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duthieliby@cardiff.ac.uk

TUMOUR –ASSOCIATED ANGIOGENESIS IN THE DEVELOPMENT AND METASTASIS OF HUMAN COLORECTAL CANCER.

By

Dr Khaled Ali Ramadan Rmali
MD

Supervised by

Professor Wen G Jiang MB BCh MD
Mr Malcolm CA Puntis FRCS PhD

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University Department of Surgery
Wales College of Medicine
Cardiff University
Cardiff, UK

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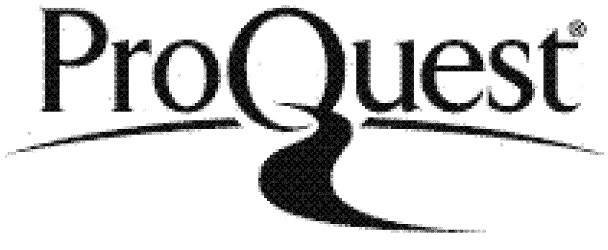

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I dedicate this thesis to my parents, my wife; Ala and my children; Ali and Abeer.

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CONTENTS

List of Figures

List of Tables

Abbreviations

aa	amino acid
Ab	antibody
Ag	antigen
AER	apical ectodermal ridge
Ala	alanine
APC	Adenomatous polyposis coli
APS	Ammonium persulfate
bp	base pair
b-FGF	basic fibroblast growth factor
BSA	bovine serum albumin
BSS	Balanced Salt Solution
CAM	cell adhesion molecule
CAM	chicken chorioallantoic membrane
CD	Cluster of differentiation
cDNA	complementary Deoxyribonucleic acid
cm	centimetre
CO_2	carbon dioxide
CS	chondroitin sulphate
CTL	cytotoxic T-lymphocytes
ddH2O	double-distilled water
DEPC	Diethyl pyrocarbonate
DiI	1,1'–dioctadecyl-3, 3, 3'3'-tetra-methylindocarbocyaninine
DMEM	Dulbecco's modified eagles medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DsDNA	double-stranded deoxyribonucleic acid
ECL	enhanced chemiluminescence
ECM	extracellular matrix
E. coli	Escherichia coli
EDTA	ethylene diaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunoabsorbant assay
Fab	antibody binding site fragment
FCS	Foetal calf serum
FITC	Fluorescein Isothiocyanate
g	gravity (unit of relative centrifugal force)
G418	Geneticin
GAG	glycosaminoglycan
GF	growth factor
GSK-3β	Glycogen synthase kinase-3–beta
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HEPES	N-hydroxyethylpiperazine-N'-2-ethansulphoxide
HER	Human Epidermal growth factor receptor
HEV	High endothelial venules
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
hr	hour
HRP	horseradish peroxidase
HS	heparan sulphate
HSPG	Heparan sulphate proteoglycan

HUVEC	human umbilical vein endothelial cells
ICAM	Intercellular adhesion molecule
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	Interleukin
Kb	kilo-base
kDa	Kilo-dalton
KS	keratan sulfate
LB	Luria–Bertani
LEF-1	leukocyte enhancer factor-1
LFA	lymphocyte function-associated antigen
m	Metre
M	molar
mA	milli-amp
mAb	monoclonal antibody
-ME	-mercaptoethanol
mg	milligram
MHC	Major histocompatibility complex
mAb	Monoclonal antibody
min	minute
ml	Milli litre
mM	milli molar
MMP	matrix metalloproteinase
MT1-MMP	membrane type 1 matrix metalloproteinase
MW	Molecular weight
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NaN3	sodium azide
NaOH	sodium hydroxide
NF2	Neurofibromatosis type II
ng	nano-gramme
nM	nano molar
nm	nanometre
NO	nitric oxide
No.	number
NP-40	Nonidet P-40
°C	degrees Celsius
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PKC	Protein kinase C
PMA	phorbol myristate acetate
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	reverse transcription
SD	standard deviation
SDS	sodium dodecyl sulfate
sec	second
TAE	Tris/acetate/EDTA electrophoresis buffer
Tcf	T-cell factor
TBE	Tris/Borate/EDTA electrophoresis buffer

TBS	Tris-buffered saline
TE	Tris/EDTA buffer
TEM	Tumour Endothelial Marker
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-b	Transforming growth factor -beta
TNF-α	tumour necrosis factor - alpha
tPA	tissue plasminogen activator
TRE	tet responsive element
TRIS	Tris-(hydoxymethyl)-aminomethane
TRITC	Tetra-Rhodamine Isothiocyanate
μF	micro Farads
μg	microgram
μl	microlitre
μM	micro molar
uPA	urokinase Plasminogen Activator
U	units
UV	ultraviolet
V	volts
Wnt	wingless-type
WT	wild type.

CHAPTER ONE

INTRODUCTION

1.1.ANGIOGENESIS

Since the first description of the term 'angiogenesis' (new blood vessels growth) by the British surgeon Dr. Jone Hunter in 1787, 'angiogenesis' has become an area of great clinical and scientific interest. as this biological process bears a great importance in normal physiology as well in pathological conditions. Angiogenesis (angio'gen'esis) is defined as the sprouting of new capillaries from pre-existing vessels characterized by expansion of the endothelium which is undergoing proliferation, migration and remodelling processes.

Angiogenesis is a dynamic multi-step process, which involves retraction of pericytes from the abluminal surface of the capillary, release of proteases from the activated endothelial cells, degradation of the extracellular matrix (ECM) surrounding the pre-existing vessels, endothelial cell migration toward an angiogenic stimulus followed by their proliferation, formation of tube-like structures, fusion of the formed vessels and initiation of blood flow.

Growth of the vascular system is primarily a developmental process occuring during embryogenesis and only to a limited extend in postnatal life. In the adult, endothelial cells are among those exhibiting the lowest replication level in the body (only 0.01% of the whole population engaged in cell division) with angiogenesis being almost completely down regulated, except for the female reproductive system, and during pathological tissue growth (wound healing, tumour growth, diabetic retinopathy, rheumatoid arthritis, psoriasis, and more than 70 other conditions).

Soluble mediators from the surrounding tissues induce the switch from the quiescent to an activated endothelial cell. The construction of a vascular network is organized into the following steps of the angiogenic cascade:

- induction of proteases and degradation of the basement membrane
- migration of the endothelial cells into the interstitial space
- endothelial cell proliferation

- lumen formation

- generation of new basement membrane with the recruitment of pericytes

- fusion of the newly formed vessels, and initiation of blood flow.

The hypothesis that tumour growth is dependent upon angiogenesis was first proposed by Judah Folkman in 1971, based on the observations that expansion of a tumour mass was limited in the absence of angiogenesis and tumours require blood supply for expansive growth. With increasing distance from vessels, hypoxic tumour cells produce angiogenic factors that induce the formation of neovessels (Folkman 1971, Folkman 1972, Folkman *et al* 2002 .).These are different from vessels of normal tissue at the morphologic and molecular levels (Ruoslahti *et al* 2002, Bompais *et al.* 2004). It has been found that solid tumours after reaching a size of 2-3 mm^3, become dependent on the angiogenic process in order to gain an adequate supply of oxygen and nutrients and removal of waste products.

Alteration in adhesion molecules, angiogenesis, and matrix metalloproteinases has been associated with metastasis and intravasation of tumour cells.

Angiogenesis plays an important role in the pathogenesis of a variety of disorders such as cancer, proliferative retinopathies, and rheumatoid arthritis (Folkman *et al .,* 1992, Folkman 1991, Klagsbrun 1991). Accumulating evidence indicates that for most tumours, the switch to the angiogenic phenotype depends upon the outcome of a balance between angiogenic stimuili and angiogenic inhibitors, both of which may be produced by tumour cells and perhaps by some host cells (Folkman 1992).

Angiogenesis can be induced by tumour cells after implantation of tumour cells in the host. The induction can begin at a very early stage, i.e., when the tumour mass contains roughly 100-300 cells. Identification of chemotactic signals that initiate tumour cell migration toward the existing vasculature may provide valuable targets for preventing tumour progression and/or metastases (Li *et al .,* 2000). Tumour cells may pass directly into the blood vascular system via veno-lymphatic communications. The mechanisms

determining whether regional lymph nodes or other sites first develop metastases remain poorly understood. In fact, most disseminated tumour cells have a limited life span in the blood stream. While many surviving cancer cells remain dormant in the host tissues, only a few develop into clinically detectable micrometastases. However, identification of those occult tumours cells and prevention of their re-growth would be of great clinical significance.

Tumourgenesis in humans is a multi-step process, and these steps reflect the genetic alterations that drive the progressive transformation of normal cells to cancer cells. Contrary to normal cells, cancer cells have defective regulatory circuits that control normal proliferation and homeostasis. While normal cells require mitogenic signals to proliferate, malignant cells are self-sufficient for the growth signals and insensitive to the growth-inhibitory signals. Therefore, tumour cells are independent in generating their own growth signals. It has been well established that a complex series of cellular interactions between several types of cells like fibroblasts, immune cells and endothelial cells as well as malignant cells within the tumour tissues can lead to cancer cells growth and metastasis (Hanahan *et al* ., 2000). In addition to the ability to synthesize their own growth factors leading to autocrine stimulation, cancer cells could indeed induce the stimulation of other cells like endothelial cells via a paracrine mechanism, by soluble factors, thus generating neovascularization in the local tumour microenvironment.

As stated above, angiogenesis implies two distinct mechanisms, endothelial sprouting and intussusceptive microvascular growth (IMG). Angiogenesis establishes the circulation in so far avascular regions. Regarding the formation of vascular networks in the forming organs, some vessels are established by vasculogenesis, e.g., in the lungs and spleen, while others, like in the brain, are derived from angiogenesis. In the following the distinct cellular mechanisms of angiogenesis will be discussed in detail along with their molecular regulation in physiological and pathological conditions.

1.1.2 Angiogenesis in the physiological state.

1.1.2.1 The cellular mechanisms of angiogenesis

Blood vessels in the embryo are formed through vasculogenesis; that is, through *in situ* differentiation of undifferentiated precursor cells (angioblasts) to endothelial cells that assemble into a vascular labyrinth (Figure 1.1) (Risau 1997). Historically, the term angiogenesis was first used to describe the growth of endothelial sprouts from preexisting postcapillary venules Figure.1.1. More recently, this term has been used to generally denote the growth and remodelling process of the primitive vascular network into a complex vascular network. This involves the enlargement of venules, which sprout or become divided by pillars of periendothelial cells (intussusception) or by transendothelial cell bridges, which then split into individual capillaries.

One of the first descriptions of the sprouting process dates back to the Greek physician Galen (c. 130–200 A.D.). He compared the developing embryo to a plant that grows along with the umbilical veins. After these have reached the skin of the embryo the cells divided, branched and proliferated into sets of progressively smaller divisions between which the flesh of the liver was deposited (Galen, De foetuum formatione) (Harris CRS 1973). The first *in vivo* observations of sprouting capillaries have been made in the chicken chorioallantoic membrane (CAM) and in the transparent tails of amphibian larvae (Danchakoff 1917, Clark 1915, Clark 1918, Clark *et al* ., 1939). More recently, a precise concept consistent with sprout formation has been elaborated using a variety of models. The most important ones are the chicken CAM and the corneal pocket (Ausprunk *et al* ., 1977, Gille *et al* ., 2001).

