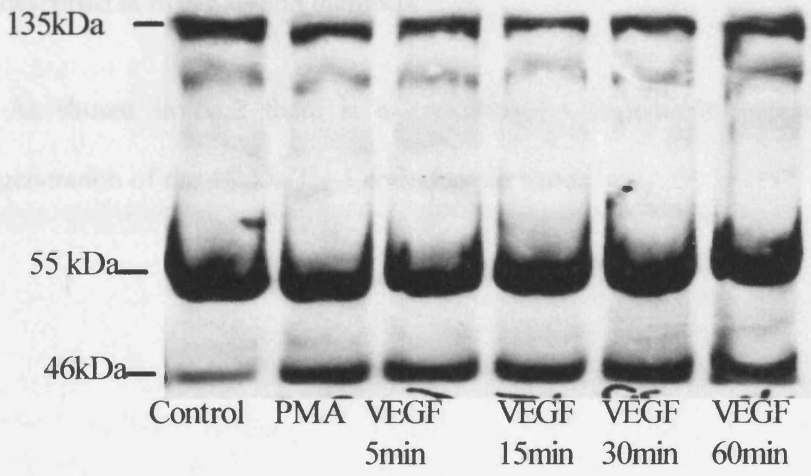


Fig. 1: Activation of endothelial cells with PMA and VEGF results to Tie-1 endodomain generation.

HUVECs were challenged with control vehicle for 60 min, 10 ng/ml PMA for 20 min, 100ng/ml VEGF for 5min, 15 min, 30 min and 60 min. Following the prescribed times of treatment cells were lysed, proteins were resolved by SDS/PAGE and tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments.

2.3.2 VEGF-regulated Tie-1 endodomain production is dose dependant.

To determine the concentration dependence of VEGF induced endodomain production, HUVECs were activated with increasing doses of VEGF: 0.1, 1, 10, 100 and 200ng/ml for 60 min. The cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting as described in materials and methods.

As shown in fig.2 there is a concentration dependent increase in the generation of the 46kDa Tie-1 endodomain band.

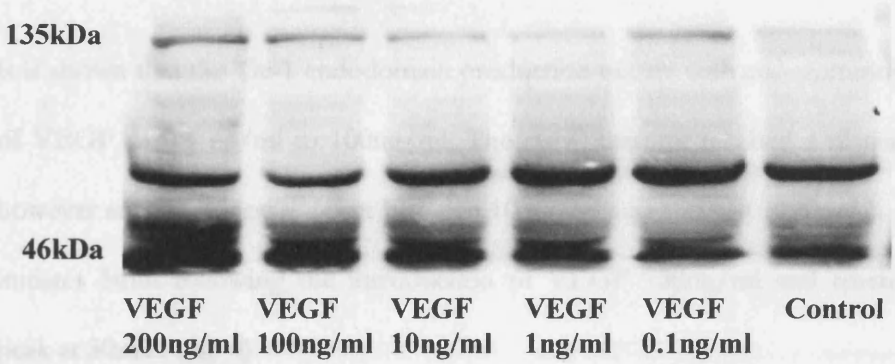


Fig. 2: VEGF-regulated Tie-1 truncation is dose related.

HUVECs were challenged with 200ng/ml VEGF for 30min, 100 ng/ml VEGF, 10 ng/ml VEGF , 1 ng/ml VEGF, 0.1 ng/ml VEGF and control vehicle After 30 min of treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments.

A series of independent experiments were performed to detect concentration and time dependence of tie-1 endodomain production. The blots were scanned and optical density of individual bands was determined. The results were normalised for loading using the non-specific 55kDa band.

It is shown that the Tie-1 endodomain production occurs with concentrations of VEGF of 0.1 ng/ml to 100ng/ml. The curve has not reached a plateau, however smaller increases occur between 10 ng/ml and 100 ng/ml.(fig. 3). It initiates 5min following the introduction of VEGF 100ng/ml and reaches peak at 30min. (fig. 4)

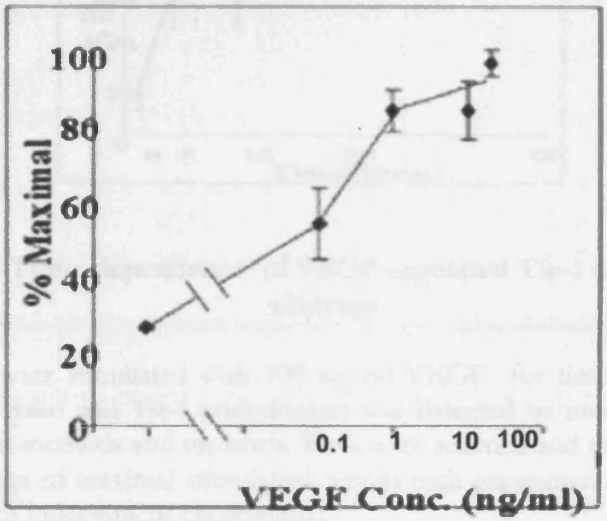


Fig. 3: Concentration dependence of VEGF-regulated Tie-1 ectodomain cleavage.

HUVECs were stimulated with VEGF concentrations as indicated. Cells were lysed and Tie-1 endodomain was detected by immunoblotting as described in methods and materials. Blots were scanned and results, expressed as percentage of maximal stimulation within each experiment are means +/- SD for three independent experiments.

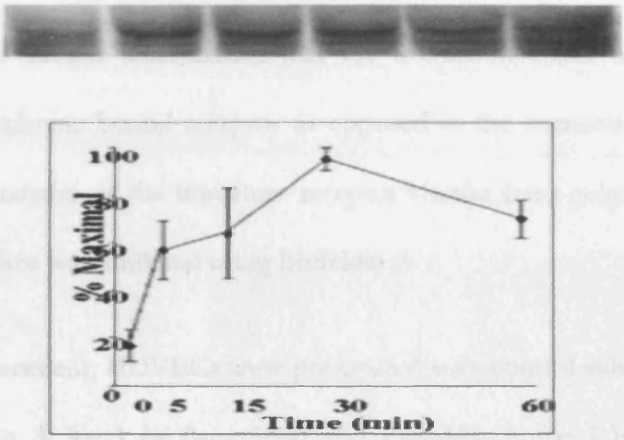


Fig. 4: Time dependence of VEGF-regulated Tie-1 ectodomain cleavage.

HUVECs were stimulated with 100 ng/ml VEGF for times as indicated. Cells were lysed and Tie-1 endodomain was detected by immunoblotting as described in methods and materials. Blots were scanned and results, expressed as percentage of maximal stimulation within each experiment are means +/- SD for three independent experiments.

2.3.3 Kinetics of Tie-1 replacement.

In order to further demonstrate that the truncation effect occurs on the mature membrane bound receptor as opposed to the immature intracellular form, the transfer of the immature receptor via the trans-golgi apparatus to the cell surface was inhibited using Brefeldin A.

In this experiment, HUVECs were pre-treated with control vehicle (lanes 1& 2), Brefeldin A for 1 hr (lanes3&4) and Brefeldin A for 3 hr (lanes5&6). HUVECs were then activated with VEGF 100ng/ml for 60min (lanes 2, 4 & 6). They were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting as described in materials and methods.

Here we show that pre-treatment with ethanol or brefeldin-A does not alter the Tie-1 ectodomain cleavage state. Addition of VEGF causes Tie-1 ectodomain cleavage. This is more pronounced in the brefeldin A pre-treated cells since new full Tie-1 molecules are prevented from reaching the cell membrane (lanes 3 & 5). **(Fig 5)**

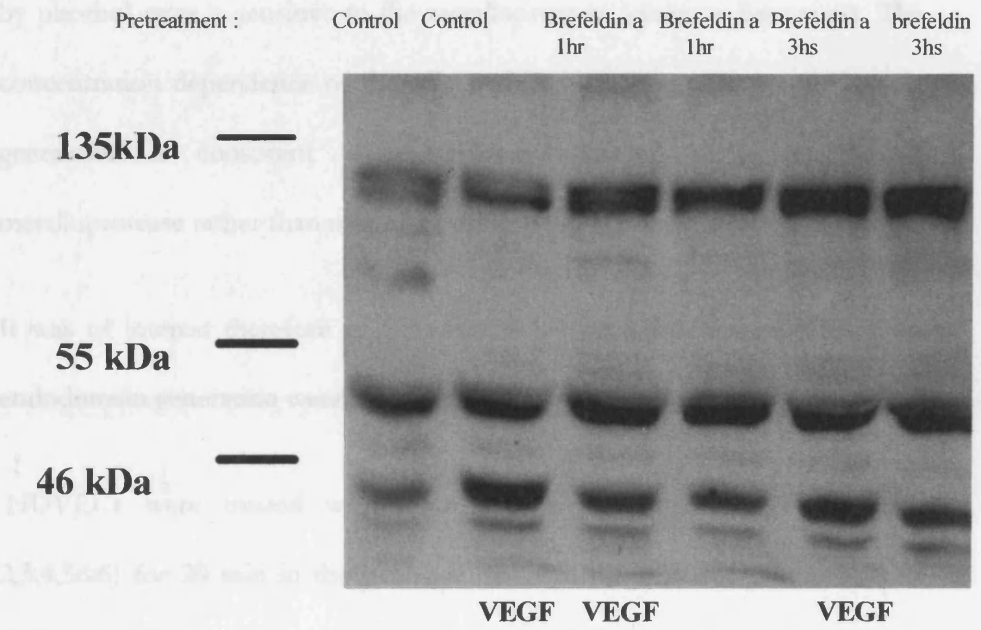


Fig. 5: Kinetics of VEGF-regulated Tie-1 ectodomain cleavage.

HUVECs were challenged with 100 ng/ml VEGF for 60 min in the presence of ethanol or brefeldin-a for 1 and 3 hours (lanes 2,3,6) or control vehicle in the presence of brefeldin-a for 1 and 3 hours(lanes 1, 3, 5). After treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments

2.3.4 Involvement of metalloproteases in VEGF induced Tie-1 endodomain production.

Previous studies [147] have shown that Tie-1 endodomain generation induced by phorbol ester is sensitive to the metalloprotease inhibitor Batimastat. The concentration dependence of the effect of Batimastat on Tie-1 endodomain generation is consistent with involvement of a membrane bound metalloprotease rather than matrix metalloprotease.

It was of interest therefore to determine whether the effects of VEGF on endodomain generation were also sensitive to this inhibitor.

HUVECs were treated with control (lane 1) PMA (20ng/ml) (lanes 2,3,4,5&6) for 20 min in the presence of an MMP inhibitor (Batimastat) in decreasing concentrations ranging: 10μM (lane 3), 1μM (lane 4), 100nM (lane 5), 10nM (lane 6). Cells were then lysed and proteins were resolved by SDS/PAGE. Following transfer to nitrocellulose membranes Tie-1 was detected by immunoblotting as described in materials and methods.

As shown in figure 6 Batimastat in the concentration range 1 to 10 μm inhibited endodomain production. This is in accordance with previous data.

VEGF induced endodomain generation was inhibited by Batimastat in a similar concentration of 1 microM (fig 7). In addition to generation of 46kDa endodomain, figure 8 shows loss of mature 135 kDa receptor when endodomain is produced. This is consistent with both VEGF and PMA

induced endodomain generation by metalloprotease sensitive cleavage of full length receptor.

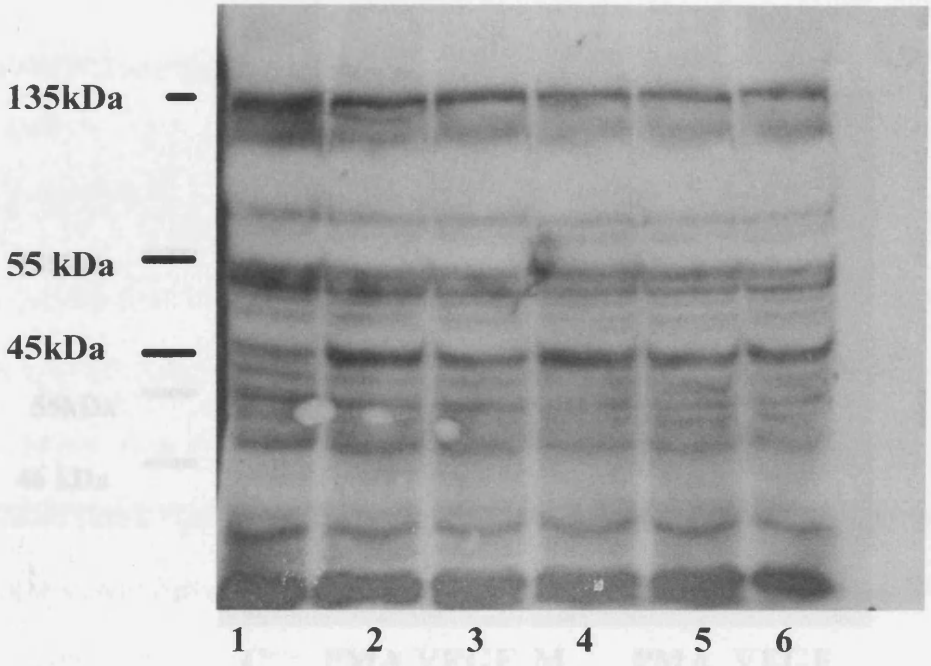


Fig. 6: PMA activated Tie-1 ectodomain cleavage is mediated by a metalloprotease.

HUVECs were challenged with control vehicle (lane 1) or 20 ng/ml of PMA (lane 2) in the presence of decreasing concentrations of Batimastat (lanes 3 to 6). After 30 min of treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments.

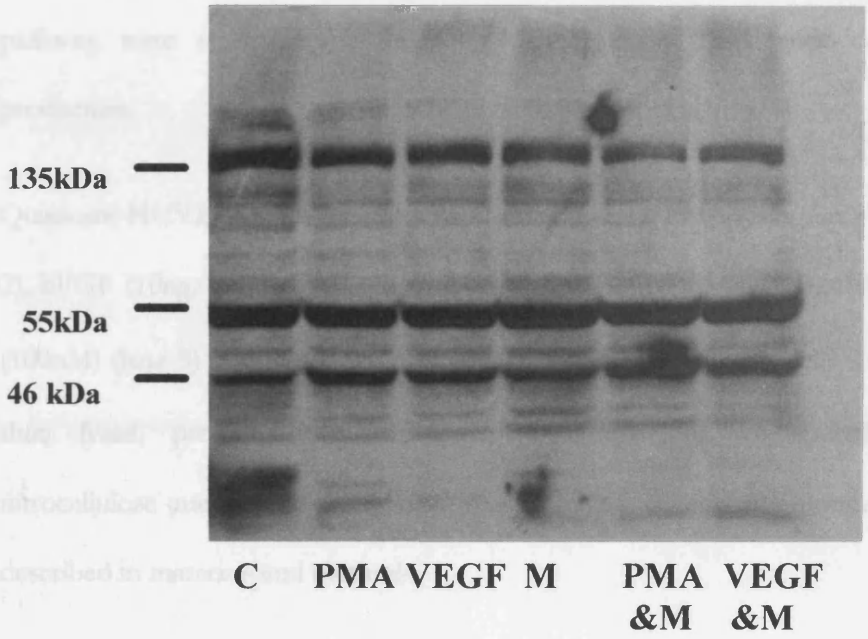


Fig. 7: VEGF-regulated Tie-1 ectodomain cleavage is mediated by a metalloprotease.

HUVECs were challenged with 20ng/ml of PMA or 200ng/ml VEGF for 30min,in the absence or in the presence of 1μ M batimastat as indicated. After 30 min of treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments.

2.3.5 Tie-1 endodomain production is VEGF-specific

In order to determine whether the Tie-1 truncation phenomenon is specific for VEGF, other agonists known to signal through the Protein Kinase C pathway were screened for their ability to induce Tie-1 endodomain production.

Quiescent HUVECs were treated with control (lane1), PMA (10ng/ml) (lane 2), bFGF (10ng/ml) (lane 3), Thrombin (0 .1 units/ml) (lane 4), Bradykinin (100nM) (lane 5) and Angiotensin II (1μm) (lane 6) for 60 min. Cells were then lysed, proteins were resolved by SDS/PAGE, transferred to nitrocellulose membranes and Tie-1 was detected by immunoblotting as described in materials and methods

It is shown that only PMA and none of the above agonists regulate Tie-1 ectodomain cleavage, as shown by the generation of the 46 kDa band in lane 2. (Fig 8)

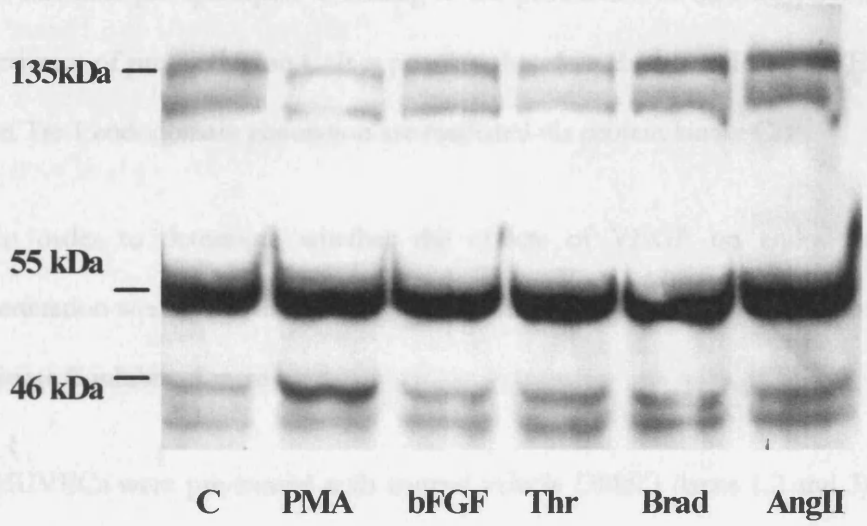


Fig. 8: Tie-1 ectodomain cleavage is VEGF-specific.

Quiescent HUVECs were treated with control (lane1), PMA (10ng/ml) (lane 2), bFGF (10ng/ml) (lane 3), Thrombin (0 .1 units/ml) (lane 4), Bradykinin (100nM) (lane 5) and Angiotensin II (1μm) (lane 6). After 60 min of treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments.

2.3.6 The role of protein kinase C in VEGF induced Tie-1 endodomain generation.

Phorbol esters activate protein kinase C. In addition, VEGF has been shown to stimulate phospholipase C leading to the production of diacyloglycerol, an activator of protein kinase C. It is possible therefore that the effects of VEGF on Tie-1 endodomain generation are mediated via protein kinase C.

In order to determine whether the effects of VEGF on endodomain generation were mediated via VEGF activation of PKC, two different protein kinase C inhibitors were used.

HUVECs were pre-treated with control vehicle DMSO (lanes 1,2 and 3) or the protein kinase C inhibitor Stauvrosporine (lanes 4,5,6) for 15 min followed by activation with control (lanes1& 4), PMA (lanes 2& 5) or VEGF (lanes3 &6) Cells were then lysed, proteins were dissolved by SDS/PAGE and Tie-1 was detected by immunoblotting as described in materials and methods

Both VEGF and PMA induced endodomain generation in DMSO treated cells. However, stavrosporine inhibited the effects of PMA on Tie-1 endodomain formation. In contrast, the effects of VEGF were not inhibited by Stauvrosporin. **(Fig 9)**

Moreover, similar experiments using HA and H7 as PKC-inhibitor in doses ID50 (lane3) and 2xID50 (lane 4) and subsequent activation with VEGF

(lanes 2,3 and 4) showed that HA inhibited the VEGF regulated Tie-1 endodomain formation only in very high, non-specific for PKC doses. **(Fig. 10)**

These results suggest that VEGF induced Tie-1 endodomain production is not mediated by the PKC pathway. Therefore, alternative signalling pathways should be explored.

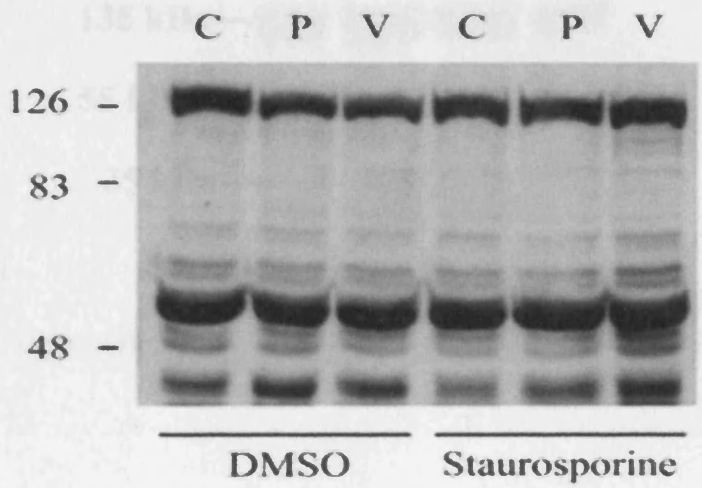


Fig. 9: Involvement of protein kinase C activity in the formation of Tie-1 endodomain.

HUVECs were treated for 30 min with control vehicle (C), 10ng/ml PMA (P), or 100ng/ml VEGF (V) as indicated in the presence of control vehicle (DMSO) or Stavrosporine. After treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments

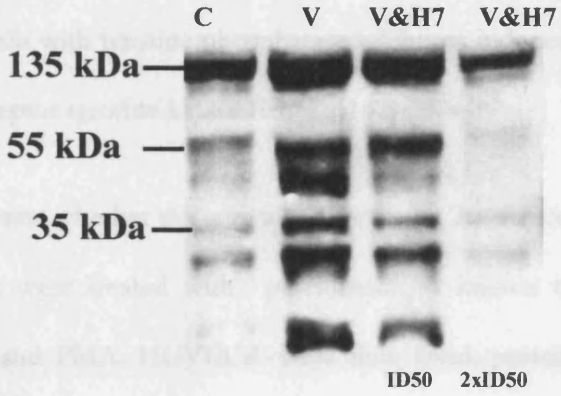


Fig. 10: Involvement of protein kinase C activity in theVEGF regulated formation of Tie-1 endodomain.

HUVECs were treated for 30 min with contol vehicle (C), 100ng/ml VEGF, in the presence of control vehicle or H7 at ID50 and 2X ID 50 as indicated . After treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments

2.3.7 Inhibition of tyrosine phosphatases induces Tie-1 endodomain production.

It has been previously shown that inhibition of tyrosine phosphorylation by treating cells with tyrosine phosphatase inhibitors induces proteolytic cleavage of the receptor tyrosine kinase Erb-4 . [147]

To determine whether this was also true to the Tie-1 receptor tyrosine kinase, HUVECs were treated with pervanadate, a known tyrosine phosphatase inhibitor and PMA. HUVECs were then lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting as described in materials and methods.

As shown in figure 11, pervanadate caused a substantial increase in the intensity of the 46kDa Tie-1 endodomain band. This is consistent with pervanadate activation of Tie-1 cleavage.

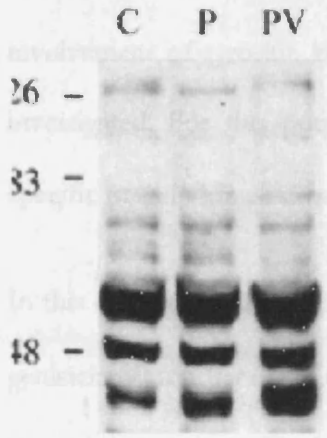


Fig. 11: Involvement of tyrosine activity in the formation of Tie-1 endodomain

HUVECs were treated for 30 min with contol vehicle (C), 10ng/ml PMA (P), or 100nM pervanadate (PV) as indicated. After treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments

2.3.8 Tyrosine kinases control VEGF regulated Tie-1 ectodomain cleavage.

Binding of VEGF to its receptors stimulates their intrinsic tyrosine kinase activityas and initiates phosphotyrosine signalling cascades[148, 149]. The involvement of tyrosine kinase activity in tie-1 endodomain generation was investigated. For this purpose, Genistein, a specific inhibitor of tyrosine-specific protein kinases was used. [150]

In this experiment, HUVECs were pre-treated with control (lanes 1,2,3,), and genistein 40 μm (lanes 4,5,6) for 15 min. This was followed by activation with control (lanes1& 4), PMA (lanes 2& 5) or VEGF (lanes3 &6) Cells were then lysed, proteins were resolved by SDS/PAGE, transferred to nitrocellulose membrane and Tie-1 was detected by immunoblotting as described in materials and methods

Both VEGF and PMA induced endodomain generation in DMSO treated cells. However, genistein inhibited the effects of VEGF on Tie-1 endodomain formation. In contrast, the effects of PMA were not inhibited by genistein. (Fig 12)

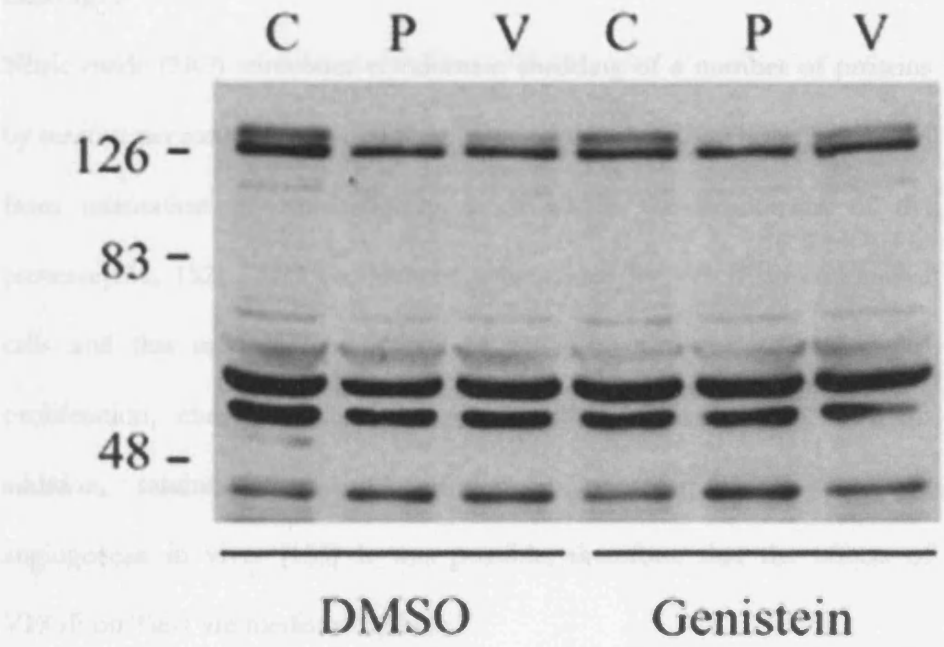


Fig. 12: Involvement of tyrosine activity in the formation of Tie-1 endodomain

HUVECs were treated for 15 min with 40 μm genistein before exposure to contol vehicle (C), 10ng.ml PMA (P), or 100ng/ml VEGF (V). After treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments

2.3.9 Nitric oxide is not involved in VEGF regulated Tie-1 ectodomain cleavage.

Nitric oxide (NO) stimulates ectodomain shedding of a number of proteins by tumour necrosis factor –a converting enzyme[151, 152]. This effect results from nitrosation of an inhibitory motif within the prodomain of the protease[151, 152]. NO production is stimulated by VEGF in endothelial cells and this mediates the effects of the growth factor on endothelial proliferation, chemotaxis and in vitro capillary formation.[153, 154] In addition, inhibition of NO production prevents VEGF-stimulated angiogenesis in vivo. [153] It was possible, therefore, that the effects of VEGF on Tie-1 are mediated by NO.

In this experiment, cells were pre-treated for 15 min with control, or 500μM of Nω-nitro-L-arginine (L-NAME) for 15 min. This was followed by activation with control, PMA 10 ng/ml or VEGF 100ng/ml. Cells were then lysed, proteins were resolved by SDS/PAGE, transferred to nitrocellulose membranes and Tie-1 was detected by immunoblotting as described in materials and methods

Both VEGF and PMA induced endodomain generation in DMSO treated cells. However, Nϖ-nitro-L-arginine (L-NAME) failed to inhibit the effects of VEGF or PMA on Tie-1 endodomain formation. (Fig 13)

Moreover, in the next experiment cells were activated with control , PMA and the molar doses of sodium nitropruside 10^{-6} (lane 3), 10^{-5} (lane 4) 10^{-4} (lane 5). Cells were then lysed, proteins were resolved by SDS/PAGE, transferred to nitrocellulose membranes and Tie-1 was detected by immunoblotting as described in materials and methods.

NO donors (Sodium nitropruside) failed to induce Tie-1 endodomain formation, suggesting that increase in NO would not result in endodomain generation. (Fig. 14)

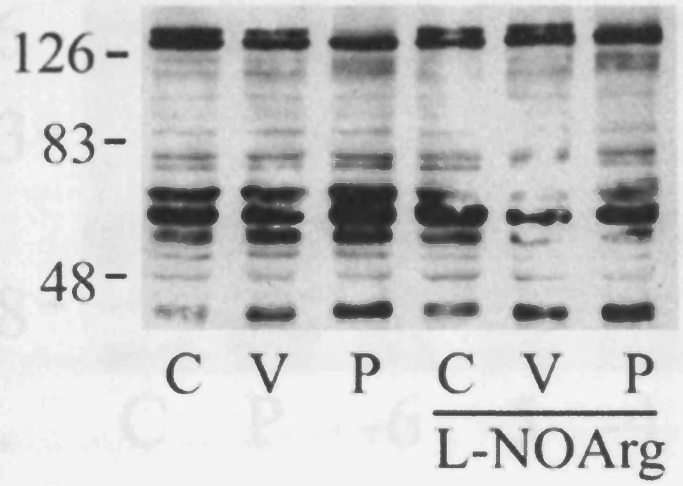


Fig. 13: Role of NO in formation of Tie-1 endodomain.

HUVECs untreated or pretreated for 15 min with 500μM Nω–nitro-L-arginine (L-NOArg) were callenged with contol vehicle (C), 10ng.ml PMA (P), or 100ng/ml VEGF (V) as indicated for 30 min. After treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments

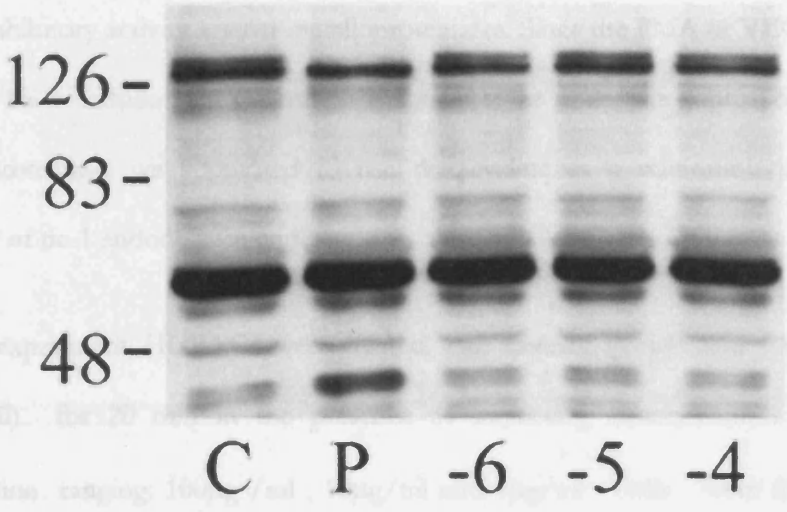


Fig. 14: Role of NO in formation of Tie-1 endodomain.

HUVECs were challenged with control vehicle (C), 10ng/ml PMA (P), or molar concentrations of sodium nitroprusside (SNP) indicated for 30 min. After treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiment

2.3.10 PMA activated Tie-1 ectodomain cleavage is inhibited by doxacycline.

A number of tetracyclines, including doxycycline, are known to possess potent inhibitory activity against metalloproteinases. Since the PMA or VEGF induced Tie-1 endodomain generation is shown to be under the control of a metalloproteinase, we attempted to use doxycycline as a micromolecular inhibitor of tie-1 endodomain generation.