The development of the shell free culture system of chicken embryos in petri dishes (Auerbach *et al* ., 1974) facilitates *in vivo* microscopy of the CAM and application of polymer pellets for controlled release of growth factors or their inhibitors (Langer *et al* ., 1976). Angiogenesis research has been developed into a new field after Folkman's proposal

that inhibition of angiogenesis could interfere with the growth of tumours (Folkman *et al* ., 1963, Folkman 1971, Folkman 1972). The first culture and cloning of capillary endothelial cells to study angiogenesis *in vitro* was reported subsequently after Folkman's initial observations (Folkman *et al* ., 1979, Folkman *et al* ., 1980, Madri *et al* ., 1983, Montesano 1983) . The isolation of the first angiogenic growth factor soon followed (Shing *et al* ., 1983, Shing *et al* ., 1984). The sprouting process consists of several consecutive steps that have been described by Ausprunk and Folkman (Ausprunk . 1977) and many other authors.

The process can be summarised into the following steps:

1). New capillaries originate from small venules or from other capillaries.

2). Local degradation of the basement membrane on the side of the venule closest to the angiogenic stimulus (collagenase, plasminogen activators, etc.).

3). Migration of endothelial cells toward the angiogenic stimulus.

4). Alignment of endothelial cells in bipolar mode.

5). Formation of a lumen (intra-cellular from vacuoles or intercellular) and endothelial cell mitosis distant to the leading tip of the sprout.

6). Loop formation by connection of individual sprouts (how sprouts manage to find each other remains unknown).

7). Flow begins after loops have formed.

8). Pericytes or smooth muscle cells eventually align along the endothelial cells outside the capillary (vessel wall maturation).

9). New basement membrane is formed.

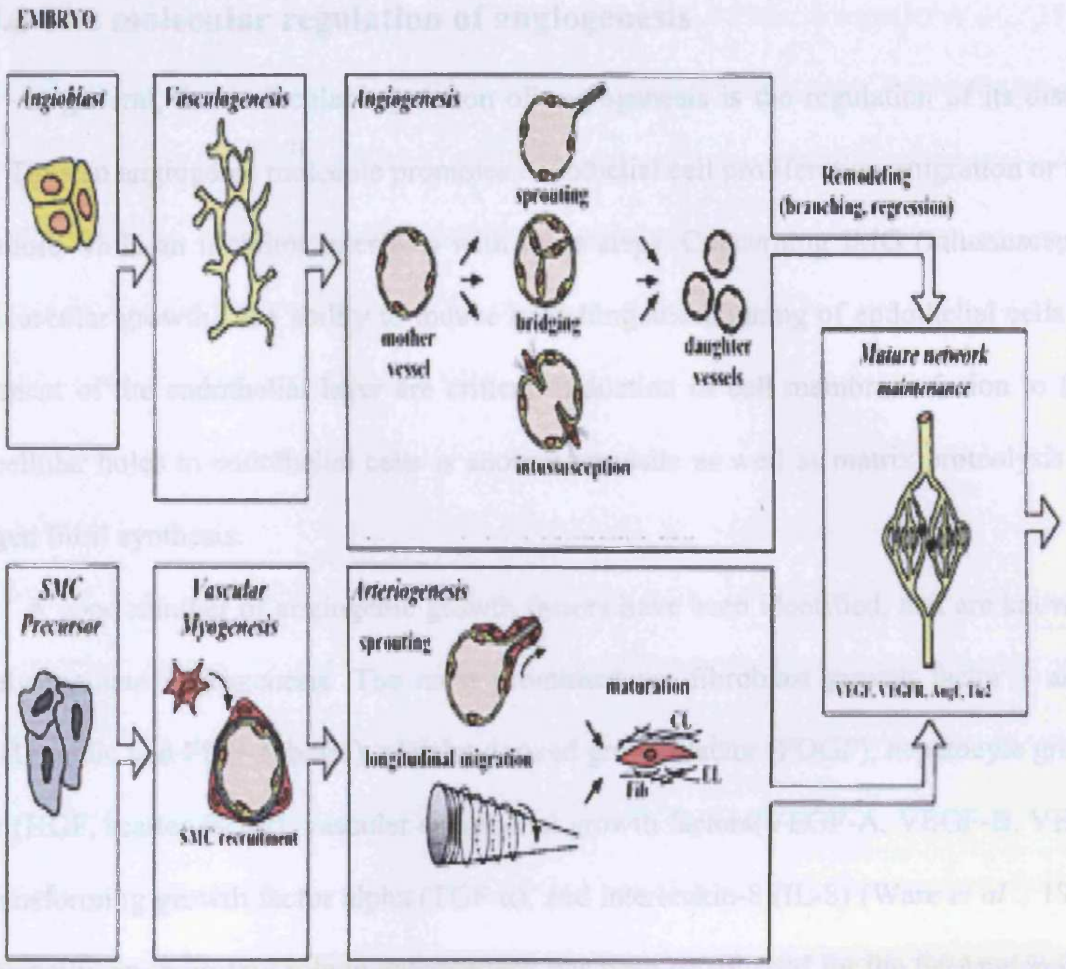

Figure 1.1. Endothelial precursors (angioblasts) in the embryo assemble in a primitive network (vasculogenesis), that expands and remodels (angiogenesis).

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1.1.2.2 The molecular regulation of angiogenesis

In general, the molecular regulation of angiogenesis is the regulation of its distinct steps. Thus an angiogenic molecule promotes endothelial cell proliferation, migration or tube formation, while an inhibitor interferes with these steps. Concerning IMG (intussusceptive microvascular growth), the ability to induce stretching and thinning of endothelial cells and movement of the endothelial layer are critical. Induction of cell membrane fusion to form transcellular holes in endothelial cells is another requisite as well as matrix proteolysis and collagen fibril synthesis.

A good number of angiogenic growth factors have been identified, that are known to actively regulate angiogenesis. The most prominent are fibroblast growth factor 1 and 2 (FGF-1, acidic and FGF-2, basic), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF, scatter factor), vascular endothelial growth factors(VEGF-A, VEGF-B, VEGF-C), transforming growth factor alpha (TGF-α), and interleukin-8 (IL-8) (Ware *et al .,* 1997). Additionally, an important role in angiogenesis has been established for the tie/Angiopoietin and the Eph-B/ephrin-B system of tyrosine kinase receptors and their ligands (Suri *et al .,* 1996, Davis *et al .,* 1996, Adams *et al .,* 1999, Wang *et al .,* 1998).

VEGF (also referred to as VEGF-A) and its receptors (flk-1 and flt-1), known for their role in vasculogenesis, are also responsible for the regulation of angiogenesis. Many studies have demonstrated that VEGF-A promotes migration, proliferation and tube formation in endothelial cells, *in vitro*. VEGF-A is one member of the VEGF family, whose membership also applies to VEGF-B, VEGF-C, VEGF-D and PlGF. The VEGF family members have differing capabilities to promote these functions (Keyt *et al .,* 1996a, Carmeliet *et al .,* 1999). VEGF-C deficient embryos die between day 11 and 12 from ubiquitous defects in their vasculature. These defects include abnormally large vessels in the yolk sac and inside the embryo accompanied by tissue necrosis, a lack of fusion of the vitelline veins with the yolk sac vessels, and failing growth of vessels from the perineural

plexus into the neuroepithelium of the brain (Ferrara *et al .*, 1996a, Carmeliet *et al .*, 1996). This confirms the importance of VEGF-C for embryonic angiogenesis. Interestingly, high expression levels of VEGF-C protiens are detected in organs where vasculogenesis is the leading mechanism of vascularization, while lower levels are found in organs that receive their vascular supply by angiogenesis (Miquerol *et al .*, 1999). While the flk-1 receptor is dominant during early vasculogenesis, flt-1 (VEGFR-1) is prominent during remodeling of the primary vascular plexus and subsequent angiogenesis. Flt-1 transgenic mice thus exhibit large, dilated vessels in the yolk sac and throughout the embryo instead of a plexus of smaller ones.

The homozygous embryos die between day 8.0 and 9.0. As mentioned before, these flt-1 vessels contain endothelial folds and ITS (intervascular tissues structures)-like structures more frequently as compared to the control (Fong *et al .*, 1995). This may indicate that vessel division is not occurring in a normal way under this circumstance and it is attempting to speculate that IMG could eventually be affected. Flt-1 expression can also be upregulated by VEGF (Barleon *et al .*, 1997).

1.1.3 Angiogenesis In pathological conditions

1.1.3.1 The cellular mechanisms

Angiogenesis is induced in many pathological states, such as wound healing, chronic inflammation, restenosis, atherosclerosis and solid tumours. New vessels in the adult arise mainly through angiogenesis, although vasculogenesis also may occur (Figure 1.2). Because vasculogenesis only leads to an immature, poorly functional vasculature, angiogenesis, which yields fully functional vessels, is therefore more likely to be a therapeutic target. As the cellular and molecular mechanisms of angiogenesis differ in various tissues (vessels in psoriatic skin enlarge, but they sprout in ischaemic retina), the therapeutic stimulation or inhibition of angiogenesis should be adjusted to the target tissue.

The number of vessels in tumour sections reflects the extent of angiogenesis and can be considered as an important independent predictor of outcome in colorectal cancer.

As mentioned above, the development of blood vessels within tumours has been extensively investigated, based on the suggestion that tumour development and spread are angiogenesis dependent (Folkman 1971, Algire *et al* ., 1945, Greenblatt *et al* ., 1968) . Furthermore, it was demonstrated that tumours switch to an angiogenic phenotype by the release of angiogenic growth factors (Folkman *et al* ., 1989). So far, tumour angiogenesis along with angiogenesis in other pathological states is thought to be implemented by the process of normal angiogenesis, i.e. by endothelial sprouting (Folkman 1985). However, the fact that tumour vessel growth is continuously perpetuated (Folkman 1985) can only be explained, if one assumes that the normal physiological process of vessel growth is abnormally regulated. What is the difference between physiological angiogenesis and angiogenesis in tumours?

The switch to the angiogenic phenotype and the recent discovery of potent anti-angiogenic agents such as thrombospondin (Good *et al* ., 1990, Dipietro 1997), angiostatin (O'Reilly *et al* ., 1994), endostatin (O'Reilly *et al* ., 1997) , and vasostatin (Pike *et al* ., 1998) implicate that a net balance of promoters and inhibitors regulates the growth of blood vessels. This balance can shift toward a pathological situation, typically detected in tumours, in which negative regulators are decreased while positive ones prevail (Bouck NP 1990, Rastinejad *et al* ., 1989, Folkman 1995, Hanahan *et al* ., 1996) . This would explain why tumour angiogenesis is an unlimited process. However, what is the nature of the imbalance between promoters and inhibitors of angiogenesis? Is there a structural correlation to the unbalanced vessel growth or are blood vessels just more frequently formed in tumours?