In this experiment HUVECs were treated with control vehicle and PMA (20ng/ml) for 20 min in the presence of increasing concentrations of doxacycline ranging: 100μg /ml , 10μg/ml and 1μg/ml . Cells were then lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting as described in materials and methods

Here we show that PMA caused endodomain generation as demonstrated by the loss of the upper part of the Tie-1 doublet. This phenomenon is inhibited by doxycycline at the lowest concentration. (Fig 15)

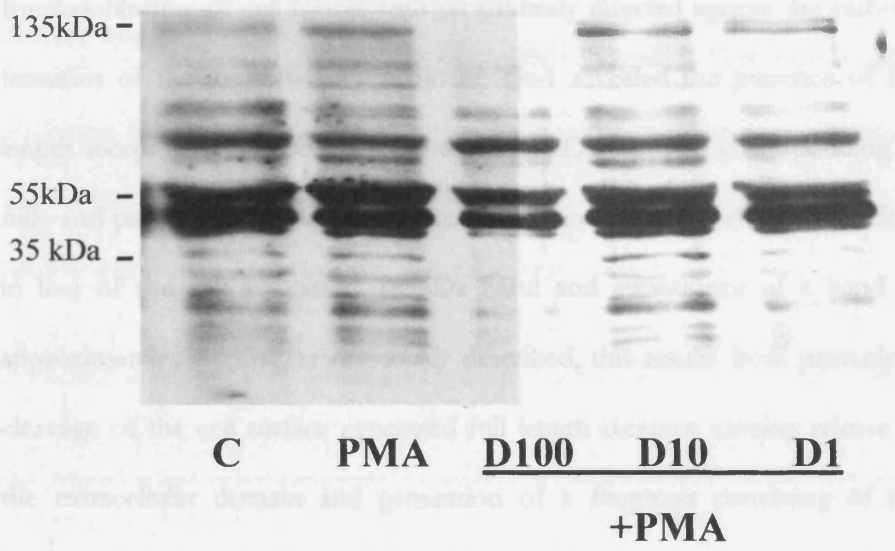


Fig. 15: Inhibition of Tie-1 truncation by doxacycline.

HUVECs were treated with control vehicle and PMA (20ng/ml) for 20 min in the presence of increasing concentrations of doxacycline ranging 100µg /ml , 10µg/ml and 1µg/ml. After treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments

2.3 Discussion

Immunoblotting of cell lysates with an antibody directed against the carboxy terminus of the intracellular domain of Tie-1 revealed the presence of full length receptor as a doublet of 135 and 125 kDa (Fig 1A) corresponding to fully and partially glycosylated protein. Stimulation of cells with PMA resulted in loss of the cell associated 135kDa band and appearance of a band of approximately 46 kDa. As previously described, this results from proteolytic cleavage of the cell surface expressed full length receptor causing release of the extracellular domain and generation of a fragment consisting of the transmembrane and intracellular domains[132]. In addition to bands corresponding to holoreceptor and endodomain, the antibody used in this study also recognises a protein of approximately 55kDa. As shown in figure 1 immunoreactivity with 135/125 and 40kDa proteins was blocked in the presence of the Tie-1 peptide to which the antibody is directed. The 55kDa protein, however, still appeared to react suggesting it was a non-specific reaction of the antibody.

The type of protease responsible for regulated ectodomain cleavage of Tie-1 is not known. Phorbol ester activated Tie-1 cleavage has been shown to be insensitive to inhibitors of serine-, aspartate-, cysteine- or trypsin-proteases [132]. To test the possible involvement of metalloproteases in mediating PMA activated Tie-1 cleavage, therefore, we examined the effects of the metalloprotease inhibitor Batimastat. This compound blocked PMA activated

cleavage in a concentration dependent manner in the micromolar range (fig 7). Similar concentrations of this compound have been shown to be required to suppress angiotensin converting enzyme secretase activity whereas inhibition of matrix metalloproteases occurs with nanomolar concentrtations of Batimastat.[155] These data suggest that regulated cleavage of Tie-1 is mediated via a metalloprotease as previously reported.

The endothelial receptor tyrosine kinase Tie-1 is believed to play an important role in the latter stages of angiogenesis contributing to vessel maturation. Tie-1 is essential for the correct formation of a vessel contributing to the stability and integrity of newly formed vessels[39]. It is believed that stabilisation and maturation of the new vessels requires activation of additional signalling pathways than those used in initiating angiogenesis. The paracrine actions of a variety of polypeptide growth factors, including platelet-derived growth factor, vascular endothelial growth factor, transforming growth factor-beta and the angiopoietins, appear to be orchestrated in a complex sequence of steps that lead to the development of the adult vascular system. [15]

Our results showed that exposure of endothelial cells to the known angiogenic factor VEGF results to proteolytic truncation of the endothelial receptor tyrosine kinase receptor Tie-1. The truncation occurs at the level of cell membrane and leads to the production a free diffusible fragment and an intracellular fragment of molecular weight of about 46kDa with simultaneous loss of the full membrane receptor. The truncation initiates within the first

five minutes of the application of VEGF and increases according to exposure time. It is not mediated via the protein kinase C pathway. VEGF-activated Tie-1 endodomain generation is inhibited by the metalloprotease inhibitor Batimastat over a similar concentration range to that which inhibits PMA-stimulated endodomain generation. These data suggest both activators induce endodomain generation in a mettaloprotease sensitive manner.

The enzyme responsible for extracellular cleavage of tymour necrosis factor – a , designated TACE (tumour necrosis factor a converting enzyme) has been cloned recently and been found to be a membrane bound metalloproteinase of the metalloprotease/disindegrin/cysteine-rich (MDC) family The metalloproteinases are involved in proteolytic shedding of multiple cell surface proteins[156]. Recent studies have shown that the addition of a mettaloproteinase inhibitor such as Batimastat or Marimastat inhibits the proteolytic cleavage of several membrane bound proteins such as human amphiregulin precursor[157], cell surface CD23[158], human EGF precursor[159], membrane-associated ICAM-1[160] or membrane-bound angiotensin-converting enzyme[155]. It has been suggested that this post-translational modification is mediated by one or more MDC enzymes.[144, 155, 161]These results are in accordance with our own suggesting that a member of this family catalyses the proteolytic cleavage of the extracellular domain of Tie-1 initiated by VEGF or PMA.

Similar biochemical pathways involving PKC and metalloproteinases in the proteolytic shedding of the ectodomain of membrane anchored receptors have been reported recently. In one of the cases it has been shown that activated PKCδ binds to the cytoplasmic domain of MDC9/meltrin-γ /ADAM9, a member of the mettaloproteinase-disintegrin family , resulting to shedding of the shedding of the ectodomain of the heparin-binding EGF-like growth factor[162]. Similar biochemical pathways are likely to occur in the VEGF or PMA induced truncation of the ectodomain of the Tie-1 receptor.

In the course of this work, Yabkowitz reported that VEGF induces loss of Tie-1 extracellullar domain from endothelial cells. These data are consistent with the findings reported here and suggest VEGF induces cleavage of Tie-1 leading to ectodomain release and endodomain generation. Moreover, extracellular truncated Tie-1 domain has been recently detected in maternal and blood cord of healthy and pre-eclamptic pregnant women[163] suggesting that the phenomenon occurs in vivo. Previous associations between VEGF and Tie-1 have also been reported: Tie-1 is upregulated in arteriovenous malformations where levels of VEGF are known to be high. Moreover, VEGF was found to increase the level of Tie-1 protein expressed in bovine aortic endothelial cells in a time-dependent manner from one to twenty-four hours. Experiments with actinomycin D indicated that this activator regulates Tie-1 at the transcriptional level.[141]

Protein kinase C represents a structurally homologous group of proteins similar in size, structure and mechanism of activation Through phosphorylation PKC modulates the functional activity of many different intracellular signalling systems which transport extracellular messages from the membrane to the nucleus. Protein kinase C participates in one of the major signal transduction systems triggered by the external stimulation of cells by various ligands including hormones, neurotransmitters and growth factors. Hydrolysis of membrane inositol phospholipids by phospholipase C or of phosphatidylcholine generates 2-diacylglycerol considered the physiological activator of this kinase. Activation of protein kinase C by Phorbol esters and related compounds is not physiological and may be responsible, at least in part, for their tumour-promoting activity. In our series of experiments PKC pathway was not shown to regulate VEGF induced Tie-1 endodomain generation. [164, 165]

In conclusion, our experiments suggest that VEGF induces truncation of the extracellular domain of the endothelial cell surface tyrosine kinase Tie-1. This phenomenon, results to the release of a soluble protein with possible paracrine activity, the reduction of the number of ligand activable cell surface receptors resulting in possible reduction of activation of endothelial cells from the Tie-1 ligand and the creation of an intracellular domain which possible important physiological significance which is currently under investigation. The truncation phenomenon occurs at the level of the cell membrane requiring the presence of active metalloproteinases. The next series of

experiments are currently were undertaken in order to investigate the physiological significance of the Tie-1 truncation.

CHAPTER 3: THE PHYSIOLOGICAL ROLE OF TIE-1 ENDODOMAIN GENERATION.

3.1 Introduction

The previous chapter describes experiments demonstrating that cell surface expression of Tie-1 is regulated by VEGF by the mechanism of proteolytic release of the Tie-1 extrcellular domain.

In this chapter, potential physiological roles of Tie-1 endodomain in endothelial adhesion, proliferation and survival are examined.

3.2 Materials & Methods

Materials

As previously described.

Methods

3.2.1 Cell Immunostaining

Endothelial cells were placed onto sterile glass cover slips and cultured in Endothelial Cell Complete Medium for 24 hours. The cells were then washed with PBS (times 3) and fixed in 70% ethanol for 30 minutes at room temperature. Following fixation, cells were incubated at 37°C for 30 min with anti-Tie-1 goat polyclonal antibody recognising the extracellular domain of Tie-1 diluted 1:10 with PBS. Coverslips were then washed 3 times with PBS followed by incubation with anti-mouse FITC-conjugated secondary antibody diluted 1: 10 in PBS for 30mins at 37°C. The coverslips were washed twice in PBS followed by one wash in ddH20, mounted in DABCO and sealed onto slides for examination by fluoerecence microscopy. Five random fields were cointed on each coverslip

3.2.3 Endothelial Cell DNA Synthesis via 5-bromo-2-deoxy-uridune incorporation

The endothelial cells were placed onto sterile glass cover slips and cultured in complete medium for 24 h. The cells were washed 3 times with PBS and incubated in Endothelial Cell Basal Medium (serum free) for 1 hr. After 1 hr, agonists were added together with BrdU to final concentration of 10μM and the incubation continued for a further 24 hours. Cells were then washed in PBS and fixed in 70% ethanol for 30mins at room temperature. Following treatment in 2M HCL for 20 min. at 37 ^{0}C the cells were neutralised in PBS PH5.8 followed by two washes in PBS pH 7.4.After fixation, cells were incubated at 37 ^{0}C for 30 min with anti BrdU mouse monoclonal antibody diluted 1:10 with PBS. The cells were again washed 3 times followed by incubation with anti-mouse Rhodamine-conjugated secondary antibody diluted 1: 10 in PBS for 30mins at 37 ^{0}C.The coverslips were washed twice in 1XPBS followed by one wash in ddH20 mounted in DABCO and sealed onto slides for fluorescent microscopy. Five random fields of cells were counted on each coverslip and the percentage of BrDU positive nuclei calculated.

3.2.4 Transfection of endothelial cells

Cells were grown to confluence of 20-40%. 2.5 μg DNA (kindly provided by Dr M Marron and Dr P Morris) per well of six well plate were used. Briefly, 2.5 μg DNA were added to 150 μl of Endothelial Cell Basal Medium (serum free). 10μls of Superfect (4μl per μg DNA) were added to the solution. The

cells were incubated for 2hs in room temperature and the solution was mixed by slow hand-rotation of the culture plate. Cells were then washed with PBS and Complete Endothelial cell medium was added .They were then returned to the incubator.

The author performed all the following experiments personally.

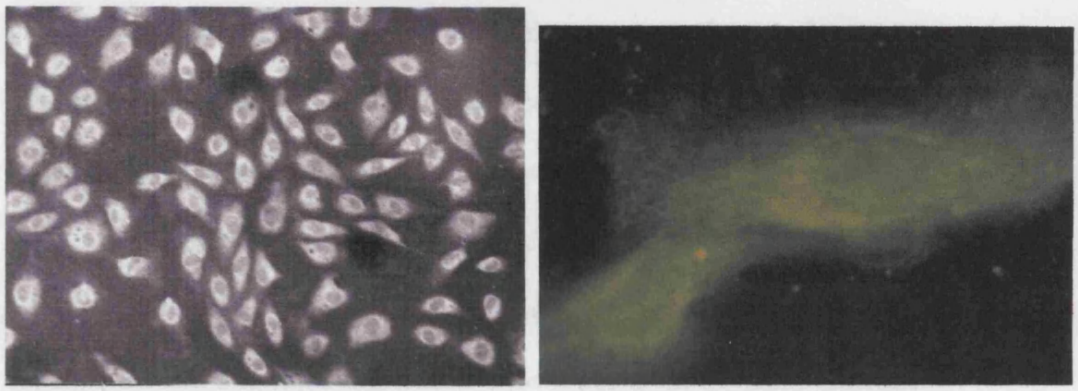
3.3 Results

3.3.1 Cell surface expression of Tie-1

The extracellular domain of Tie-1 is large and contains three fibronectin III repeats. One potential function of the ectodomain could be to act as an intercellular adhesion molecule. If this was the case it would be expected that the receptor be found at cell to cell contacts. To test this possibility Tie-1 was immunodetected in cultured endothelial cells.

HUVECs were cultured to 40% confluence. They were then fixed as described in M&M. Various fixation techniques had been attempted in order to prevent destruction of the TIE-1 receptor at that stage. Cells were then immunostained for Tie-1 as described in methods and materials.

As shown in figure 16 Tie-1 is uniformly expressed outside the cell nucleus. It does not show the characteristics of an endothelial adhesion molecule.



40x

80x

Fig. 16: Cell surface expression of Tie-1.

The receptor does not have the characteristics of an adhesion molecule. Endothelial cells were placed onto sterile glass cover slips and cultured to 40% confluence. Th cells were then washed and fixed in 70% ethanol for 30 minutes. Following fixation, cells were incubated with anti-Tie-1 antibody recognising the extracellular domain of Tie-1 followed by incubation with anti-mouse FITC-conjugated secondary antibody and examined by fluoerecence microscopy . Cells treated with anti-mouse FITC-conjugated secondary antibody only were used as controls (C).

3.3.2 Metalloproteases regulate endothelial DNA synthesis.

In this experiment, cells were cultured to 20-30% confluence in complete endothelial cell growth Medium. The medium was then replaced with Endothelial Cell Basal Medium and the cells were activated with VEGF (100ng/ml) only or VEGF (100ng/ml) in the presence of a metalloprotease inhibitor (Batimastat 10^{-5} M). Untreated cells and Batimastat 10^{-5} M only treated cells were used as controls. BrDU was added to the Endothelial Cell Basal Medium immediately prior to the addition of the activators as a DNA synthesis indicator as described in methods and materials. Cells were cultured for 24 hours and then fixed and treated as described in methods and materials

Cells were microscoped with fluorescence settings suitable for FITC. BrDU positive nuclei show intense red fluorescence indicating high uptake of BrDU and therefore active DNA synthesis **(Fig. 17)**

In a series of subsequent experiments the percentage of BrDU positive nuclei per visual field was counted in the presence of VEGF +/- Batimastat. It is shown that the basal proliferation rate of endothelial cells is 10%. Addition of VEGF increases the proliferation rate to 53%. Batimastat does not alter the basal proliferation rate at a statistically significant level. However, Batimastat in the presence of VEGF suppresses the endothelial cell proliferation rate to 32% ($p<0.05$). **(Fig. 18)**

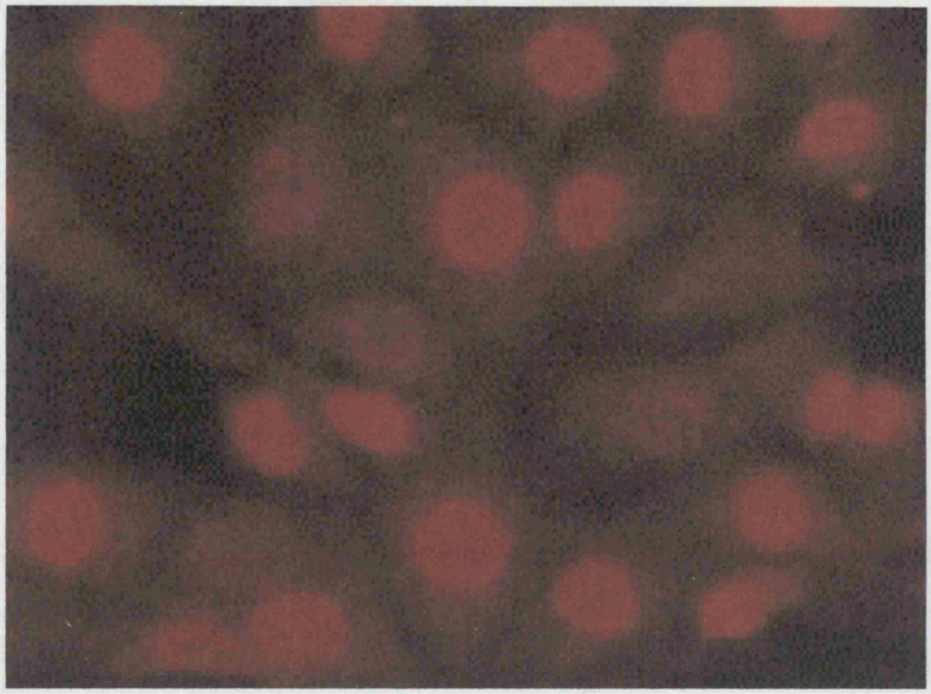


Fig. 17: BRDU proliferation assay.

The endothelial cells were cultured in the presence of BrdU to final concentration of 10μM for 24 hours. Cells were then washed in PBS and fixed. Following fixation, cells were incubated with anti BrdU mouse monoclonal antibody followed by incubation with anti-mouse Rhodamine-conjugated secondary antibody. Five random fields of cells were counted on each coverslip under fluorescent microscopy and the percentage of BrDU positive nuclei was calculated.

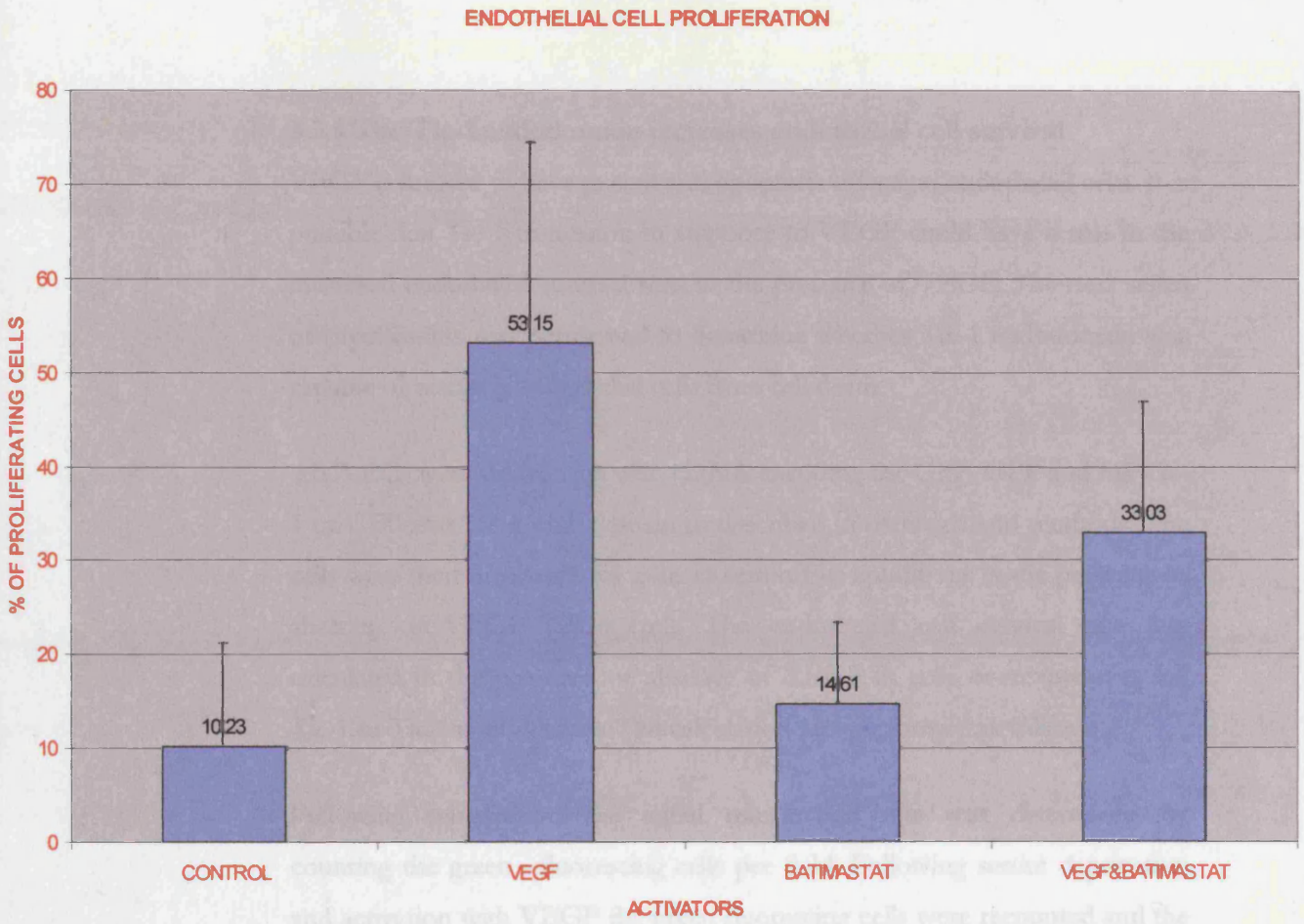


Fig. 18: Regulation of endothelial DNA synthesis by VEGF and metalloproteases.

Endothelial cells were either untreated or activated with 100ng/ml VEGF, 10-5 M Batimastat or 100ng/ml VEGF with batimastat 10-5 BrDU was added to the Endothelial Cell Basal Medium immediately prior to the addition of the activators as a DNA synthesis indicator as described in M&M. Cells were cultured for 24 hours and then fixed and treated as described in M&M. Five random fields of cells were counted on each coverslip under fluorescent microscopy and the percentage of BrDU positive nuclei was calculated.. Data representative of 5 independent experiments are presented. Data are shown as mean + SD.

3.3.3 The Tie-1 endodomain increases endothelial cell survival

VEGF is known to have potent anti-apoptotic effects in endothelial cells. It is possible that Tie-1 truncation in response to VEGF could have a role in the increased endothelial survival seen in the presence of VEGF. The next series of experiments was performed to determine whether Tie-1 endodomain was capable of rescuing endothelial cells from cell death.

HUVECs were transfected with cDNA encoding for GFP, GFP and full Tie-1 or GFP and Tie-1 endodomain as described in materials and methods. The cells were then incubated for 24hs in serum free conditions in the presence or absence of VEGF (100ng/ml). The endothelial cell survival rate was calculated in the presence or absence of VEGF in cells overexpressing full Tie-1 or Tie-1 endodomain. The calculation was performed as follows:

Following transfection the initial transfection rate was determined by counting the green –fluorescing cells per field. Following serum deprivation and activation with VEGF the green fluorescing cells were recounted and the differences calculated and plotted. Smaller differences indicated increased endothelial cell survival and vice versa. **(Fig. 19)**

It is shown that endothelial cell loss of 46% in 24 hs in the control (c) group does not differ significantly with the group overexpressing full length TIE-1 (FL) (42%) or overexpressing TIE-1 endodomain (E) (40%). Addition of VEGF 100ng/ml (C&VEGF) increases endothelial cell survival (35%). The increase becomes more attenuated when Tie-1 endodomain is overexpressed (E&VEGF) (18%) while overexpression of the full TIE-1 does not control VEGF regulated endothelial cell survival (35%) ($p<0.05$).

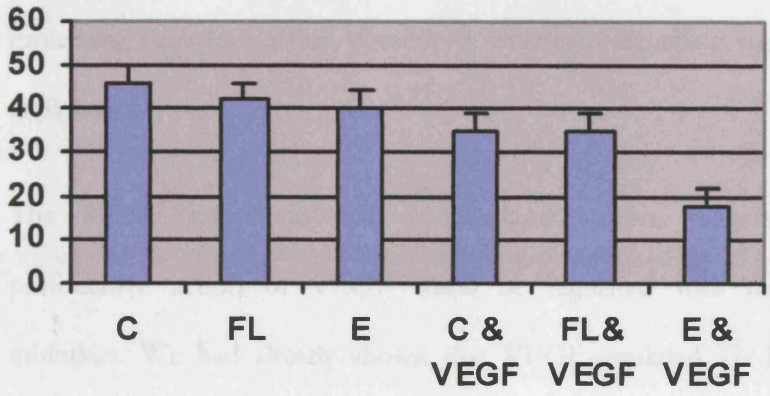


Fig. 19: Expression of truncated Tie-1 results to increase endothelial cell survival in the presence of VEGF:

HUVECs were transfected with cDNA expressing GFP and full (FL) Tie-1 or Tie-1 endodomain (E) and cultured ihn serum-free conditions in the presence or absence of VEGF. Here we show that overexpression oftie-1 endodomain leads to increased endothelial cell survival in the presence of VEGF. Results are shown as % of nonsurving cells at 24 hours (mean + SD).

3.4 Discussion

The potential role of Tie-1 ectodomain cleavage was investigated in this series of experiments.

Our experiments did not demonstrate a role for Tie-1 as an adhesion molecule. Therefore, other possible downstream signalling significance was explored.

The BRDU endothelial cell proliferation studies suggested that the proliferative action of VEGF could be regulated with metalloprotease inhibitors. We had already shown that VEGF-regulated Tie-1 ectodomain cleavage is mediated by a metalloprotease. It is therefore possible that the full Tie-1 receptor, in the absence of angiogenic cytokines, contributes to the quiescence of the non-activated endothelium contributing therefore to vessel stabilisation following angiogenesis. Activation of the endothelium with VEGF leads to increased proliferative state prior to angiogenesis. This is associated with early Tie-1 ectodomain release. This may contribute to vessel destabilisation prior to angiogenesis. However, further experiments would be required to support this hypothesis investigating the Tie-1 status during various stages of angiogenesis and VEGF activation in the in vivo situation.

Moreover, in our endothelial cell survival series, we show that over expression of full Tie-1 or Tie-1 endodomain *per se* do not regulate endothelial cell survival. However, endothelial cell activation with VEGF results to increased cell survival in the presence of Tie-1 endodomain. This phenomenon may

have an important role in the initiation of angiogenesis when activated endothelial cells loose their basic membrane support. Increased survival capabilities are of obvious benefit prior to proliferation and migration.

A role therefore can be stipulated for Tie-1 in both the quiescent and activated epithelium. In the quiescent epithelium Tie-1 signalling probably participates in vessel stability and endothelial cell quiescence. Activation with VEGF leads to early loss of the extracellular domain and therefore modifying or inhibiting the signalling capabilities of the receptor leading to endothelial cell proliferation, increased survival and vessel destabilisation. When the angiogenic process is complete and the levels of VEGF decrease the Tie-1 receptor regains its full form contributing to the maturation and stabilisation of the newly formed vessel. However, further substantiation of this hypothesis will require the study of Tie-1 –1 in an active in vivo system using specific activators and inhibitors of Tie-1 signalling.

Chapter 4

CHAPTER 4: THE ROLE OF TIE-1 ENDODOMAIN IN VESSEL STABILITY

4.1 Introduction

A variety of in vitro models have been established that may mimic certain cellular and biochemical events during the angiogenic cascade. Some of these models include the matrigel tube-forming assay, the fibrin and collagen gel-cord forming assays, the aortic ring model, the human placental outgrowth model and a number of cell proliferation assays. However the cellular events contributing to angiogenesis do not occur in isolation but are co-ordinately and spatially regulated. Therefore, in order to gain a more detailed understanding of angiogenesis in a true physiological microenvironment a number of in vivo models have been developed. Examples are: the rat, mouse and rabbit corneal pocket assays, the primate iris neovascularisation model, the murine matrigel plug and the Chick Chorioallantoic Membrane (CAM) assay.[4, 166, 167]

The Chick embryo CAM is composed by two separate membranes –the somatic mesoderm derived chorion and the splachnic mesoderm derived

allantois- that fuse early (days 4 to 5) during embryonic development. The CAM is the major respiratory structure for the exchanges of gases and nutrients during embryonic development until the time of hatching and thus it becomes higly vascularised. Development of the blood vessels within the CAM begins in the centre and migrate to the periphery. It is believed that the vascular development begins to slow and become essentially completed by the day 11-14 of development extending over virtually the entire inner aspect of the cell. In this regard it provides an ideal microenvironment in which to study angiogenesis. [168] [169]

The CAM model is an in vivo model that is relatively inexpensive, rapid and utilises a microenvironment in which angiogenesis naturally occurs. We therefore used it to further investigate the role of Tie-1 in physiological angiogenesis.

A particular advantage of this model is the accessibility of the growing vessels. To gain insight into the involvement of Tie-1 and Tie-1 truncation in normal developmental vascularisation , therefore, expression of truncation state of the receptor was examined in the CAM model.

4.2 Methods & materials

4.2.1 The CAM assay method

Fertilised white Leghorns were received at incubation age day-0. The eggs were cleaned with Hibitane and were directly incubated horizontally at 37oC and 80% relative humidity or stored at 0-5°C for future use. Exposure of the CAM was performed either with the open dish or the window technique.[170, 171]

4.2.1.1 The open dish technique

The eggs were cleaned with Hibitane and cracked open under laminar flow on the 3rd day of the incubation into 100mm X 20mm glass Petri dishes. The dishes were sealed and then returned to the incubator for further growth.

4.2.1.2 The window technique

On day 3 of the incubation the eggs were removed from the incubator and cleaned with Hibitane. With the use of a motor tool fitted with a spherical emery wheel point an abrasion was made through the shell at the apex without damaging the cell membrane. A 10ml syringe equipped with an 18G needle was used to aspirate 1-2 mls of albumin. Care was taken to avoid puncturing the yolk by aiming the syringe downwards. Next, a window was cut on the upper surface of the egg. The motor-tool was fitted with a cutting disc dipped in Hibitane. A slit about 0.6 mm long was made through the shell into the air sac. Then a window of approximately 1.5 x 1.5 cm was cut ¾ of

the thickness of the shell over the embryo. Special care was taken to avoid damage to the shell membrane and underlying egg mass.

With the use of curved forceps this piece of shell was removed followed by the underlying shell membrane. The window was sealed with a piece of clear tape and returned to the incubator.

4.2.2 Modulation of CAM Angiogenesis

4.2.2.1 Application of growth factors and antibodies

A 15mm clear plastic tissue culture disc was used. The sterile, dialysed, lyophilised test material was suspended in sterile glass distilled water at the desired test amount in a volume of 10µl.

Each disk received a centrally deposited 10µl drop of the test material solution. On the underside of the disc, directly beneath the sample drop, a small dot had been applied to mark the sample site using permanent ink from a fine marker.

The disks were dried in laminar flow hood for 2hs. With a curved forceps the disc was placed sample down on the CAM.

4.2.2.2 Incorporation of exogenous endothelial cells.

The model of the CAM has been frequently used for studies concerned with tissue invasion.

Bovine aortic endothelial cells were cultured to confluence in Gelatin coated T-80 in Completd Endothelial Cell Medium.The medium was removed and cells were washed with PBS. Warmed trypsin (2.5 mls) was added and the cells returned to the incubator for 3min. After ensuring that most cells were free, 10 mls of Complete Endothelial Cell Medium was added washing the surface gently. The solution was centrifuged at 300xg for 6min. The cells were resuspended in 2mls Endothelial Cell Basal Medium. They were then directly pipetted on the surface of the day 7 CAM.The eggs were incubated for additional 48hs.

4.2.3 Cell labelling

4.2.3.1 X-gal screening

Tissue was fixed for 60 minutes in 0.2%(v/v) formaldehyde , 0.2% glutaraldehyde in PBS PH7.4 then rinsed 3 times with PBS. It was then stained for 24 hours at 4^0 C in x-gal solution (1mg/ml X-gal, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$,2mM $MgCl_2$,0.02%(v/v) NP-40 0.01 % (w/v) sodium deoxycholate in PBS) and rinsed with 3.0% DMSO in PBS. Tissue was washed thoroughly with PBS and observed as appropriate.

4.2.3.2 PKH26 red fluorescent cell linker (Sigma)

This technique uses fluorescent cell linker technology to incorporate aliphatic reporter molecules into the cell membrane by selective partitioning. Briefly, cells were trypsinized and put into single cell suspension into serum-free medium. The cells were centrifuged 400xg for 5min into a loose pellet. After

centrifuging, the supernatant was aspirated and the cells were resuspended in Diluent C.1 ml of the cell solution was added to 1 ml of $4X10^{-6}$ PKH26 dye and incubated at 25°C for 3min.The reaction was stopped by adding equal volume of serum. The solution was centrifuged 400xg 10 min to remove cells from the staining solution. The cells were washed for three times. The stained sample was checked for cell recovery, cell viability and fluorescence intensity.