The pathological parameters of tumour circulation (Jain 1988), suggest that blood vessel growth in tumours is a dysregulated process. The comparison between healing wounds and growing tumours in respect to the composition of their stroma revealed many

similarities and differences of both systems and lead to the characterization of tumours as 'wounds that do not heal' (Dvorak 1986). Recently, *in vivo* video microscopy demonstrated that intussusceptive microvascular growth is an alternative mechanism of tumour angiogenesis (Patan *et al .*, 1996a) . In these studies it is shown that tumour angiogenesis follows the normal principles of intussusceptive growth, but also has pathological differences. The formation of tissue pillars and intervascular tissues structures (ITSs) occurs more frequently and includes sequences of contradictory steps (formation of segments followed by their occlusion) that are implemented in much shorter periods of time as compared to the situation in the embryo (Patan *et al .*, 1996, Patan *et al .*, 1996b).

In the tissues surrounding the tumour or adjacent to the wound suture in wound healing, venules and smaller veins expanded from the previously existing vascular network by a process that has been termed segmentation. Segmentation leads to the formation of tissue folds that were connected in the centre of the vessel lumen in a spoke-wheel like pattern. ITSs (intervascular tissues structures) frequently separate from these folds. Thus segmentation is based on intussusceptive microvascular growth (IMG). A similar process had been described by Nagy et al, in ascites from tumours in the peritoneum (Nagy *et al .*, 1995).

Interestingly, the growth of the pre-existent networks establishes connections to the newly formed loop systems. In the tumour, but not in wound healing, segmentation also consists of pathological variations that cause formation of blind ending tubules (Patan *et al .*, 2001). Thus the wound, unlike the tumour, forms a network of perfectly connected vessels that facilitates perfusion and reoxygenation. Hypoxia induced angiogenesis subsequently ceases in wound healing. In the tumours, the pathological structure of the vascular network facilitates abnormal circulatory conditions and likely perpetuates hypoxia driven angiogenesis. These observations demonstrate that the pathophysiological conditions of the tumour circulation are based on an abnormal vascular network structure that is derived from

pathological mechanisms of vessel formation and growth (Patan *et al* ., 2001). The latter study also demonstrates that the recanalization process of large thrombotic lesions can follow the mechanisms of intussusceptive microvascular growth (IMG) (Patan *et al* ., 2001).

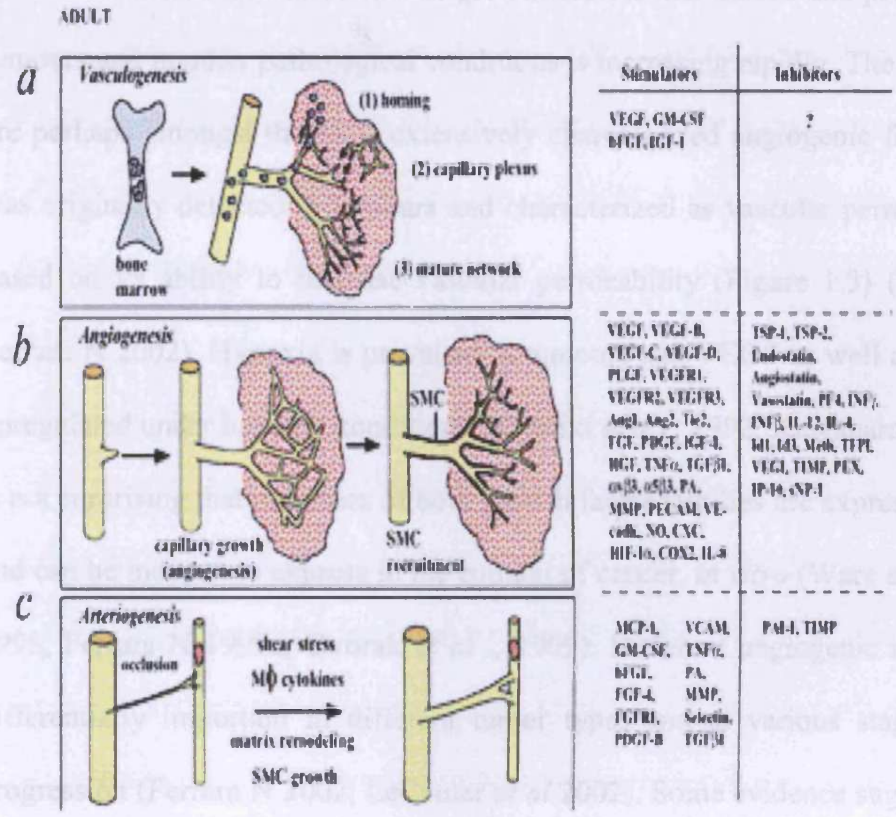

Figure 1.2: Pathological vascular growth in the adult may occur via vasculogenesis (angioblast mobilization), angiogenesis (sprouting) or arteriogenesis (collateral growth).http://www.nature.com.uk

1.1.3.2 The molecular regulation.

Literature on the role of growth factors that induce and promote angiogenesis in tumours and in other pathological conditions is increasing rapidly. The VEGFs and the FGFs are perhaps amongst the most extensively characterized angiogenic factors. In fact, VEGF was originally detected in tumours and characterized as vascular permeability factor (VPF) based on its ability to increase vascular permeability (Figure 1.3) (Senger *et al .,* 1983, Ferrara N 2002). Hypoxia is prevalent in tumours and VEGF as well as FGF expression are upregulated under hypoxic conditions (Shweiki *et al .,* 1992, Kuwabara *et al .,* 1995). Thus it is not surprising that members of both growth factor families are expressed by many tumours and can be induced to express in the context of cancer, *in vitro* (Ware and M. 1997, Folkman 1995, Ferrara N 1999a, Dvorak *et al .,* 1999). Different angiogenic mechanisms might be differentially important in different tumor types and at various stages of the neoplastic progression (Ferrara N 2002, LeCouter *et al* 2002). Some evidence suggests that VEGF may be especially critical during the initial stages. Such a notion may be crucial for the design of further clinical trials.

Furthermore, interfering with the VEGF pathway in tumours by application of neutralizing antibodies (Kim *et al .,* 1993) or by retrovirus-mediated expression of a dominant negative flk-1 mutant, suppresses the growth of many tumour cell lines *in vivo* (Millauer *et al .,* 1994, Millauer *et al .,* 1996). Similarly, high local expression of soluble flt-1, which able to block the function of VEGF, inhibits tumour growth and metastases (Kong *et al .,* 1998, Goldman *et al .,* 1998). Another major finding which attracted interest in the field was that reported by Weidner *et al.* (Weidner *et al .,* 1991) who found that the greater the degree of angiogenesis detected in a primary tumour, the worse the prognosis. This established a direct relationship between tumour progression and angiogenesis. It was first shown in breast cancer and subsequently in a large and diverse array of other tumours,

including melanomas, gliomas, lung, bladder and prostate cancers, and indeed a few other tumour types (Weidner N 1995).

Recently, angiopoietins and tie receptors have also been implicated in angiogenesis in the pathological state. Angiopoietins are a family of vascular growth factors that collaborate with members of the vascular endothelial growth factor family to regulate vascular and lymphatic vessel growth (Davis *et al .,* 1996, Valenzuela *et al .,* 1999, Maisonpierre *et al .,* 1997), acting *via* the endothelial receptor tyrosine kinase Tie2. Although angiopoietin-1 (Ang-1) seems to be an obligate activator of the Tie2 receptor, angiopoietin-2 (Ang-2) seems to have context-specific effects, activating this receptor on some cells while blocking Tie2 activation on other cells or under different conditions. It has been demonstrated that targeting the tie2 receptor in tumours using a soluble tie2 receptor delivered by an adenoviral vector reduces tumour growth and metastases (Lin *et al .,*1998).Additionally, it was shown that in melanomas the VEGF receptor pathway and the tie2 pathway are essential for melanoma growth. However, since the inhibition of one pathway could not be compensated by the other, it was suggested that both systems are independent mediators of angiogenesis in melanoma (Siemeister *et al .,* 1999).

During early tumour development, Ang-2 is detected in host vessels that are coopted by tumour cells and subsequently regress. In a later stage, Ang-2 is coexpressed with VEGF at the tumour margin where angiogenesis is dominant (Holash *et al .,* 1999) . Interestingly, the Tie1 receptor is expressed during hypoxia and as a response to VEGF *in vitro* (McCarthy *et al .,* 1998). Tie1 was also detected in large vessels close to the wound suture on day 3 in wound healing and on day 7 in the small vessels of the neovasculature that course throughout the wound (Peters *et al .,* 1993). Flt-1 showed a corresponding expression pattern in respect to time and location (Korhonen *et al .,* 1992). These findings match the onset of formation of loops in the wall of the large vein and their further remodeling (Patan *et al .,* 1996b). Concerning angiogenesis in ischemic

tissues, bFGF and VEGF-A with its two tyrosine kinase receptors are increased in myocytes and macrophages of the ischemic myocardium (Banai *et al .*, 1994, Li *et al .*, 1996, Shyu *et al.,* 1998). A few clinical trials using VEGF and FGF to induce neovascularization in ischaemic tissues are in progress (Baumgartner *et al .*, 1998, Boehm *et al .*, 1997). It thus appears that the most important regulators of embryonic angiogenesis play very similar roles in angiogenesis in the adult under pathological conditions.

However, the precise role of these growth factors in respect to the mechanisms of physiological and pathological angiogenesis has still to be determined. Furthermore, the detection of potent angiogenic inhibitors in tumours that interfere with tumour growth and metastasis (O'Reilly *et al .*, 1994, O'Reilly *et al .*, 1997, O'Reilly *et al .*, 1999) enhances the chances that the imbalance between promoters and inhibitors of angiogenesis, is key to suppressing tumour growth spread.

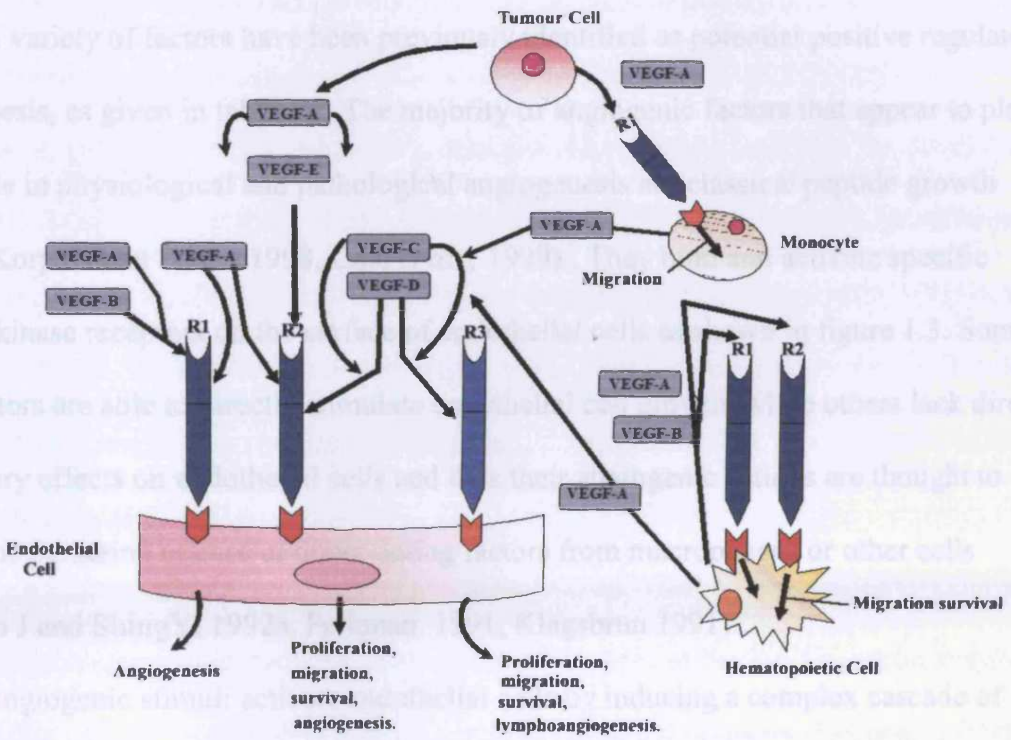

Figure 1.3 Tumour cells induce angiogenic factors, resulting in tumour proliferation, migration and angiogensis.