The CAMs were examined using standard filter setup for fluorosceine or rhodamine

4.2.4 Histology & Immunohistopathology

4.2.4.1 Preparation of CAMs

CAMs were harvested at the allocated times and days and fast frozen in liquid nitrogen or preserved in 4% formaldehyde. Fast frozen sections were mounted onto cork and sliced. Formalin preserved CAMs were either examined directly or were embedded in paraffin and sectioned.

For immunoblotting purposes, CAM slices, approx 1cm x 1 cm were washed, pulverised and lysed by direct addition of Laemli sample buffer containing 100 mM DTT. The lysates were boiled for 10 minutes at 95^{O} C, sonicated for 30 sec and centrifuged for 10 min at 12000g. Western blotting was performed as described previously.

4.2.4.2 Recording of the obtained image

For image recording, each exposed CAM was captured using manual photography:

For this purpose an Olympus OM-4 Camera equipped with an extension tube mounted on a tripod was used. The resulting final magnification was 1:5. This allows observation and study of the whole vasculature at the level of arterioles and venules. It is of great importance that a high-resolution film is used in order to achieve high quality results. Special care was taken in order to maintain standard conditions of illumination and sample placement. Also care was taken in order to keep the pre-marked centre of the application disk at the centre of the optic field. For reasons of comparison, a picture of a standard grid was taken at the beginning and the end of each photographic session. The photographs were subsequently analysed manually or scanned and converted into a digital image, which allows quantification with appropriate software.

The author performed all the following experiments personally.

4.3 Results

4.3.1 The CAM in the open dish and window models

Both the open dish technique as well as the window techniques was originally utilised. The open dish offers the advantages of advanced speed and easier access for manipulation of angiogenesis. Alternatively, the window technique is more tedious in preparation but offers the advantage of minimal disruption in the environment of the growing embryo.

The open dish technique (Fig. 20) involved culture of the embryo in a bacteriological glass petri dish or equivalent vessel. Although survival of the embryos until day 7 was achieved, this technique was finally abandoned due to very high embryo mortality (>90%) despite attempts with various vessels and culture settings.

The window technique was therefore used as described in methods and materials (fig. 21) using this technique we achieved survival of embryos >70% in consecutive series and therefore was chosen as more suitable for further use.

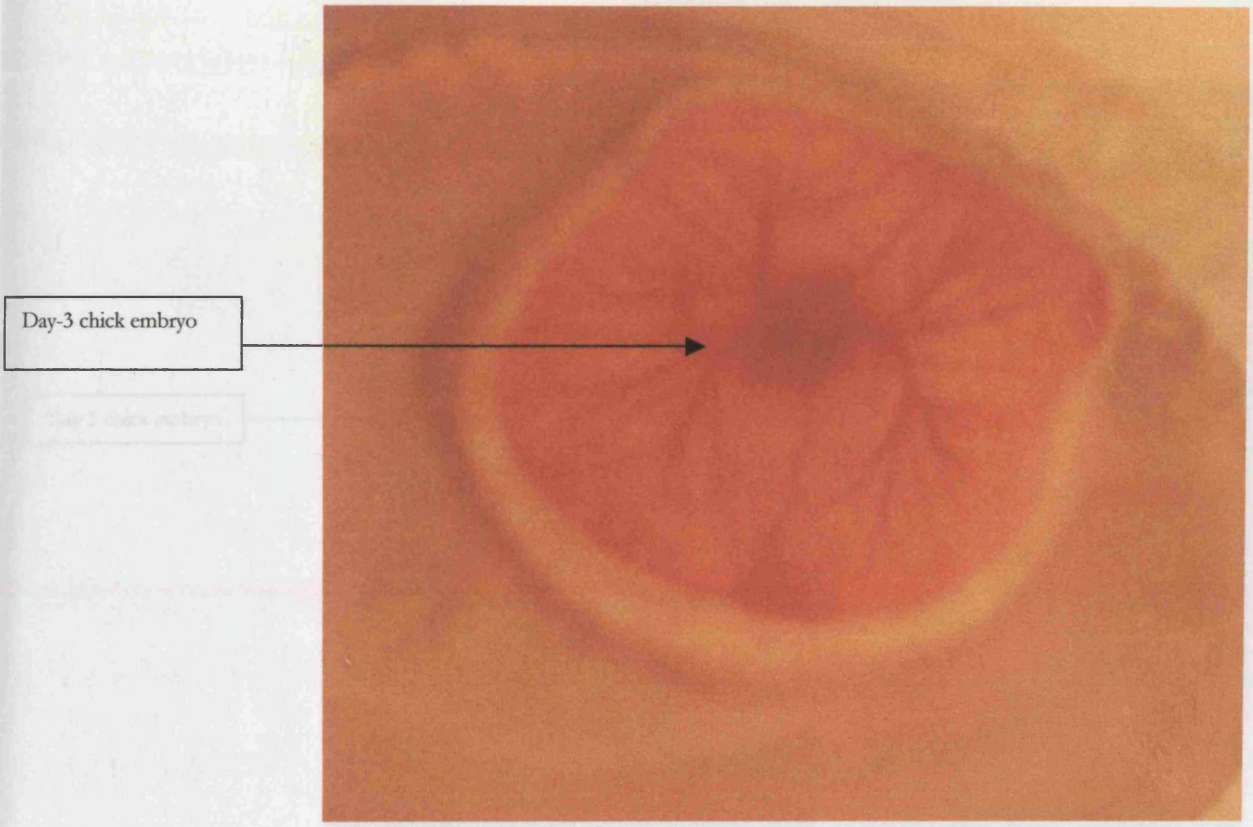


Fig. 20: The CAM open dish technique: Chick embryo day 3.

Fertilised white Leghorns were cracked open under laminar flow on the 3rd day of the incubation into 100mm X 20mm glass Petri dishes. The dishes were sealed and then returned to the incubator for further growth.

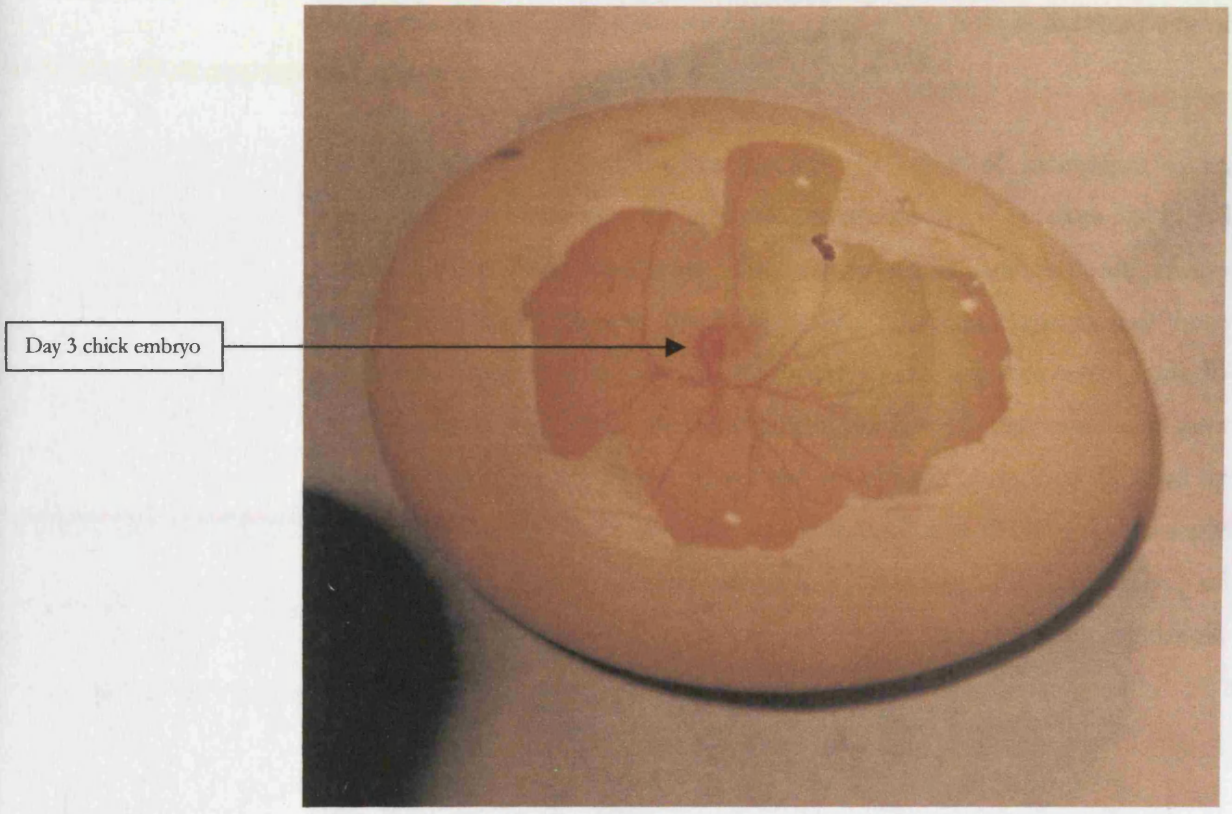


Fig. 21: The CAM window technique: chick embryo day 3.

Fertilised white Leghorns were used at incubation age day-3. A window of approximately 1.5 x 1.5 cm was cut ¾ of the thickness of the cell over the embryo with the use of a motor tool fitted with a spherical emery wheel. Special care was taken to avoid damage to the cell membrane and underlying egg mass. This piece of cell was removed followed by the underlying cell membrane. The window was sealed and returned to the incubator.

4.3.2 Angiogenesis during development of the CAM

During this series we show the successive steps in the normal development of the CAM from day 3 to day 11. (Fig 22)

The CAM becomes visible after the first 72 hours of incubation as an expanding disk overlying the embryo. Anatomically, it receives its blood supply directly from the heart. Two major vessel branches are always identified initially developing in anti-diametrical positions. Expansion of those vessels and branching to second, third and fourth degree vessels leads to development of the CAM. The expansion of the CAM is very rapid until days 7-8 , gradually slowing down to cover the entire inner surface of the shell by day 11.By day 7 onwards the membrane is maturing showing increasingly more complex microvascular network. Therefore, different days of development are chosen depending on the nature of the angiogenic phenomena to be studied.

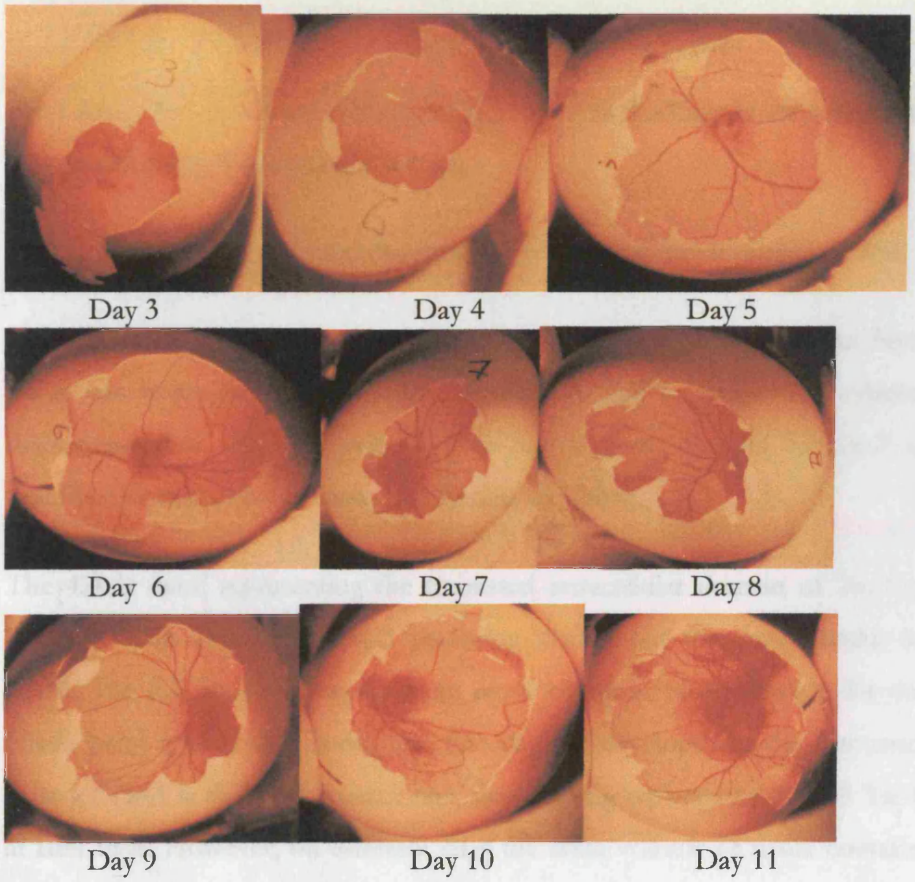


Fig. 22: Normal development of CAM from day 3 to day 11:

Fertilised white Leghorns were incubated until day 11. The CAM was exposed using the window technique on day 3. Here we show that the CAM undergoes rapid development in size and in vessel network complexity during embryonic development.

4.3.2 Tie-1 is expressed in the developing CAM.

Tie-1 expression and truncation pattern of Tie-1 in the Cam During days 3 to 8 were determined by immunoblotting.

During this series we detected the presence of Tie-1 in the developing CAM.

Fresh sections of CAM were taken of approximately 1X1 cm^2. The fresh tissue was homogenised as decribed in methods and materials. The extracts underwent SDS –PAGE electrophoresis and western blotting for Tie-1 as described in methods and materials. Results are shown.

The 42kda band representing the truncated intracellular domain of Tie-1 is detected from embryonic day-3 persisting throughout the development of CAM. The full Tie-1 is detected from day 7 onwards. Normalisation for the 50kda band reveals that during the first days of development the truncated form of Tie-1 is the predominant one, slowly being replaced by the full Tie-1 at later days. However, on different days the same volume of tissue contains different amount of endothelial cells therefore the total amount of Tie-1 detected is related to the the number of endithelial cells present in the specimen, nevertheless relative distribution between full and truncated Tie-1 could be accessed. (Fig. 23 & 24). Moreover, differences inn the D3 and D4 on different blots could be attributed to different tissue loading, biological variation and non specific binding of the antibody.

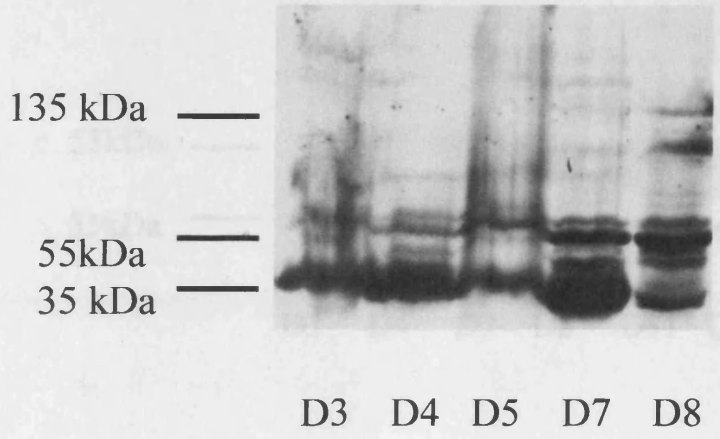


Fig. 23: Tie-1 Expression in the developing CAM.

Fertilized white Leghorns were incubated until day 8 using the window technique. Fresh sections of CAM were taken of approximately 1*1 cm^2. Fresh tissue was homogenised as described in methods and materials. Extracts underwent SDS –PAGE electrophoresis and western blotting for Tie-1 as described in methods and materials. Results representative of four independent experiments are shown.

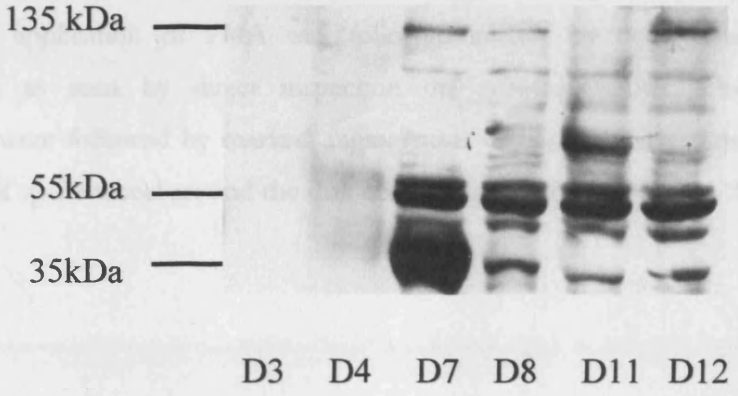


Fig. 24: Tie-1 Expression in the developing CAM (later days).