1.1.4. Positive and negative regulators of angiogenesis

Recently, tumour angiogenesis has undergone an increasing interest in a prognostic and therapuatic maters in a variety of solid tumours.

1.1.4.1. Positive regulators of angiogenesis

A variety of factors have been previously identified as potential positive regulators of angiogenesis, as given in table 1.1. The majority of angiogenic factors that appear to play a major role in physiological and pathological angiogenesis are classical peptide growth factors (Korpelainen *et al .,* 1998, Gale *et al .,* 1999) . They bind and activate specific tyrosine kinase receptors on the surface of endothelial cells as shown in figure 1.3. Some of these factors are able to directly stimulate endothelial cell growth, while others lack direct stimulatory effects on endothelial cells and thus their angiogenic actions are thought to require the paracrine release of direct-acting factors from macrophages or other cells (Folkman J and ShingY. 1992a, Folkman 1991, Klagsbrun 1991).

Angiogenic stimuli activate endothelial cells by inducing a complex cascade of events that lead to the modulation of endothelial cell proliferation, migration, survival, extracellular proteolysis, differentiation, and morphogenesis (Risau 1997, Hanahan *et al.* 1996, Bouck *et al .,* 1996). The rapid activation of otherwise quiescent endothelial cells is tightly regulated by a dynamic balance between angiogenesis stimulators and inhibitors (Hanahan *et al* . 1996, Iruela-Arispe *et al .,* 1997).

The best studied example is the family of vascular endothelial growth factors (VEGFs) consisting of four members (VEGF A, B, C, and D) (Gale NW and Yancopoulos G.D. 1999, Ferrara N and Davis-Smyth T ., 1997). They bind three different endothelial cell-specific VEGF receptors as stated above (VEGFR 1–3) with varying affinity (Korpelainen EI and Alitalo K. 1998, Ferrara N and Davis-Smyth T. 1997). The functional involvement of

VEGF in physiological and pathological angiogenesis has been clearly demonstrated (Ferrara *et al* 1996a). A novel family of angiogenic factors, named angiopoietins, are the ligands for the endothelial cell specific tyrosine kinase receptor Tie-2 (Gale *et al.* 1999).

The role of angiopoietins and their receptor in angiogenesis has been demonstrated in genetically modified mice. Depletion of angiopoietin or Tie-2 function in knockout mice and over expression of angiopoietins in transgenic mouse models provided clear evidence that the angiopoietin system, rather than being involved in the early phases of vascular development, is crucial for the subsequent remodelling and stabilization of the developing vasculature (Maisonpierre *et al .,* 1997, Suri *et al .,* 1996, Sato *et al .,* 1995). In addition, both *in vitro* and *in vivo* studies revealed that the angiopoietin family consists of agonists and antagonists of the Tie-2 receptor. For example, while angiopoietin-1 activates the Tie-2 tyrosine kinase receptor on endothelial cells, angiopoietin-2 inhibits Tie-2 receptor activation (Maisonpierre *et al .,* 1997).

Angiopoietins and their receptor also appear to be required for tumour angiogenesis, supported by evidence that the expression of a soluble form of the Tie-2 receptor resulted in the inhibition of tumour growth in tumour transplantation experiments (Lin *et al .,* 1998).

Members of the CXC chemokine family that contain a Glu–Leu–Arg motif, such as interleukin 8, also exhibit angiogenic activity. They are mostly macrophage-derived and could act directly on endothelial cells. In contrast, CXC chemokines lacking the Glu–Leu–Arg motif, such as platelet factor 4 (PF 4), interferon gamma-induced protein 10 (IP 10), or *GRO*, are inhibitors of angiogenesis (see below). Other classical growth factors that have been reported to stimulate endothelial cell proliferation are transforming growth factor alpha (TGF-α) and platelet-derived growth factor BB (PDGF BB).

Protein factors, such as pleiotrophin, angiogenin, angiotropin, platelet activating factor (PAF), and the HIV *tat* gene product are able to induce proliferation, migration, or morphogenesis of endothelial cells (Bouck *et al .,* 1996). Some angiogenic factors that are

able to induce angiogenesis *in vivo*, for example tumour necrosis factor alpha (TNF-α) or transforming growth factor beta (TGF-β), do not directly interact with endothelial cells; they rather attract other cell types, e.g. macrophages, to the location of ongoing angiogenesis and trigger the production and release of true angiogenic factors (Bouck *et al .*, 1996).

In addition to peptide angiogenic factors, a number of low molecular weight compounds have been described that stimulate angiogenesis. Examples are butyryl glycerol, prostaglandin E1 and E2, nicotinamide, and related compounds such as adenosine, copper complexes (Cu–Gly–His–Lys), ceruloplasmin, and heparin. These molecules are beyond the topic of this thesis.

Table 1.1. List of Known Angiogenic Growth Factors

Angiogenin
Angiopoietin-1
Del-1
Fibroblast growth factors: acidic (aFGF) and basic (bFGF)
Follistatin
Granulocyte colony-stimulating factor (G-CSF)
Hepatocyte growth factor (HGF) /scatter factor (SF)
Interleukin-8 (IL-8)
Leptin
Midkine
Placental growth factor
Platelet-derived endothelial cell growth factor (PD-ECGF)
Platelet-derived growth factor-BB (PDGF-BB)
Pleiotrophin (PTN)
Progranulin
Proliferin
Transforming growth factor-alpha (TGF-alpha)
Transforming growth factor-beta (TGF-beta)
Tumor necrosis factor-alpha (TNF-alpha)
Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF)

1.1.4.2 Negative regulators of angiogenesis

The existence of naturally occurring molecules with anti-angiogenic activity has been known for decades (Folkman *et al.* 1992b). Anti-angiogenic factors belong to different classes of molecules and the mechanisms by which they interfere with angiogenesis may vary from one to another as shown in (Table 1.2). Moreover, some inhibitors specifically inhibit endothelial cells, while others may also affect other cell types. Examples of such pleiotropic angiogenesis inhibitors include cytokines, such as interferon (Sidky *et al.*, 1987) and interleukin-12 (Voest *et al.*, 1995), and some chemokines, such as GRO (pro-inflammatory "chemokine" superfamily of chemotactic cytokines) (Cao *et al.*, 1995) and IP-10 (Angiolillo *et al.*, 1995).

Two main approaches have been taken to identify compounds that are able to inhibit endothelial cell proliferation *in vitro* and neovascularization *in vivo*: (1) isolation of angiostatic compounds from tissues that exhibit reduced vascularization (e.g. cartilage) or ongoing regulated angiogenesis (e.g. active tumour progression versus dormant metastasis) and (2) rational design of compounds that interfere with mechanisms known to be involved in the angiogenic process, such as the activity of angiogenic factors or endothelial cell adhesion. An example for the latter approach is the development of neutralizing antibodies to VEGF and the discovery of soluble VEGF receptors (Ferrara N and Davis-Smyth T . 1997).

Another approach towards the identification of angiostatic factors was based on the purification of compounds that inhibit endothelial cell proliferation or migration. Some of the factors identified by this method were small chemical entities, such as TNP 470 (a fumagillin-derivative), cyclodextrin in conjunction with hydrocortisone, 2-methoxyoestradiol, thalidomide, D-penicillamine, gold thiomalate, and vitamin D3 analogues (Folkman 1995, Hanahan *et al.* 1996).

Several components of the extracellular matrix and/or proteolytic fragments have been reported to inhibit angiogenesis. For example, heparin-binding fragments of fibronectin

are able to prevent endothelial cell proliferation (Homandberg *et al.*, 1985). Interestingly, fibronectin itself promotes angiogenesis, likely by supporting endothelial cell adhesion, migration, and remodeling (Grant *et al.*, 1990). Therefore, the generation of proteolytic fragments during ECM degradation by endothelial cells might trigger a feedback mechanism controlling capillary morphogenesis. Amongst ECM components, the matricellular protein thrombospondin-1 (TSP-1) has been extensively characterized as an angiogenesis inhibitor. TSP-1 affects several properties of endothelial cells, including proliferation, motility, cell-to-cell interaction, and morphogenesis (Dawson *et al.*, 1999).

Recent studies have indicated that most effects of TSP-1 on endothelial cells are mediated by its binding to CD36, a cell surface receptor expressed by microvascular endothelial cells (Dawson *et al.*, 1997). However, TSP-1 is also able to influence the activity of several modulators of angiogenesis, such as hepatocyte growth factor (HGF), FGF2, and TGF-α1. Therefore, TSP-1 appears to regulate angiogenesis both by directly affecting endothelial cell functions and by controlling the activity of angiogenesis modulators (Dawson *et al.*, 1999).

It has been demonstrated that primary tumours generate proteolytic activities that cleave plasminogen and collagen XVIII, and that the proteolytic products, a 38 kD fragment named angiostatin and a 20 kD fragment named endostatin, respectively, repress vascularization of secondary tumours or distant metastases (O'Reilly *et al.*, 1994, O'Reilly *et al.*, 1997). Upon removal of the primary tumours, serum levels of angiostatin and endostatin drop resulting in the outgrowth of metastases. Conversely, purified angiostatin and endostatin have been shown to inhibit angiogenesis and thus tumour growth in mouse tumour transplantation experiments.

Angiostatin inhibits endothelial cell migration and proliferation most likely by its ability to bind a cell surface ATP synthase (Moser *et al.*, 1999). Angiostatin also inhibits ECM-stimulated plasminogen activation, resulting in decreased endothelial cell invasion

(Stack *et al.*, 1999), and specifically represses the activation of the mitogen-activated protein kinase (MAPK) pathway by activating a tyrosine phosphatase (Redlitz *et al.*, 1999). However, no inhibition of FGF2-induced activation of MAPKs was found in bovine capillary endothelial cells treated with angiostatin (Claesson-Welsh *et al.*, 1998) indicating that angiostatin may affect different pathways in endothelial cells depending on the particular endothelial cell type. Finally, many studies have revealed that angiostatin induces apoptosis of endothelial cells (Claesson-Welsh *et al.*, 1998, Lucas *et al.*, 1998).

The results suggest that the anti-angiogenic activity of angiostatin is mediated not only by blocking the stimulatory effects of angiogenic factors, but also by direct cytotoxic effects on endothelial cells. Endostatin shares with angiostatin most of the effects on endothelial cells, such as inhibition of growth factor-induced proliferation and migration (Taddei *et al.*, 1999) as well as the induction of apoptosis (Dhanabal *et al.*, 1999). Nevertheless, its mode of action is still unclear. The ability of endostatin to bind to ECM components accounts for its wide tissue distribution.

Binding to heparan sulphate proteoglycans has been proposed to be involved in its anti-angiogenic activity, possibly by competing with FGF2 for the binding to these cell surface co receptors (Sasaki *et al.*, 1999). The interaction between endostatin and heparan sulphate proteoglycans seems to be dispensable for the storage of endostatin in basement membranes (Miosge *et al.*, 1999, Chang *et al.*, 1999). Moreover, endostatin mutants unable to bind heparan sulphate proteoglycans still inhibit VEGF-induced migration of endothelial cells (Yamaguchi *et al.*, 1999).

Table 1.2. Some endogenous inhibitors of angiogenesis

Name	Description
Thrombospondin-1 and internal fragments of thrombospondin-1	Thrombospondin is a 180 kDa, large, modular extracellular matrix protein (Tolsma ss et al 1993)
Angiostatin	A 38 kDa fragment of plasminogen involving either kringle domains 1–3, or smaller kringle 5 fragments (O'Reilly MS et al 1994, and Cao Y et al 1997)
Endostatin	A 20 kDa zinc-binding fragment of type XVIII collagen (O'Reilly MS et al 1997))
Vasostatin	An N-terminal fragment (amino acids 1–80) of calreticulin (Pike SE et al 1998)
Vascular endothelial growth factor inhibitor (VEGI)	A 174 amino acid protein with 20–30% homology to tumor necrosis factor superfamily (Zhai Y et al 1999)
Fragment of platelet factor 4 (PF4)	An N-terminal fragment of PF4 (Gupta SK et al 1999)
Derivative of prolactin	16 kDa fragment of the hormone (Clapp C et al 1993)
Restin	NC10 domain of human collagen XV (Ramchandran R et al 1999)
Proliferin-related protein (PRP)	A protein related to the pro-angiogenic proliferin molecule (Jakson D 1994)
SPARC cleavage product	Fragments of secreted protein, acid and rich in cysteine (Vazquez F et al 1999)
Osteopontin cleavage product	Thrombin-generated fragment containing an RGD sequence (Sage EH et al 1999)
Interferon α/β	Well known anti-viral proteins (Ezekowitz RA et al 1992))
Meth 1 and Meth 1	Proteins containing *m*etalloprotease and *t*hrombospondin domains, and disintegrin domains in NH_2 termini (Sage EH et al 1999)
Angiopoietin-2	Antagonist of angiopoietin-1 which binds to tie-2 receptors (Davis S et al 1996 and Maisonpierre PC et al 1997)
Anti-thrombin III fragment	A fragment missing C-terminal loop of anti-thrombin III (a member of the serpin family) (O'Reilly MS et al 1999)

1.2. The Blood Vessels System.

The initial description of putative vascular structure has been credited to Hippocrates, who described "white blood vessels" and thought they could be glands. Later, the valves of the heart were discovered by a physician of the Hippocratean School around the 4th century BC. However, their function was not properly understood. Because blood pools in the veins after death and thus arteries look empty, ancient anatomists assumed they were filled with air and that they were for transport of air. Herophius distinguished veins from arteries but thought that the pulse was a property of the arteries themselves. Erasistratus observed that arteries cut during life, bleed. He ascribed the fact to the phenomenon that air escaping from an artery being replaced with blood entering by very small vessels between veins and arteries. Thus he apparently postulated capillaries but with reversed flow of blood.

Galen in the 2nd century AD knew that blood vessels carry blood and identified venous (dark red) and arterial (brighter and thinner) blood, each with distinct and separate functions. Growth and energy were derived from venous blood created in the liver from chyle, while arterial blood gave vitality by containing pneuma (air) and originated in the heart. Blood flowed from both creating organs to all parts of the body where it was consumed, no blood returned to the heart or liver. The heart did not pump blood around, the heart's motion sucked blood in during diastole and the blood moved by the pulsation of the arteries themselves.

Galen believed that the arterial blood was created by venous blood passing from the left ventricle to the right by passing through 'pores' in the interventricular septum, air passed from the lungs via the pulmonary artery to the left side of the heart. As the arterial blood was created 'sooty' vapors were created and passed to the lungs also via the pulmonary artery to be exhaled.

Ibn Nafis in 1268 was the first person to accurately describe the process of blood circulation in the human body. Contemporary drawings of this process have survived. In 1552 Servetus described the same and Realdo Colombo proved the concept. All these results were not widely accepted however.

Finally William Harvey, a pupil of Hieronymus Fabricius (who had earlier described the valves of the veins without recognizing their function), performed a sequence of experiments and announced in 1628 the discovery of the human circulatory system as his own and published an influential book. This work, with its essentially correct exposition slowly convinced the medical world. At about the same time William Harvey described the blood circulation (Witte *et al*., 2001). Harvey was not able to identify the capillary system connecting arteries and veins; these were later described by Marcello Malpighi.

The vascular system being based on their structure and function, blood vessels are classified as arteries, capillaries, or veins, compring a one way, open-ended complex network of arteries, arterioles, capillaries, venules and veins. The main transport systems are the circulatory systems, in which substances are dissolved or suspended in liquid and carried from one part of the body to another in a system of tubes called vessels. There are two main circulatory systems: The blood circulatory system (sometimes called the cardiovascular system) and the lymphatic system. The blood circulatory system is the main method of transporting oxygen, carbon dioxide, nutrients and metabolic breakdown products, cells of the immune and other defence systems, chemical messengers (hormones), other important substances (e.g. clotting factors). The lymphatic system drains extra-cellular fluid from the tissues returning it to the blood circulatory system after passage through lymph nodes. This system is also involved in absorption of nutrients from the gut.

Blood vessel formation starts at the beginning of the third week of generation. Blood vessels first start to develop in the extraembryonic mesoderm of the yolk sac, connecting

stalk, and chorion. Blood vessels begin to develop in the embryo about two days later. The vascular system develops during embryonic development by at least two distinct processes; vasculogenesis is the development of blood vessels from in situ differentiating angioblasts and angiogenesis is the sprouting of capillaries from pre-existing vessels. The molecular mechanisms involved in the regulation of these processes are poorly understood.

Arteries and veins have a similar structure; the walls have three layers. The inner most layer is the tunica intima, or endothelium, with which we are mostly interested in this study, this endothelial cell lining whose function in larger vessels is simply to provide minimal resistance to the passage of blood. The tunica intima also includes a layer of elastic connective tissue that gives it greater strength.The middle layer of vessels, or tunica media, contains smooth muscle and another layer of elastic fibres. This muscle is responsible for manipulating the diameter of the passage within the vessel. Narrowing the passage increases the resistance to blood flow and reduces its volume. If major vessels throughout the body are constricted in a systemic response, blood pressure will be elevated. The outer layer of the vessel wall is a supporting layer of connective tissue called the tunica externa (or adventitia). It helps to anchor the blood vessel within the body. Veins differ structurally from arteries primarily in the thickness of the muscular and elastic layers. Because venous blood is under considerably less pressure, venous walls are correspondingly weaker.

The endothelium is the layer of thin, flat cells that lines the interior surface of blood vessels, forming an interface between circulating blood in the lumen and the rest of the vessel wall. Endothelial cells line the entire circulatory system, from the heart to the smallest capillary. In small blood vessels and capillaries, endothelial cells are often the only cell-type present. Endothelial cells are involved in many aspects of vascular biology, including vasoconstriction & vasodilation, and hence the control of blood pressure, blood clotting

(thrombosis & fibrinolysis), atherosclerosis, formation of new blood vessels (angiogenesis), inflammation and swelling.

The vascular system seems to be one of the most excellent pathways for malignant cells dissemination, because of the role of its inner layer (endothelium). Intravasation and extravasation of tumour cells appeared to be similar cell biological phenomena, in which two different avenues are involved (Kawaguchi *et al*., 1983, Kawaguchi *et al*., 1982): 1). Tumour cells migrate through the pores in the vascular walls which were produced by direct actions of tumour cells, and 2), endothelial cell cytoplasm enclosed the tumour cells neighboring to the vascular walls and as a result intravascular or extravascular tumour cell movement occurs.

Tumour cells arrested in a target organ are in close contact with vascular endothelial cells and further contact with the basement membrane. Extravasated tumour cells produce three different features of metastatic lesions in the combination of tumour strain and the organ affected; formation of tumour nodules accompany neovasculature, spreading of tumour cells along the perivascular tissue of an organ, and diffuse infiltration of tumour cells in an organ (Figure 1.4).

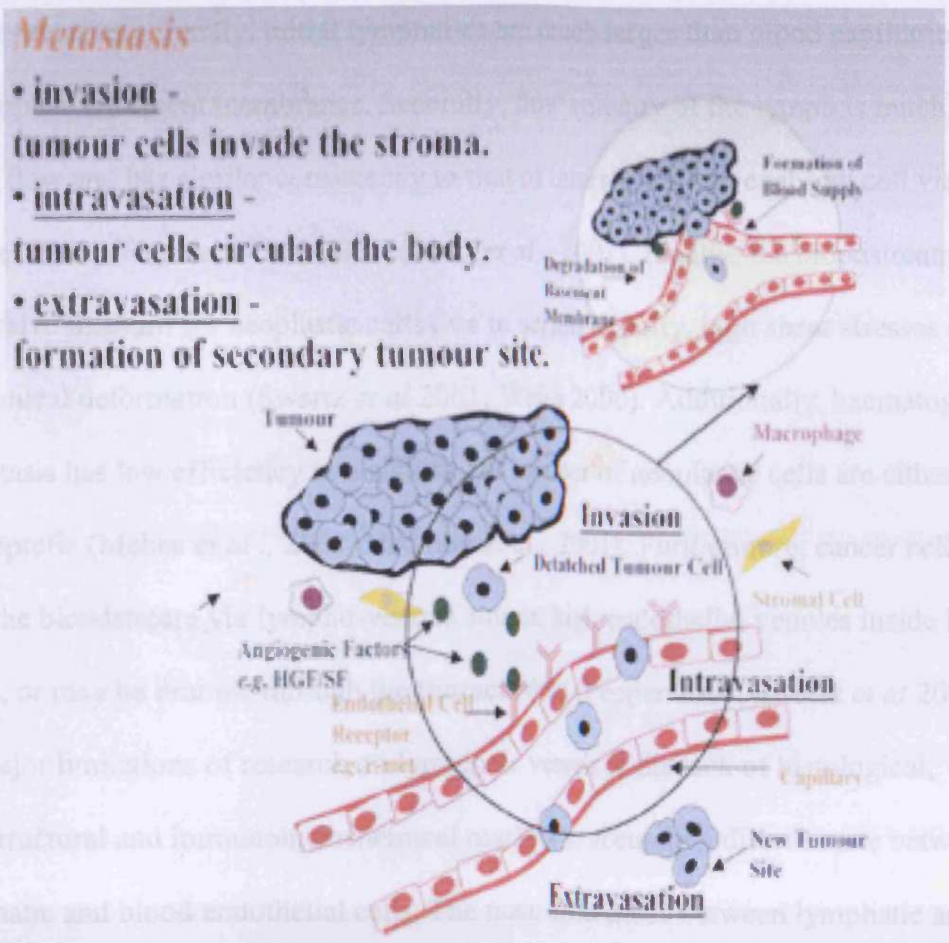

Figure 1.4. Diagram shows the extrvasation and intravasation of tumour cells through endothelial cell. (Martin TA *et al* Kluwer Academic Publication, 2001)