Fertilized white Leghorns were incubated until day 14 using the window technique. Fresh sections of CAM were taken of approximately 1*1 cm^2. Fresh tissue was homogenised as described in methods and materials. Extracts underwent SDS –PAGE electrophoresis and western blotting for Tie-1 as described in methods and materials. Results representative of four independent experiments are shown.

4.3.3 Vessel destabilisation following PMA application

It has been shown previously that PMA is a potent angiogenic agent .In an attempt to chemically manipulate angiogenesis. PMA was applied on day 5 CAMs. The application of PMA was followed initially by oedema and haemorrhage as seen by direct inspection on growing CAMs. These phenomena were followed by marked angiogenesis taking the characteristic appearance of spoke wheel around the disk containing the activator. (Fig. 25)

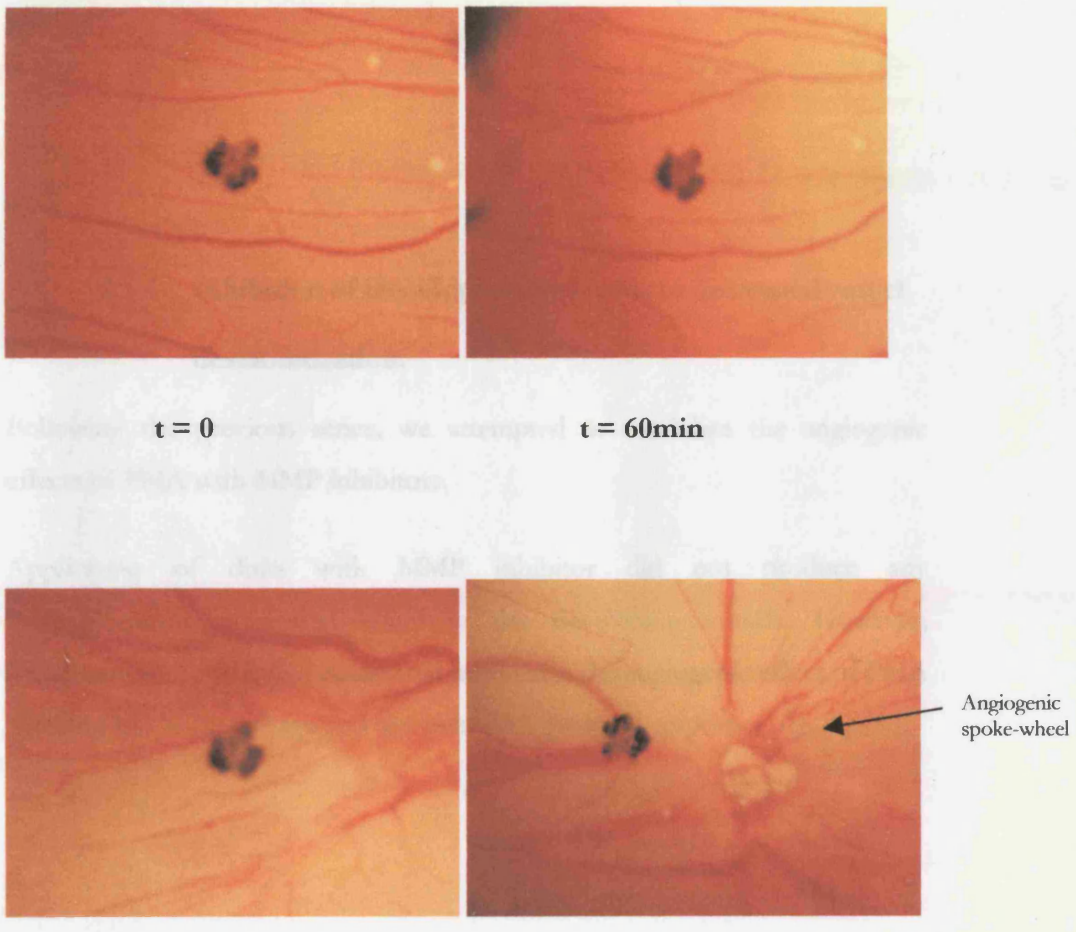


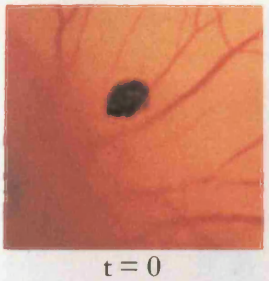
Fig. 25: Vessel destabilisation following PMA application:

Fertilized white Leghorns were incubated until day 7 using the window technique. Sterile disc containing lyophilised 20μg/ml PMA was applied. Serial photographs were taken at time point 0, 60 minutes, 12 hours and 24 hours. Signs of vessel destabilization such as leakage, oedema, thrombosis, and vessel architectural changes as seen. Black dot represents marker on superior surface of disc. Results representative of four independent experiments are shown.

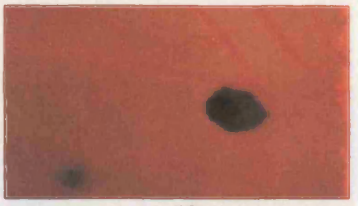
4.3.4 Inhibition of metalloproteases leads to decreased vessel destabilitisation.

Following the previous series, we attempted to modulate the angiogenic effects of PMA with MMP inhibitors.

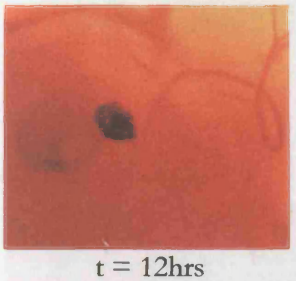
Application of disks with MMP inhibitor did not produce any macroscopically detectable effect in the developing vessels. However, combination of PMA and Batimastat modulates the angiogenic effect of PMA showing less haemorrhage and less marked angiogenic response. (fig.26).



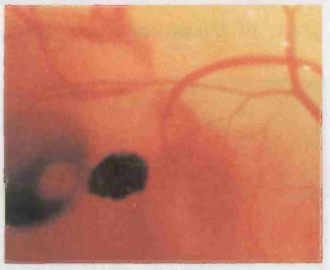
t = 0
batimastat $10^{-3}\mu$ m



t = 60
batimastat $10^{-3}\mu$ m

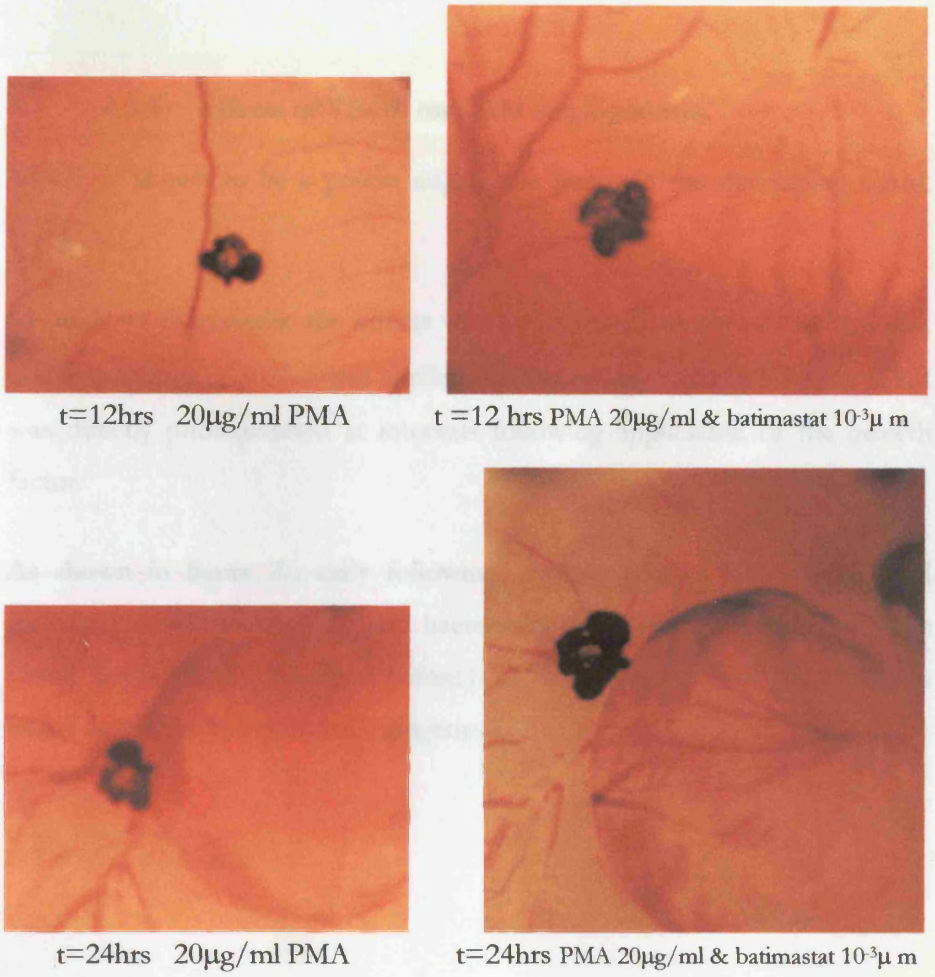


t = 12hrs
batimastat $10^{-3}\mu$ m



t = 24hrs
batimastat $10^{-3}\mu$ m

CAMs activated with batimastat $10^{-3}\mu$ m



CAMs activated with:

20µg/ml PMA & batimastat 10^{-3}µ m **20µg/ml PMA**

Fig. 26: Vessel destabilisation following PMA application. Inhibition of metalloproteases leads to decreased vessel destabilization.

Fertilized white Leghorns were incubated until day 7 using the window technique. Sterile disc containing lyophilised 20µg/ml PMA, batimastat 10^{-3}µ m and PMA 20µg/ml with batimastat 10^{-3}µ m was applied. Serial photographs were taken at time point 0, 60 minutes, 12 hours and 24 hours. Signs of vessel destabilization such as leakage, oedema, thrombosis, and vessel architectural changes as seen. Batimastat on its own does not cause any effect on the developing CAM. How ever, Batimastat reduces the distabilizating effect of PMA on the CAM. Black dot represents marker on superior surface of disc. Results representative of four independent experiments are shown.

4.3.5 Effects of VEGF on CAM angiogenesis.

VEGF is shown to be a potent angiogenic factor in the developing CAM. [172]

In order to characterise the effects of VEGF in our model of CAM a disk containing 2μgs of VEGF was applied directly on day 7 CAM. The live CAM was directly photographed at intervals following application of the growth factor.

As shown in figure 27, early following application of VEGF on CAM increased vessel permeability and haemorrhage are evident as a brush –like vessel formation as described before.[172, 173] This is most likely due to vessel destabilisation prior to angiogenesis.[2]

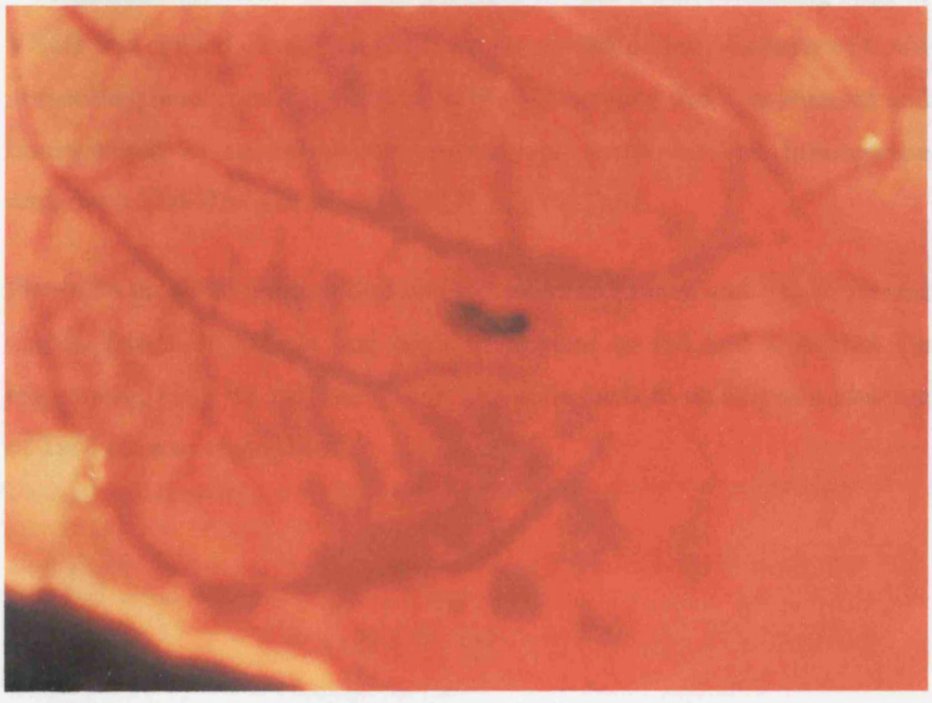


Fig. 27: Effects of VEGF on CAM angiogenesis.

Fertilized white Leghorns were incubated until day 7 using the window technique. Sterile disc containing lyophilised 2µg of VEGF. Serial photographs were taken at time point 0 and 24 hours. Signs of vessel destabilization such as leakage, oedema, as seen. Black dot represents marker on superior surface of disc. Results representative of four independent experiments are shown.

4.3.7 VEGF activation leads to proteolytic cleavage of Tie-1 extracellular domain in the developing CAM.

To determine the effect of VEGF on Tie-1 in vitro a disk containing 2μgs of VEGF was applied directly on day 7 CAM. A disk of 1cm diameter of CAM immediately underneath the applied VEGF containing disk was resected and homogenised as described in materials and methods. The homogenate underwent SDS-PAGE and western blotting for Tie-1.

Figure 28 shows an immunoblot of Tie-1 in control trated and VEGF treated CAMs. Bands of 126kda and 42 kDa respond to full and truncated Tie respectively. Here we see that VEGF activation leads to ectodomain cleavage of Tie-1 prior to angiogenesis.

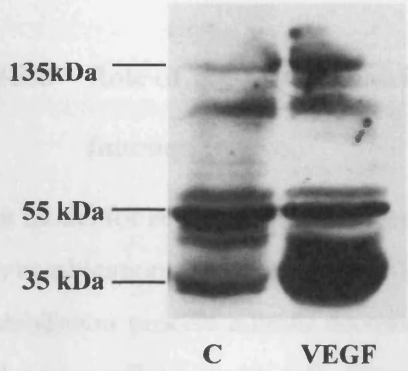


Fig. 28: VEGF activation leads to proteolytic cleavage of Tie-1 extracellular domain in the developing CAM.

Fertilized white Leghorns were incubated until day 7 using the window technique. Sterile disc containing lyophilised 2μg of VEGF. Fresh sections of approximately 1*1 cm^2 of the underlying CAM were taken Fresh tissue was homogenised as described in methods and materials. Extracts underwent SDS –PAGE electrophoresis and western blotting for Tie-1 as described in methods and materials. Results representative of four independent experiments are shown.

4.3.8 Role of Tie-1 truncation in VEGF induced neovessels function in vivo.

A current model for regulation of angiogenesis by VEGF involves the growth factor, in combination with angiopoietin 2, destabilising existing microvessels. This destabilistion process entrails decreased association between endothelial cells and perivascullar support cells allowing for endoththelial cell migration and proliferation.[174-176] The pro-survival activity of VEGF ensures endothelial apoptosis does not result from removal of pericytes support. As described on chapter I VEGF stimulates Tie-1 endodomain formation, most likely by increased cleavage of full length Tie-1 It was of interest therefore to determine whether Tie-1 cleavage has any roles in VEGF induced angiogenesis in vivo.To do this the effects of of VEGF on CAM angiogenesis were analysed and the truncation state of Tie-1 determined. To determine the effects of inhibiting Tie-1 cleavage attempts were made to develop peptide based antagonists of the protease with the aim of testing their impact on VEGF induced destabilisation, neovessels formation and endothelial function in vivo.

The Tie-1 cleavage site was identified near the transmembrane region. We attempted to synthesise a peptide corresponding to the cleavage remnant and raised an antibody against it. Initial experiments utilising this antibody against the developing CAM on day 12 showed destabilisation of the vasculature. (fig 29 & 30).