The vascular system differs from the lymphatic system in its structure and its role in cancer metastasis. Firstly, initial lymphatics are much larger than blood capillaries and have in-complete basement membranes. Secondly, flow velocity of the lymph is much slower than blood flow and has similar consistency to that of interstitial fluid enabling cell viability (Pepper 2001, Sleeman *et al*., 2001, Swart *et al*., 2001). Finally, the bloodstream is a highly aggressive medium for neoplastic cells due to serum toxicity, high shear stresses and mechanical deformation (Swartz *et al* 2001, Weiss 2000). Additionally, haematogeneous metastasis has low efficiency as a significant number of neoplastic cells are either quiescent or apoptotic (Mehes *et al*., 2001, Naumov *et al*., 2001). Furthermore, cancer cells may pass in to the bloodstream via lympho-venous shunts, high endothelial venules inside lymph nodes, or may be drained through the thoracic duct (Pepper 2001, Swartz *et al* 2001). One of the major limitations of research on lymphatic vessels is the lack of histological, ultrastructural and immunohistochemical markers to accurately discriminate between lymphatic and blood endothelial cells. The main differences between lymphatic and blood vascular endothelium are listed in table 1.3.

Vascular capillaries are identified by the fact that they are lined by a single layer of endothelial cells surrounded by basement membrane. This is characterized by a large surface area, having poorly developed junctions with frequent large gaps between cells and relatively high permeability (especially at intercellular clefts) to fluid and macromolecules, making capillaries permit the passage of large biological macromolecules, pathogens and migrating cells, and these characteristics not found in lymphatic capillaries.. However these anatomical differences do not provide a practical way in the differentiation between blood and lymphatic vessels, particularly in regard to studies involving vasculature.

Table 1.3 Summary of the main differences between lymphatic and vascular endothelium. ICAM: Intracellular adhesion molecule. JAM: Junctional adhesion molecule. VEGFR-3: Vascular endothelial growth factor receptor-3. Prox-1: Prospero related homeobox gene-1. LYVE-1: Lymphatic vessel endothelial receptor-1. SLC: Secondary lymphoid chemokine. IP-10: IFN-γ-inducible protein-10

	Blood vessel	***Lymphatic vessel***
Cell surface molecules	Von Willebrand factor (Factor VIII), VE-cadherin, ICAM, CD31, JAM1/JAM2, PAL-E (absent from arterioles and some capillaries) and others	VEGFR-3, prox-1, podoplanin, LYVE-1
Basement membrane	Present and continuous	Absent or incomplete basement membrane
Junction types	Tight junctions / Adherens / gap junctions.	Overlapping Loose junctions readily permit the passage of macromolecules, pathogens and migrating cells (not for larger ducts)
Enzymes	Presence of alkaline phosphatase and lack of 5'-nucleotidase	Lack of alkaline phosphatase and presence of 5'-nucleotidase
Chemokines	SLC	IP-10, Eotaxin
Pericytes	Mostly present (unreliable)	Mostly present

1.3. Specific vascular endothelial markers

Interest in grading tumour angiogenesis was initially rekindled with the advent of non-specific endothelial markers (Mlynek *et al*., 1985, Srivastava *et al*., 1988, Porschen *et al*., 1994) but it has been only in the last decade as more specific endothelial markers have become available that quantitation studies have been performed. Angiogenesis in tumours may be assessed by several endothelial markers, including factor VIII (von Willebrand factor) (Teo et al ., 2002), PECAM-1 or CD31 (Solovey *et al*., 2001), pan endothelial marker or CD34, VE-cadherin (Cadherin-5) (Croix Brad et al ., 2000), P1H12 (Solovey et al ., 2001), VEGFR2 (Eleanor et al ., 2001) and Tie receptors (Croix Brad et al ., 2000). These markers have been shown to be expressed in endothelial cells from both normal and tumour tissues.

The ideal vascular endothelial marker would have particular characteristics. It would be exclusively found (positive marker) on or excluded from (negative marker) vascular endothelial cells, rather than depending on relative differences in expression level between perivascular and vascular vessels (Sleeman *et al* ., 2001). In perivascular tumours histopathology technique is usually very characteristic, making diagnostic confusion unlikely here. Very cellular tumours, however, can be mistaken for adnexal tumours. Adnexal tumours express cytokeratins, carcinoembryonic antigen (CEA), or epithelial membrane antigen (EMA), markers not found in glomus tumours or hemangiopericytomas. Naevi can be distinguished from perivascular tumours by their expression of S-100. Glomus tumours express vimentin and muscle-actin isoforms and, to a variable degree, desmin. Laminin and collagen IV, constituents of basal lamina, can be found outlining cells or groups of cells. Hemangiopericytomas variably express vimentin, CD34, and factor XIIIa, but they do not express factor VIII, Ulex europaeus, or smooth muscle actin. Occasional

expression of S-100, Leu-7, and myelin-associated glycoprotein has also been reported (Govender *et al*., 2002).

On the other hand, in tumours, vascular markers such as CD34, factor VIII-associated protein, or Ulex europaeus are variably expressed. Most hemangioendotheliomas express factor VIII, although the Kaposi form type may not express either this marker or Ulex europaeus while still expressing CD34. These markers are sometimes not expressed by the more aggressive angiosarcomas. For these tumours, CD31 has been reported to be more specific. Lymphatic endothelium expresses factor VIII, CD31, and Ulex europaeus, thus complicating the differential diagnosis of lymphangioma/lymphangiosarcomas and hemangioma/angiosarcoma (Miettinen *et al*., 1994).

Although vascular endothelial cells share certain common functions, it is well known that considerable heterogeneity exists both structurally and functionally along the length of the vascular tree, displaying tight continuous monolayers in organs like the brain or discontinuous layers with intercellular gaps or fenestrae, for example, in the kidney or spleen (Risau W 1995, Garlanda *et al*., 1997, Cines *et al*., 1998). In addition, there are clear differences between macro- and microvascular ECs within the vascular beds of the same organ (Hewett *et al*., 1993).They should be highly stable, specific, and sensitive.

Previously, many specific vascular endothelial markers have been used, most commonly, CD31, von Willebrand factor (vWF), CD34 and PECAM. Other sub-optimal (non specific) markers which have also been employed are, vimentin (Wakui *et al*., 1992), lectin (Carnochan *et al*., 1991, Svrivastava *et al*., 1988, Barnhill *et al*., 1992), alkaline phosphatase (Mlynek *et al*., 1985), and type IV collagen (Visscher *et al*., 1993, Visscher *et al*., 1994, Vesalainen *et al*., 1994), all of which suffer from lower specificity and sensitivity.

As discussed above, tumour vascularity is measured by staining tissue sections with antibodies specific (or highly specific) for antigens expressed by vascular endothelial cells

such as factor VIII (von Willibrand factor), CD-31 or CD-34 (Weidner N 1995). This is followed by counting (under high power) the number of highlighted vessels in so-called vascular 'hotspots' i.e. localized areas where there are unusually high numbers of vessels, as detected first under lower power magnification (Weidner *et al* ., 1991). The following sections discuss details of some of the main markers for vascular endothelium.

1.3.1.: Von Willebrand factor (vWF).

Von Willebrand factor (vWF) has merited extensive study since its characterization during the 1980s. This can be attributed both to its pivotal role in blood coagulation mechanisms, and to its association with the most common inherited bleeding disorder in man, von Willebrand disease (VWD). The development of an understanding of the pathophysiology of vWF and VWD originates from the description in 1926 of a family from Föglö, an island in the land archipelago of Finland. Von Willebrand (von Willebrand EA 1926) reported a family with a serious hereditary bleeding disorder which he described as a 'pseudohaemophilia'.

Von Willebrand factor (vWF), a multimeric glycoprotein, is synthesized exclusively in endothelial cells and megakaryocytes and stored in Weibel-Palade bodies in ECs and in platelet-granules (Mannucci 1995). As a carrier for coagulation factor VIII in the plasma, vWF may coordinate formation of fibrin and platelet-rich thrombi. Under physiological conditions, vWF is released from ECs into the plasma and to the abluminal cell surface, where it binds to the sub endothelium. Here it mediates initial platelet adherence to the sub endothelium by linking to specific platelet membrane receptors (glycoprotein lb-IX complex) and to constituents of sub endothelial connective tissue. In pathological conditions, ECs stimulation by agents such as thrombin, fibrin, histamine, epinephrine, vasopressin, endotoxin, cytokines, components of the complement systems, and shear stress is followed

by a rapid release of vWF from the storage granules of the endothelium into the circulation (Lopes *et al*., 1998).

The vWF gene, located on the short arm of chromosome 12 (Ginsburg *et al*., 1985, Verweij *et al*., 1985), resides within 178kb of genomic DNA. It consists of 52 exons, the largest, exon 28, spanning 1.4kb (Mancuso *et al*., 1989). The vWF gene is transcribed into a 9-kb mRNA which is translated to produce a 2813 amino acid product (Verweij *et al*., 1986), designated pre-pro-von Willebrand factor (Bonthron *et al*., 1987). This precursor polypeptide consists of a 22 amino acid signal peptide, a 741 amino acid propeptide and a 2050 residue mature subunit. The mature vWF sequence is comprised of a series of homologous sequence domains thought to have arisen as a result of gene segment duplications (Figure1.5) (Verweij *et al*., 1986, Bonthron *et al*., 1987, Shelton-Inloes *et al*., 1987). Functional sites of the vWF molecule represented by letter (C) have been localized to areas of these repeat units, as illustrated (Figure1.5) in relation to the domain structure.

Restricted to endothelial cells and megakaryocytes, it is less specific for endothelial neoplasms than CD31 and CD34. However, it is useful as a confirmatory marker, particularly in well-differentiated tumours (Millard *et al*., 1985). von Willebrand factor (vWF) is routinely used to identify vessels in tissue sections. Vessel density in tumour specimens, as determined by immuno-histochemical staining for vWF or other endothelial cell markers, is a negative prognostic factor for many solid tumours. vWF is heterogeneously distributed throughout the vasculature, transcriptional control in response to the tissue microenvironment being responsible for local variations in endothelial cell levels of vWF.

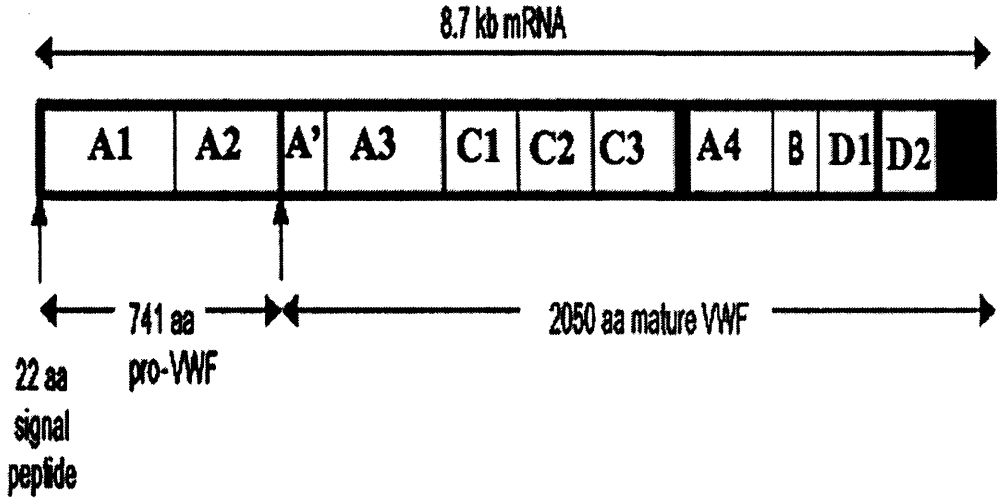

Figure 1.5. The vWF repeated homologous domain structure, representing the complete 2813 amino acid (aa) vWF protein encoded by a 8.7 kb mRNA. Sites of signal peptide and propolypeptide cleavage are indicated by vertical arrows. The lettered boxes (A1 to D2) represent repeated homologous segments. The B domain may be further subdivided into regions B1 to B3. C representation of the location of functional domains within the mature vWF subunit Numbers beneath the domains delineated by vertical bars indicate the amino acid range of each domain.

1.3.2. CD31 (PECAM-1)

The antigen, also known as GPIIa or the cellular adhesion molecule PECAM-1 (platelet endothelial cell adhesion molecule), belongs to the immunoglobulin superfamily and is expressed by some haematopoietic and endothelial cells. It has been shown to have a sensitivity and specificity of 100% for endothelial cells. It is expressed by 80% to 100% of angiosarcomas and hemangiomas. However, it is also weakly expressed by rare carcinomas and mesotheliomas and in rheumatoid arthritis (Longacre *et al*., 1994).

The 130-kDa transmembrane glycoprotein platelet endothelial cell adhesion molecule-1 (PECAM-1) of the immunoglobulin(lg) superfamily is expressed on the surface of circulating platelets, monocytes, neutrophils, and selected T-cell subsets and is a constituent of the endothelial intercellular junction.

PECAM-1 is found in large amounts on ECs and is less abundant on platelets and most leukocytes. It plays a major role in a number of cellular interactions, most notably in the adhesion cascade between ECs and polymorphonuclear leukocytes, monocytes, and lymphocytes (by heterophilic cell interactions) in inflammatory processes and between adjacent ECs (by homophilic cell interactions) during the process of inflammation and angiogenesis (Sun *et al*., 1998, Nakada *et al*., 2000). It appears to be required for neutrophil transmigration as intravenous injection of anti- PECAM-1 antibody results in a 75% decrease in neutrophil sequestration in the lung in a model of immune-complex injury (Vaporciyan *et al*., 1993).

1.3.3. Blood Group Antigens (ABO)

Ulex lectin, derived from *Ulex europaeus*, binds to the H substance of the ABO system. It seems to be more sensitive than factor VIII in the recognition of endothelium and angiosarcomas. However, it is less specific since it also recognizes a variety of normal cells and some sarcomas (Holthofer *et al*., 1982).

1.3.4 CD34

Less is known about the significance and modulation of endothelial CD34 expression. CD34, regarded as a common diagnostic endothelial marker, is a 115-kDa transmembrane glycoprotein. Present on human progenitor cells and endothelial cells, it is a very sensitive marker for endothelial differentiation. Anti-CD34 antibody stains neoplastic endothelium more strongly than normal endothelium. It is expressed by 70% of angiosarcomas, 90% of Kaposi's sarcomas, and 100% of epithelioid hemangioendotheliomas. However, CD34 has a much broader reactivity. It is expressed by certain cells around skin adnexal structures and by nerve sheath lesions, benign and malignant solitary fibrous tumours, gastrointestinal tumours, and 50% of epithelioid sarcomas (Miettinen *et al .*, 1994). The coexpression of CD34 and cytokeratin is observed in epithelioid sarcomas, epithelioid angiosarcoma, and glandular schwannoma. Also, 88% of dermatofibrosarcoma protuberans expressed CD34 compared with only rare cases of benign fibrous histiocytoma and dermatofibroma. CD34 in conjunction with Factor-13a is used in the differential diagnosis of superficial spindle-cell lesions. Both markers are expressed by Kaposi's sarcoma and are absent in keloids. In dermatofibrosarcoma protuberans, CD34 is expressed while F13a is not. The opposite is true for benign fibrous histiocytoma and dermatofibroma (Cohen *et al .*, 1994). In clinical practice, CD34 is also used as a marker for leukaemia diagnosis and subclassification, as a label for the quantity of stem/progenitor cells in blood and marrow, and therefore as an antigen for immunological purification of stem/progenitor cells for clinical transplantation (Sauter *et al .*, 1998, Krause *et al .*, 1996). Although it is reported to be up-regulated in angiogenesis (Schlingemann *et al .*, 1990), the function of endothelial CD34 remains to be clarified (Sauter *et al .*, 1998).

1.3.5. Monoclonal antibodies

Antibodies that react with endothelial cell (EC) structures (known as anti–endothelial cell antibodies AECA) were first reported in the early 1970s. These monoclonal antibodies have been raised against vascular endothelial cells with the aim to be used as a vascular endothelial-specific markers in several diseases characterized by immune-mediated vascular damage and used to differentiate between tumour and normal vessels (Table 1.4) (Del Papa *et al*., 1994). Some antibodies have been shown to bind lymphatic vessels as well. However, a double stain with collagen type-IV antibodies could be used to differentiate lymphatic from blood vessels (Ezaki *et al*., 1990).

Table 1.4 . Some selective markers of Endothelium

Antibody	Antigen	Distribution of Ec staining	Reference
TEC 11	Endoglin	tumour>normal vessels	*Burrows FJ et al 1994*
E-9	p96kD dimer	tumour>normal vessels	*Wang JM et al 1993*
FB5	Endosialin	mostly tumour vessels	*Rettig WJ et al 1992*
EN 7/44	P30.5 kD	Stain tumours and inflammation	*Hagemeier HH et al 1986*
PAL-E	?	Normal small vessels and tumour	*Schlingemann RO et al 1985*
4A11	blood group	Preferential tumour and inflammation	*Koch AE et al 1994*

1.3.6. Gap junctions.

The intercellular junctions between vascular endothelial cells have distinguishing features, including overlapping, interdigitated and attenuated interconnections which are open to provide large gaps through which macromolecules and circulating cells can pass (Roberto Bruzzone *et al* ., 1993). This junction is composed of connexin (Cx) proteins, of which there are at least 20 being identified (Willecke *et al* ., 2002, Kumar *et al* ., 1996). More over, the presence of gap junctions has been documented among and between vascular cells (Larson *et al* ., 1987, Little 1995). Conflicting reports of the exact Cx composition in cells and tissues suggest that Cx distribution varies among vascular beds and vessel types and is differentially regulated in response to physiological and pathological conditions (such as cancer). Endothelial cells predominantly express Cx37 and Cx40, both of which have been suggested as a possible marker for small vascular capillaries (Bastide *et al* ., 1993, Hennemann *et al* ., 1992, Beyer 1992, Gabriels *et al* ., 1998, Bruzzone *et al* ., 1993). However, Cx 43 has been found expressed in vascular endothelium (Bruzzone *et al* ., 1993). Cx37 and Cx40 can also be detected in the junctions between cultivated lymphatic vessel endothelial cells (Jaehyuk *et al* ., 2004). Therefore, connexin cannot be considered as a specific marker.

1.3.7. Additional molecular markers in newly formed blood vessels, especially integrins and cell adhesion molecules.

The discovery of VEGF receptors and their upregulation in newly formed blood vessels highlights the fact that indeed, there can be major phenotypic differences between mature, quiescent vessels, and their newly formed counterparts. Such differences are essential in avoiding unwanted toxicity to normal vessels when using anti-angiogenic drugs, and thus achieving a sufficient therapeutic index. A number of such differences are now

known, and include a very significant elevation of expression in ECM-binding integrin receptors, such as $\alpha_v \beta_3$ or $\alpha_v \beta_5$ (Brooks *et al .*, 1994, Stromblad *et al .*, 1996). This was first reported by Cheresh and colleagues (Brooks *et al .*, 1994) who exploited such differences using specific neutralizing antibodies or small molecule peptide antagonists to block angiogenesis, which occurs, at least in part, by induction of endothelial cell apoptosis.

Other markers that are upregulated in activated endothelial cells include adhesion molecules such as E-selectin (Bischoff 1995), endoglin (Burrows *et al .*, 1995), glycoproteins such as `prostate-specific' membrane antigen (Chang *et al .*, 1999), the ED-B domain of fibronectin (Castellani *et al .*, 1994, Neri *et al .*, 1997) and various proteases (Hiraoka *et al .*, 1998). Many of these can be exploited not only as potential therapeutic targets but also for detection of cancer by nuclear medicine based medical imaging techniques (Neri *et al .*, 1997).

1.4. The vascular endothelial growth factors and their receptors

The detection of vascular endothelial growth factors (VEGFs) started with the discovery of VEGF in 1989 (Keck *et al .*, 1989, Leung *et al .*, 1989). VEGF was initially termed vascular permeability factor (VPF), and still retains this term (Klagsbrun *et al .*, 1993, Veikkola *et al .*, 1999) . Its first function (vascular permeability) was discovered by Dvorak and colleagues (Senger *et al .*, 1993, Dvorak *et al .*, 1995) and was molecularly defined by Ferrara *et al* (Ferrara N 1999b, Ferrara N and Davis-Smyth T., 1997). There are a number of the VEGF family members being identified. The VEGF family currently consists mainly of VEGF -A, -B, -C and –D (Figure 1.6) (Lee J 1996, Olofsson *et al .*, 1999, Joukov V 1996, Orlandini *et al .*, 1996), of which VEGF (or VEGF-A as it is sometimes called) is the most important one. There are several VEGF isoforms and a number (e.g. $VEGF_{121}$ and $VEGF_{165}$) are readily secreted.

Unlike bFGF, VEGF is a very specific mitogen for vascular endothelial cells. It also functions as a potent pro-survival (anti-apoptotic) factor for endothelial cells in newly formed vessels (Alon *et al .*, 1995, Benjamin *et al .*, 1997, Benjamin *et al .*, 1999). Indeed, this may be one of its most significant functions. VEGF is expressed by the vast majority of cancers (Senger *et al .*, 1993, Dvorak *et al .*, 1995), often at elevated levels. Blocking its activity, e.g. by specific neutralizing antibodies to VEGF or to VEGF receptors expressed by 'activated' endothelial cells, can inhibit experimental tumour growth *in vivo*, but not *in vitro* (Kim *et al .*, 1993, Witte *et al .*, 1998). This is because of the highly elevated and restricted expression of two receptor tyrosine kinases, called flk-1/KDR (also known as VEGF-receptor-2) and flt-1 (also known as VEGF receptor-1), by the endothelial cells of newly formed blood vessels, which bind VEGF with high affinity (Neufeld *et al .*, 1999).