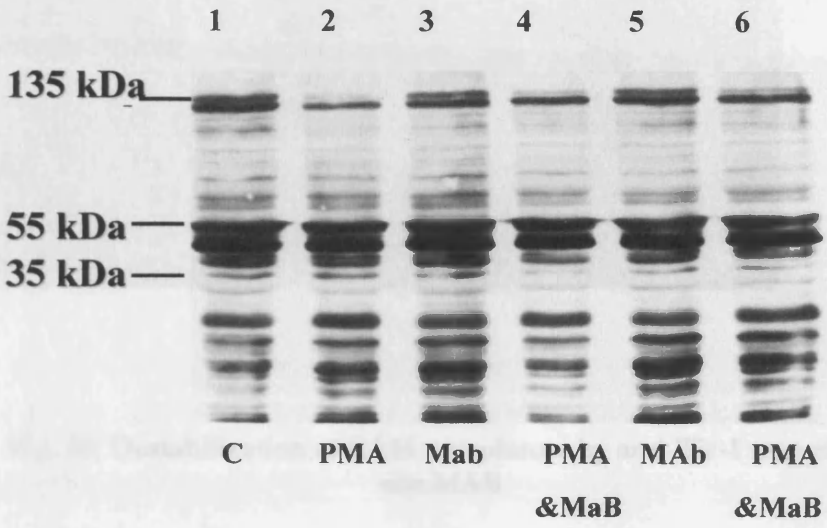


Fig. 29: Regulation of PMA-induced Tie-1 ectodomain cleavage by anti-Tie-1 truncation site Mab.

HUVECs were challenged with control vehicle for 60 min, 10 ng/ml PMA for 20 min anti Tie-1 truncation site Mab for 1 hour or PMA 20ng/ml on pretreated Ecs with anti-Tie-1 truncation site Mab for 1 hour. Following the prescribed times of treatment cells were lysed, proteins were resolved by SDS/PAGE and Tie-1 was detected by immunoblotting. The positions of full and Tie-1 endodomain are indicated with their predicted molecular weights. A non-specific immunoreactive band is seen at approximately 55kda. Blot representative of five independent experiments.
Here we see that PMA induces truncation of Tie-1 as indicated by the loss of the Tie-1 doublet at 135kDa (lane 1). Use of Mab only does not change the morphology of the doublet (lanes 3 & 5). However, PMA-induced Tie-1 trucation in the presence of Mab is reduced as demopnstated by the relative preservation of the superior part of the doublet (lane 6)

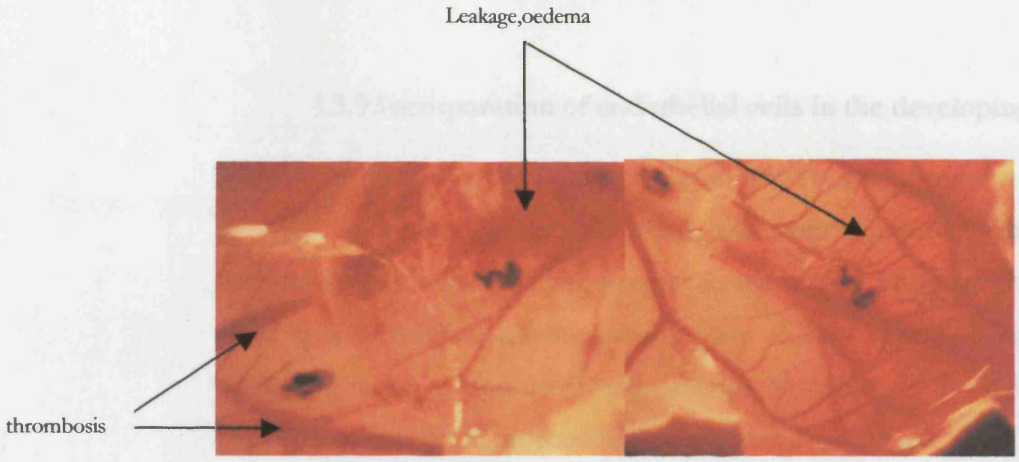


Fig. 30: Destabilization of CAM vasculature by anti-Tie-1 truncation site MAB.

Fertilized white Leghorns were incubated until day 10 using the window technique. Sterile disc containing lyophilised 20µl of anti-Tie-1 truncation site MAb PMA was applied. This photograph is taken at time point 24 hours. Signs of vessel destabilization such as leakage, oedema, thrombosis, and vessel architectural changes as seen. Black dot represents marker on superior surface of disc. Results representative of three independent experiments are shown.

4.3.9 Incorporation of endothelial cells in the developing CAM

The aim of these experiments was either to test the role of truncated receptor in vivo by incorporating endothelial cells with truncated receptor and comparing with non-cleavable receptor in neovessels. Moreover, it could be possible to incorporate a non-cleavable form of Tie-1 in endothelial cells that are incorporated in neovessels and examine the effect of not being able to cleave Tie-1 on VEGF induced re-modelling of these vessels.

In an attempt to manipulate angiogenesis in the developing CAM, exogenous endothelial cells were directly transferred onto the CAM on day 5.

Bovine aortic endothelial cells were cultured to confluence and directly applied on the developing CAM as described in methods and Materials. Following 24hs of incubation, the CAMs were harvested.

Confocal microscopy shows exogenous fluorescent endothelial cells aligning around developing vessels. Moreover, tissue section and staining for x-gal shows incorporation of endothelial cells in the CAM.

Although incorporation of endothelial cells proved to be feasible, this method was not used for further study due to extensive variability on the transfection rate and survival of the embryos. (Fig. 31 & Fig 32)

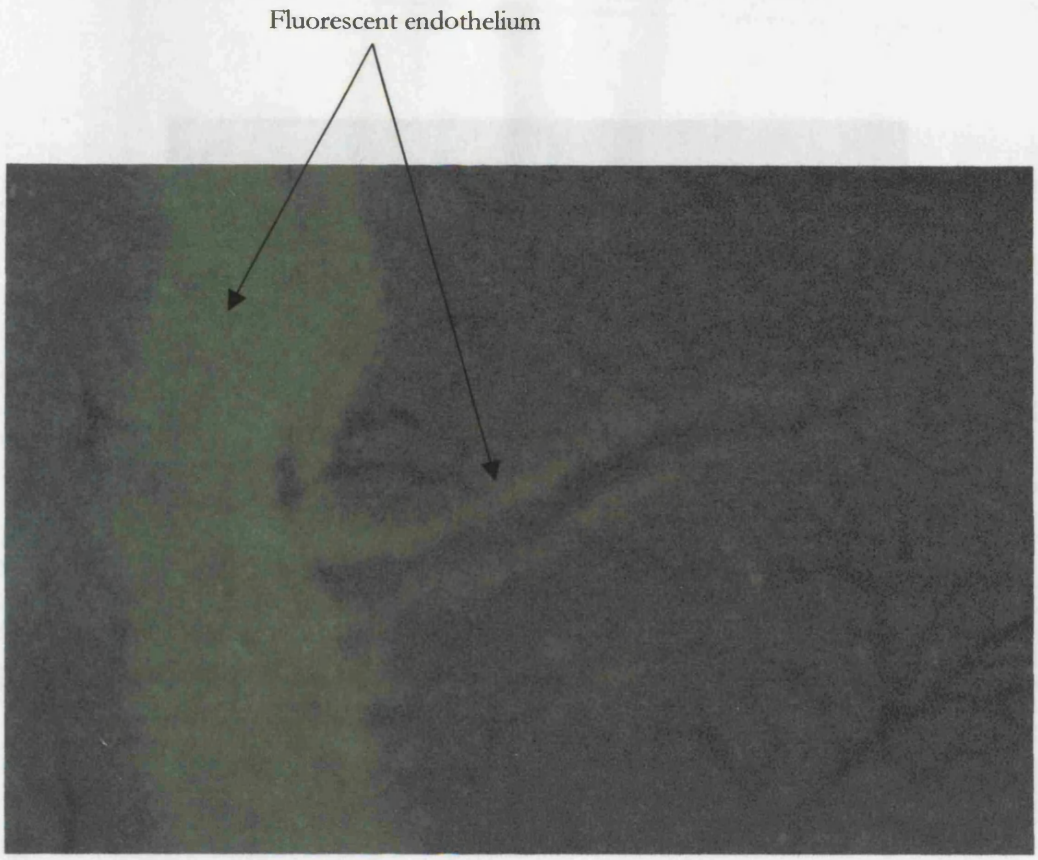


Fig. 31: Incorporation of endothelial cells in the developing CAM.

Bovine endothelial cells stained usiong the PKH26 red fluorescent cell linker (Sigma) were directly applied on the developing CAM . CAMs were examined using standard steromicroscope 5x with filter setup for fluorosceine or Rhodamine

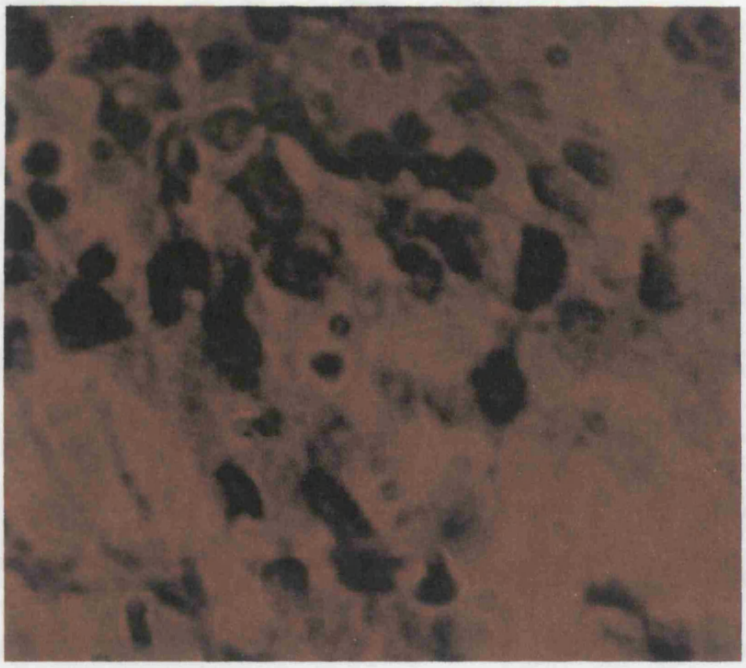


Fig. 32: Incorporation of endothelial cells in the developing CAM.

Bovine endothelial cells stained using B-gal cell marker applied on the developing CAM. Following 24 hs CAMs were harvested and fixed. . CAMs waere then stained for 24 hours at 4^0 C in x-gal solution an then were embedded in paraffin and sectioned.Direct microscopy at 20x. Result representative of four independent experiments.

4.4 Discussion

Normal embryonic vessel development can be studied using the CAM model. Embryonic angiogenesis in these circumstances is tightly regulated and is complete by day 12 to 14 of gestation.

Incorporation of exogenous endothelial cells in the developing cells is a very attractive research possibility. Direct application of cells leads to variable incorporation of the cells to the developing vessel. Possibly, microinjection of cells into the vessel lumen could improve the incorporation rate. Transfection of those cells with c-DNA could help research in angiogenic factors and receptors.

Activation with PMA or VEGF leads initially to vessel destabilisation. This presents macroscopically as oedema and haemorrhage. Angiogenesis soon follows. This cascade of phenomena is regulated by metalloproteinase and can be controlled by MMP inhibitors.

Tie-1 is detected form the very early days of CAM development. During the first days, when vessel development is active, Tie-1 is mainly detected in the truncated form. After day 7 or 8, when the angiogenic activity of the CAM starts to subside, the Tie-1 molecule is detected in the full form. Activation with VEGF leads to ectodomain cleavage of Tie-1, as was predicted by our previous in vitro experiments. During that stage, the vessels are destabilised

before angiogenesis. A potential physiological role therefore can be stipulated for Tie-1 in maintaining vessel stability when in full form. Ectodomain cleavage possibly is a prerequisite for angiogenesis leading vessel destabilisation and thus allowing the angiogenic factors to activate the endothelial cells. The lack of known ligands makes further substantiation of the hypothesis difficult.

However, preliminary experiments with mabs against the cleavage site suggest vessel destabilisation when interfering with the ability of Tie-1 to signal. Further experiments with purified mabs will be necessary to further elucidate the physiological role of Tie-1 in vessel development.

CHAPTER 5: CHARACTERISATION OF TIE-1 IN HUMAN PLATELETS.

5.1 Introduction

In the course of an investigation into Tie-1 ectodomain in serum, Tie-1 was detected in platelets. Given the number of circulating platelets this represents a significant pool of receptor. Platelet Tie-1 could regulate local concentrations of any Tie-1 ligands available to the endothelial receptor. Furthermore, it is also conceivable that Tie-1 may have a signalling role in platelets. The importance of Tie-1 in vessel formation and maintenance thereafter prompted further characterisation of the platelet receptor.

5.2 Materials and methods

5.2.1 Materials

Affinity purified goat antibodies recognizing the Tie-1 and Tie-1-2 (Tie-2) extracellular domains were obtained from R& D Systems (Abingdon, Oxon, UK) and rabbit antibody against the intracellular domain of Tie-1 from Santa Cruz Biotechnology Inc (supplied by Autogen Bioclear, Wilts, UK). Human umbilical vein endothelial cells were isolated and cultured as previously described. Blood samples were collected from healthy donors via venopuncture in the antecubital vein of an uncuffed arm.

5.2.2 Methods

5.2.2.1 Fluorescence activated flow cytometry

Expression of Tie-1 on the platelet surface was analysed using a modification of a whole blood cytometric technique, as follows:

Blood was collected from healthy donors and diluted within 10 minutes 1:10 in HEPES goat anti-Tie-1 antibody (1:20), with or without ADP (10 μmol/l), thrombin (0.8 units/ml) or PMA (10ng/ml). Samples incubated with thrombin also contained GPRP peptide (0.125 mmol/l) to prevent fibrin cross-linking and subsequent clotting. Samples were incubated for 30 minutes at 22°C after which 5μl of a 1:40 dilution of anti-goat FITC were added and samples were incubated for a further 30 min. Samples were then diluted 1:100 in 0.2% formyl saline (0.2% formaldehyde in 0.9% NaCl) and analysed, within 2 hours, in a Coulter XL flow cytometer. Platelets were identified by means of

their light scatter characteristics (forward and side scatter) and analysed for fluorescence in logarithmic mode. For each donor positive fluorescence was determined against samples incubated with the second layer antibody alone and the identity of the platelet population was confirmed with a FITC-conjugated CD42b (RFGP37). All samples were analysed in duplicate and results are expressed as Binding Index [177].

5.2.2.2 Western blotting

1 ml of ACD (0.085 trisodium citrate, 0.071 citric acid, and 0.11M glucose) was added to 6 ml of whole citrated blood. The platelet rich plasma was prepared by centrifugation at 235xg for 15 min and 2mls of PGI2 (0.2 mg/ml in HBS) was added to 2mls of platelet rich plasma. The platelets were sedimented by further centrifugation at 650xg for 15 min, the supernatant was removed, the platelets were gently resuspended in HEPES buffered saline (HBS) pH 6.0, PGI2 (0.2 mg/ml in HBS) (1µl per 1 ml PRP) was added and the solution was further centrifuged at 400xg for 15 min. This step was repeated twice. The pellet was resuspended in 10 mls HBS pH 7.4 with 1mM CaCl2 and the solution was divided in two tubes. Confirmation of platelets in the sample was performed by flow cytometry. Aliquots of resting platelets containing $2x10^7$ cells were challenged with control vehicle (resting) or activators for 30 min. Cells were then collected by centrifugation at 650xG and lysed directly or stored at -70°C for subsequent analysis. Platelets were lysed by addition of sample buffer containing 100mM dithiothrietol and heated at 105^{O} C for 4 min. Control treated or phorbol ester stimulated

endothelial cells were similarly lysed. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE) and electrotransferred to nitrocellulose membranes. Following blocking membranes were probed with antibodies and immunoreactive proteins detected using horseradish peroxidase conjugated secondary antibody and the ECL detection system (Amersham International Plc, Bucks, UK).

Statistical analysis (Student's t-test) comparing thrombotic indexes was performed with the use of Statistica software StatSoft, Inc. (1995).

The author performed all the following experiments personally. Assistance with Fluorescence activated flow cytometry by department of Biochemistry , Glenfield Hospital.

5.3 Results

5.3.1 Tie-1 is present in platelets

In the course of an investigation into circulating Tie-1 ectodomain we detected significant levels of Tie-1 associated with platelets.

Blood samples were collected from healthy donors via venopuncture in the antecubital vein of an uncuffed arm. Samples were incubated and analysed as described in methods and materials.

Using an antibody recognizing the extracellular domain of Tie-1, flow cytometric analysis reveals specific binding of the antibody to resting platelets (fig. 33)

In order to confirm the presence of Tie-1 and characterize platelet Tie-1 in more detail Western blotting was performed on platelet extracts, as described in methods and materials. The antibody used for blotting was different from that used for flow cytometry and was raised against the carboxy-terminus of Tie-1. This antibody has been described previously [178].

As shown in figure 34, Western blotting revealed a major immunoreactive Tie-1 band of molecular mass 110 kDa present in platelets. For comparison endothelial cell extract was also subject to electrophoresis and blotting under exactly the same conditions as the platelet extract. Endothelial Tie-1 was present as a doublet of 135 and 125 kDa as has been previously described.

In order to test for the presence of the closely related Tie-2 platelet extracts were also probed with an antibody raised against the extracellular domain of Tie-2. No reactivity was seen indicating that platelets do not express Tie-2 (fig. 34). The same antibody did recognize Tie-2 in blots of endothelial cell, which revealed a strongly immunoreactive band of 140 kDa corresponding to Tie-2 (fig 34).

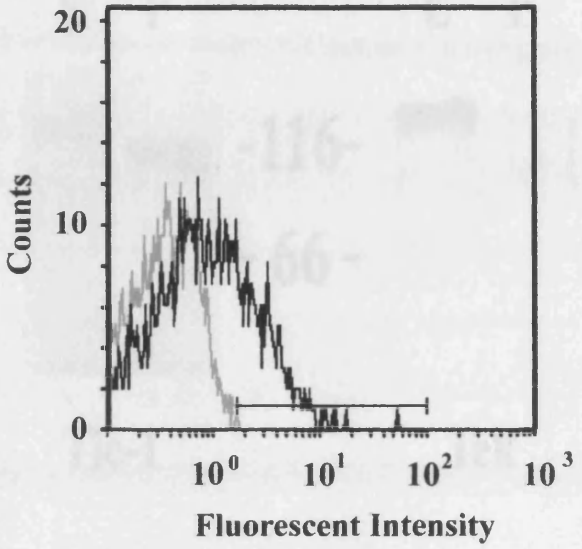


Fig. 33: Expression of Tie-1 on the platelet surface.

Whole blood was incubated with secondary antibody only (grey) or with goat anti-Tie-1 (black) followed by FITC-conjugated secondary detection antibody and analysed by flow cytometry.

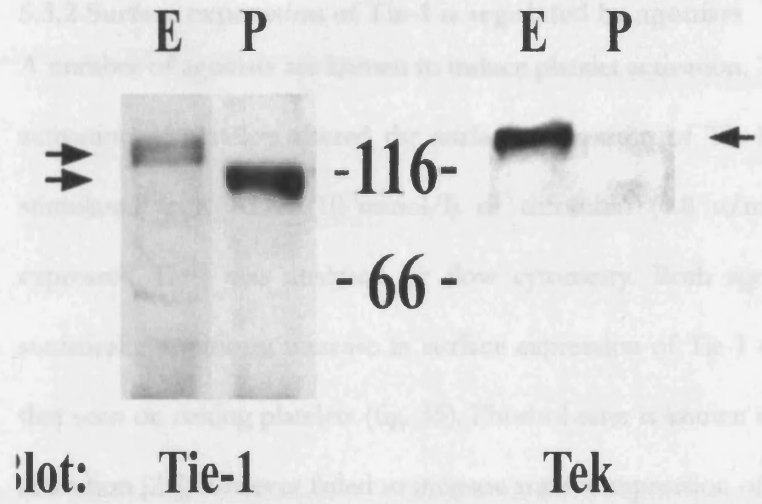


Fig. 34: Comparison of Tie-1 and Tie-1-2 in human platelets and endothelial cells.

Human platelets (P) were isolated and human umbilical vein endothelial cells (E) were cultured as detailed in Materials and Methods. Cells were lysed and proteins resolved by SDS/PAGE, transferred to membranes and Tie-1 and Tie-1-2 were detected by Western blotting. The position of Tie-1 and Tie-1-2 on blots is indicated by arrows. Relative mobility of molecular mass markers is indicated in kDa.

5.3.2 Surface expression of Tie-1 is regulated by agonists

A number of agonists are known to induce platelet activation. To test whether activation of platelets altered the surface expression of Tie-1 platelets were stimulated with ADP (10^{-5}mmol/l) or thrombin (0.8 u/ml) and surface expressed Tie-1 was analysed by flow cytometry. Both agonists caused a statistically significant increase in surface expression of Tie-1 over and above that seen on resting platelets (fig. 35). Phorbol ester is known to cause platelet activation [26], however failed to increase surface expression of Tie-1 (fig. 35).

5.3.3 Platelet Tie-1 is not truncated in response to phorbol esters

We shown previously that endothelial Tie-1 undergoes ectodomain cleavage in response to stimulation with phorbol esters, VEGF and TNF β [132, 178-180]. It was of interest therefore to determine whether platelet Tie-1 is similarly regulated.

Resting platelets were stimulated with the phorbol ester PMA for 30 min before lysis, gel electrophoresis and blotting. Blots were probed with an antibody recognizing the intracellular domain of Tie-1 (fig 36). For comparison human endothelial cells were also stimulated and Tie-1 truncation similarly examined.

Control treated endothelial cells have a 135/125 kDa doublet of full length and partially glycosylated Tie-1. PMA stimulation results in loss of the surface expressed 135kDa receptor and generation of a 45 kDa truncated form of Tie-1 (fig 4). This has previously been shown to result from metalloprotease

mediated proteolytic cleavage in the extracellular domain of Tie-1 [132, 178-180]. In contrast to endothelial cells, PMA stimulation of platelets did not result in the generation of truncated Tie-1 although the level of the full-length receptor was markedly decreased (fig 36).

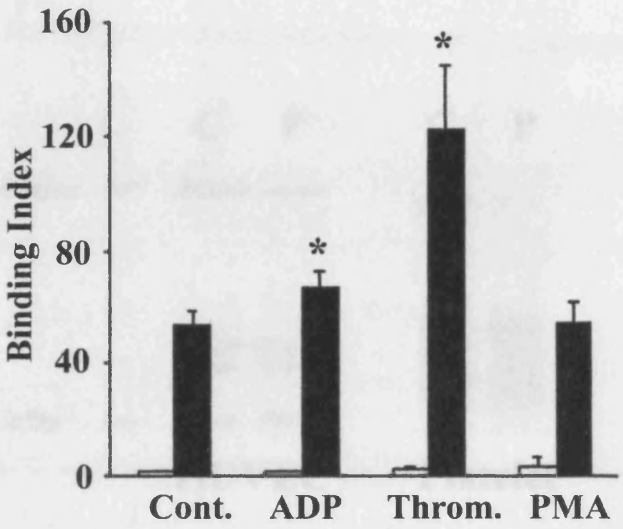


Fig. 35: Expression of Tie-1 on the surface of activated platelets.

Platelets were stimulated with control vehicle, ADP (10 µmol/l), thrombin (0.8 units/ml) or PMA (10 ng/ml) for 30 min as indicated. Tie-1 surface expression was determined by fluorescence activated flow cytometry analysis as described in Materials and Methods. Results are shown as means +/- SEM for 4 independent experiments, solid bars represent specific anti-Tie-1 binding ability and empty bars represent corresponding non-specific Ig fluorescence, * indicates $P < 0.05$, Students 't' Test.

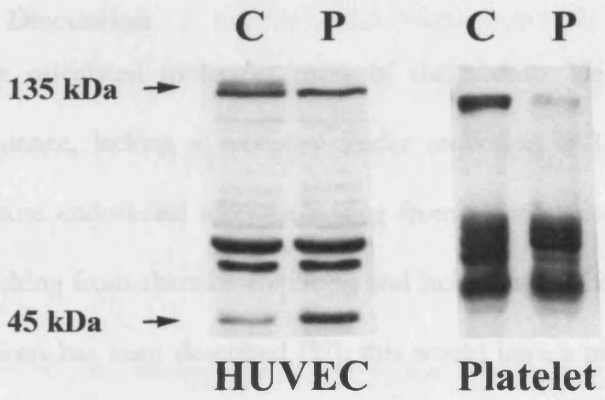


Fig. 36: Platelet Tie-1 does not undergo regulated ectodomain cleavage.

Human umbilical vein endothelial cells (HUVEC) or platelets were stimulated for 30 min with PMA (10ng/ml) before cell lysis and Western blotting as described in Materials and Methods. Arrows indicate full length and truncated Tie-1 in endothelial cells.

5.4 Discussion

The calculated molecular mass of the human Tie-1 derived amino acid sequence, lacking a secretory leader sequence, is 122.8 kDa; the 135kDa mature endothelial receptor arising from glycosylation[37]. A form of Tie-1 resulting from alternative splicing and lacking one of its three EGF-homology regions has been described [37]; this would have a predicted molecular mass of 118kDa before glycosylation. Recently another group has reported rat platelets to possess Tie-1 based on immunocytochemistry of balloon injured rat carotid arteries. [181]Thus the lower molecular mass of platelet Tie-1 is not due to decreased glycosylation or the lack of an EGF homology domain. The most likely explanation is that platelet Tie-1 is partially truncated. As the blotting antibody recognizes the carboxy-terminal sequence of Tie-1 any truncation would have to be at the extracellular amino-terminus. It is possible that partial truncation in the ectodomain of platelet Tie-1 could affect its ability to bind ligand. Determination of the functional consequences of differences in molecular mass between endothelial and platelet Tie-1 will require identification of a ligand for the receptor.

The functional importance of increased Tie-1 expression on the surface of activated platelets is currently under investigation. It is possible that the receptor could have a signalling role in controlling aspects of platelet function following stimulation. The extracellular domain of Tie-1 contains a number of

features involved in protein: protein interactions, including the EGF-homology domains. It is possible that these may be involved in binding Tie-1 to cell surfaces or matrix bound proteins and thus Tie-1 could mediate aspects of platelet adhesion following activation. Phorbol ester is known to cause platelet activation[182], however failed to increase surface expression of Tie-1 (fig. 3).

In contrast to endothelial cells, PMA stimulation of platelets did not result in the generation of truncated Tie-1 although the level of the full-length receptor was markedly decreased (fig 4). This indicates that PMA does not induce Tie-1 truncation in platelets. However, the loss of the full-length receptor suggests that it is being released from the cells. In addition to endothelial Tie-1, a number of transmembrane proteins undergo ectodomain cleavage following phorbol ester activation of cells; these include the nerve growth factor receptor TrkA, ErbB-4 and colony stimulating factor-1 receptor [143, 183]. The mechanism whereby protein kinase C activation leads to proteolytic cleavage of these proteins is not known. The finding that platelet Tie-1 is not subject to this type of modification suggests critical components of this system are lacking in these cells.

In conclusion, our data demonstrate the presence of a significant pool of the receptor tyrosine kinase Tie-1 in platelets from normal subjects. There are

important differences between the receptor expressed in endothelial cells and that on platelets. Most notably, the platelet receptor differs in mass from that of the endothelial protein and this may have functional consequences. Stimulation of platelets increases surface expression of Tie-1 however; again in contrast to the endothelial form, the receptor does not undergo truncation in response to PMA, or other platelet activators. Platelet Tie-1 has the potential for modulating endothelial function by competing for any Tie-1 ligands and could have signalling roles important in controlling aspects of platelet behaviour.

Chapter 6

CHAPTER 6: CLOSING REMARKS-FUTURE RESEARCH

In conclusion, this thesis shows that the endothelial receptor tyrosine kinase Tie-1 is regulated by the angiogenic growth factor VEGF. This growth factor induces Tie-1 endodomain formation with concomitant loss of full length receptor and the process is inhibited by metalloprotease inhibitors. This scheme is consistent with VEGF-induced Tie-1 cleavage.

Although it is reasonable to assume that this phenomenon will prevent downstream signalling via the receptor due to loss of its ligand-binding capability, the resulting intracellular fragment has previously been shown to associate with tyrosine-phosphorylated proteins and here is shown to increase endothelial cell survival in the presence of angiogenic factors. These findings are in agreement with previous observations in adult chimeras that Tie-1 is required to support the survival or proliferation of certain types of endothelial cells[38]. In the absence of any known ligand for Tie-1, the cleavage phenomenon is the only known signaling mechanism for this receptor via which is demonstrates its physiological role. Tie-1 cleavage was shown to occur in vivo during angiogenesis as well as following stimulation with exogenous angiogenic growth factors and is associated with vessel destabilisation prior to angiogenesis.

More specifically, our experiments suggest that initiation of angiogenesis that would require destabilisation of the preexisting vasculature is associated with the cleaved form of Tie-1. It is possible trhat the angiogenic factors (mainly VEGF) cleave the Tie-1 recepor at an early stage in order to allow for mobilisation and proliferation of endothelial cells. This is also suggested by the fact that cleaved Tie-1 is associated with endothelial cell proliferation, nessessary at the early stages of angiogenesis. Moreover, at the later stages of angiogenesis, during the stages of maturation and establishment of quiescent vascular bed Tie-1 resumes its full form contributing to vessel stability.

In addition, Tie-1 was identified in platelets in a different, non-cleavable isoform than the endothelial one. The cell surface expression if Tie-1 increases following platelet activation. Taking into account the large number of circulating platelets it is possible that platelet Tie-1 could regulate circulating Tie-1 ligand levels by sequestration. In addition, the expression of Tie-1 on the surfaces of both platelets and endothelial cells, similar to other angiogenesis associated molecules such as the β3 integrins places Tie-1 in a strategic position for the control of functions such as haemostasis, wound healing and angiogenesis.

Previous studies have suggested functional cooperation beween Tie-2 and Tie-1. Recently it hass been shown thatTie-2 in endothelial cells is physically associated with Tie-1 providing a direct mechanism for such functional cooperation. It is possible then for VEGF to modify the Tie-2: Tie-1 comlex

demonstrating a previously unspected level of crosstalk between VEGF , an initiator of vessel growth and remodelling and Tie-1 and Tie-2, regulators of vessel stability. Such interactions are likely to be important in coordinating control of blood vessel formation and remodelling.

The identification by others of the Tie-1 cleavage site on the extracellular domain of the receptor adjacent to the transmembrane region enabled construction of a peptide corresponding to the extracellular stump and development of a monoclonal antibody against it. The initial experiments showed such an antibody to have mild inhibitory effect on Tie-1 cleavage in cells. Future work to develop more potent inhibitors based on these antibodies, perhaps by increasing access of the antibodies by using them as Fab fragments and more detailed analysis of specificity will be required. It may also be possible to utilise some of the antibodies that react against epitopes in the 'stump' of cleaved receptor to detect truncated Tie-1 in tissue sections by immunocytochemistry.

Vessel destabilisation is associated with a variety of pathologies including acute and chronic inflammation, solid tumour development growth and metastasis as well as atherosclerotic plaque stability. Clearly our ability to understand the mechanisms with which this phenomenon is controlled will increase our knowledge in the pathogenesis of those diseases and lead to the development of new therapeutic strategies in conditions associated with vessel instability and leakage, inflammation and tumour growth and invasion.

The control of blood vessel stability, integrity and regression is important in both pathological angiogenesis and for strategies aimed at therapeutic revascularization by angiogenesis. For example, the clinical effectiveness of therapeutic revascularization is dependent on the functional integrity of the vessels formed. Initiation of angiogenesis without maturation of neovessels leads to immature, leaky vessels prone to thrombosis similar to those found in tumours [184]. Conversely, promoting vessel regression, or suppression of angiogenesis by stabilizing existing vessels, could provide ways of limiting angiogenesis dependent disease. Vessel maturation and establishment of integrity is regulated by the Tie family of receptors found on endothelial cells[15].These receptors are likely to be prime targets for manipulation of angiogenesis, however very little is known about them.

Ectodomain cleavage is under the control of angiogenic growth factors and serves to destabilize vessels prior to growth, remodelling or regression. An understanding of the control of Tie-1 truncation during vessel formation, and its role in regulating vessel integrity and stability, is important for developing more effective means of revascularizing ischaemic tissue and may lead to novel ways of preventing neovascularization or inducing vessel regression in situations such as rheumatoid arthritis and tumour growth.

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