It has been recently revealed that VEGF family members are expressed in a variety of human tumours in different ways and tumour cells have been reported to be able to secrete VEGF-A, VEGF-B, VEGF-C, and VEGF-D (Achen *et al .*, 2001, Ferrara N *and* Davis-Smyth T 1997, Salven *et al .*, 1998). However, the angiogenic switch is thought to be carefully regulated, and at least some specific genetic events in tumour progression correlate with vascular metastasis, suggesting that a "Angiogenic switch" mechanism is also a distinct possibility (Jussila *et al .*, 2002).

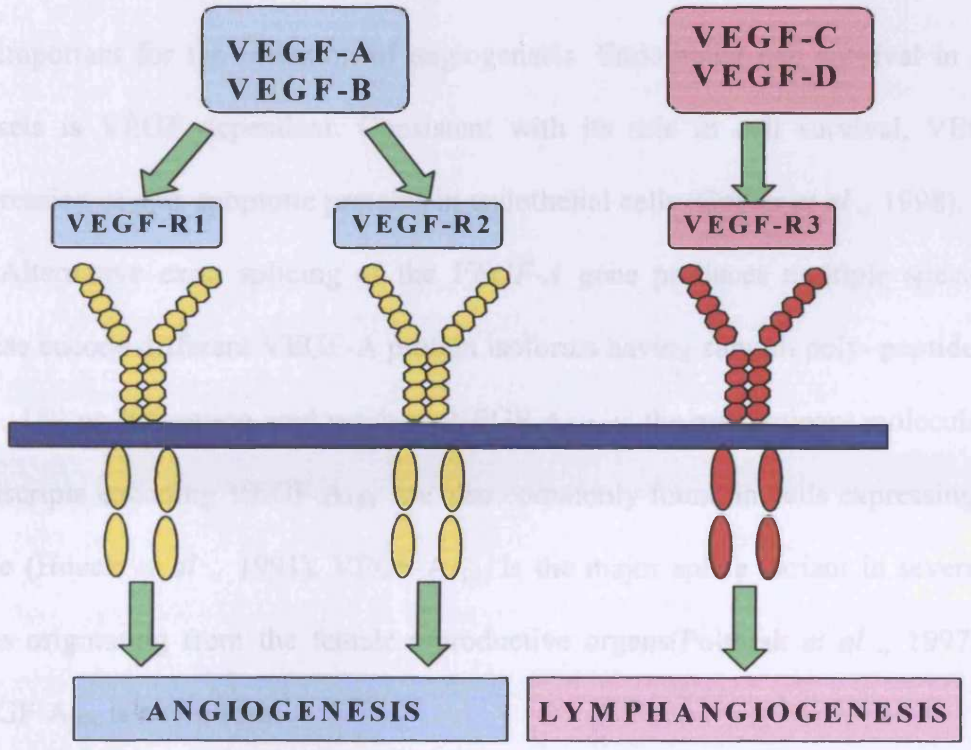

Figure 1.6. The currently known VEGFs and their receptors. VEGFR-1 and VEGFR-2 have seven extracellular immunoglobulin homology domains, but in VEGFR-3, the fifth immunoglobulin domain is cleaved on receptor processing into disulfide-linked subunits. VEGFR-1 and VEGFR-2 mediate angiogenesis, whereas VEGFR-3 is mainly involved in lymphangiogenesis.

1.4.1.VEGF-A

VEGF-A is a major regulator of both physiological and pathological neovascularization. VEGF-A is a relatively specific mitogen for vascular endothelial cells and elicits a pronounced angiogenic response in a variety of *in vivo* models. VEGF-A stimulates endothelial cells to degrade extracellular matrix (ECM), migrate and form tubules *in vitro. In vivo* VEGF-A also functions as a regulator of vascular permeability, which is considered to be important for the initiation of angiogenesis. Endothelial cell survival in newly formed vessels is VEGF dependent. Consistent with its role in cell survival, VEGF-A induces expression of anti-apoptotic proteins in endothelial cells (Gerber *et al .,* 1998).

Alternative exon splicing of the *VEGF-A* gene produces multiple species of mRNA. These encode different VEGF-A protein isoforms having subunit poly- peptides of 121, 145, 165, 189 or 206 amino acid residues. VEGF-A_{165} is the predominant molecular species, but transcripts encoding VEGF-A_{189} are also commonly found in cells expressing the VEGF-A gene (Houck *et al .,* 1991). VEGF-A $_{145}$ is the major splice variant in several tumour cell lines originating from the female reproductive organs(Poltorak *et al .,* 1997). In contrast, VEGF-A_{206} is a rare form.

The splice variants differ in their bioavailability and bioactivity: whereas VEGF-A_{121} is freely soluble and does not bind heparin, the larger isoforms contain increasingly basic and heparin-binding C-terminal domains. A significant fraction of secreted VEGF-A_{165} remains bound to the cell surface and ECM VEGF-A_{189} and VEGF-A_{206} are almost completely sequestered in the ECM (Park *et al .,* 1993) .VEGF-A_{145} binds to heparin with a similar affinity to VEGF-A_{165}. The longer isoforms can be released by plasmin cleavage, generating a proteolytic fragment containing the first 110 N-terminal VEGF-A amino acid residues (Houck *et al .,* 1992, Keyt *et al .,* 1996b).

Local VEGF-A concentrations can thus be increased during tissue growth and remodeling involving proteolysis. Compared with VEGF-A_{165} , VEGF-A_{121} and VEGF-A_{110} are 50-100-fold less potent in endothelial cell proliferation assays (Keyt *et al .,* 1996b). The greater mitogenic potency of VEGF-A_{165} may be conferred by its neuropilin-1 binding region, which is encoded by exon 7 of the VEGF-A gene. Transcription of VEGF-A mRNA is induced by a variety of growth factors and cytokines, including platelet-derived growth factor-B, epidermal growth factor, tumour necrosis factor alpha, transforming growth factor-beta1 TGF-b1 and interleukin-1β. It has been proposed that VEGF may function as a mediator for indirect-acting angiogenic agents such as TGF- b1(Pertovaara *et al .,* 1994).

1.4.2. VEGF-B.

VEGF-B is a nonglycosylated highly basic heparin-binding growth factor with close structural similarities to VEGF-A and PlGF. It exists as two alternatively spliced forms, VEGF-$B_{167}$and VEGF-B_{186} (Olofsson *et al .,* 1996, Gimmond *et al .,* 1996) , and can form heterodimers with VEGF-A (Olofsson *et al .,* 1996, Olofsson *et al .,* 1999). VEGF-B is a selective ligand for VEGFR-1. At the amino acid level VEGF-B demonstrates 45% identity with VEGF-A and approximately 30% with PlGF (Olofsson *et al .,* 1996). The VEGF-B isoforms differ in their biochemical properties.

VEGF-B_{186} has a some what hydrophobic, but O-glycosylated C-terminus, and is secreted from cells. In comparison, VEGF-B_{167} has a strongly basic heparin-binding C-terminus and thus becomes sequestered into the heparan sulphate proteoglycans of the pericellular matrix. Both forms of VEGF-B are widely expressed, being most abundant in heart and skeletal muscle (Olofsson *et al .,* 1996). VEGF-B binding VEGFR-1 on endothelial cells results in increased expression and activity of urokinase type plasminogen activator and plasminogen activator inhibitor 1 (Olofsson *et al .,* 1998). This suggests a role

for VEGF-B in the regulation of extracellular matrix degradation, cell adhesion and migration.

1.4.3. VEGF-C and VEGF-D.

VEGF-C and VEGF-D differ from other VEGF family members by the presence of long N- and C-terminal propeptides flanking the VEGF homology domain (Lee J 1996, Joukov 1996, Orlandini *et al .*, 1996, Joukov *et al .*, 1996, Lee *et al .*, 1996, Achen *et al .*, 1998, Achen 1998). The fully processed or mature forms of VEGF-C and VEGF-D consist of the VEGF homology domain (VHD), which acts as a ligand not only for VEGFR-3, but also for VEGFR-2 (Achen *et al .*, 1998, Jouko *et al .*, 1997). VEGF-C was originally cloned as a ligand for VEGFR-3(Joukov *et al .*, 1996). VEGF-C is produced as a preproprotein, consisting of a signal peptide followed by N-terminal propeptide, the VEGF-homology domain and a cysteine- rich C-terminal propeptide. Its VEGF-homology domain shows approximately 30% identity with VEGFA-$_{165}$ (Joukov *et al .*, 1996, Lee *et al .*, 1996). Proteolytic processing of the VEGF-C precursor produces a 29 to 31kDa protein. A second proteolytic step can be used to generate a 21-kDa homodimer, which binds to VEGFR-2 and VEGFR-3, and induces receptor autophosphorylation (Joukov *et al .*, 1997).

VEGF-C has been shown to induce lymphangiogenesis in transgenic mouse skin and in mature chick chorioallantoic membrane (Jeltsch *et al .*, 1997, Oh *et al .*, 1997). However, recombinant VEGF-C also promotes angiogenesis when applied to early chorioallantoic membrane of chicks, to mouse cornea or to ischaemic hindlimbs of rabbits (Cao *et al .*, 1998, Witzenbichler *et al .*, 1998). Therefore, VEGF-C is likely to play a dual role both as an angiogenic and lymphangiogenic growth factor. The partially adjacent expression patterns of VEGFR-3 and VEGF-C suggest a paracrine mode of VEGF-C action in the formation of the venous and lymphatic vascular systems (Kukk *et al .*, 1996, Kaipainen *et al .*, 1995). VEGF-

C stimulates migration and mitogenesis of cultured endothelial cells (Lee *et al* ., 1996, Cao *et al* ., 1998).

Many clinical studies correlating the levels of VEGF-C in tumours and their metastatic potential have revealed controversial results. However, a significant correlation between VEGF-C expression and distant metastasis has been observed in a variety of carcinomas including breast (Kurebayashi *et al* ., 1999), oesophageal (Kitadai *et al* ., 2001), gastric (Ichikura *et al* ., 2001, Yonemura *et al* ., 1999), colorectal (Akagi *et al* ., 2000), thyroid (Bunone *et al* ., 1999, Fellmer *et al* ., 1999), head and neck (O-charoenrat *et al* ., 2001), prostate (Tsurusaki *et al* ., 1999), and lung (Niki *et al* ., 2000, Ohta *et al* ., 2000).

VEGF-D was first cloned as a c-Fos-inducible mitogen for fibroblasts (Orlandini M*et al* ., 1996). Of the VEGF family members, VEGF-D is most closely related to VEGF-C. The VEGF homology domain VEGF-D and VEGF-C are 61% identical. Both factors have long N- and C-terminal extensions (Achen *et al* ., 1998). A derivative of VEGF-D, corresponding to the mature, proteolytically processed VEGF-C, is a ligand and an activator of VEGFR-2 and VEGFR-3 (Achen *et al* ., 1998). The fact that VEGF-D binds also VEGFR-2 has suggested that it may be possibly angiogenic. However, controversy remains as it has been shown that transgenic overexpression of VEGF-D leads to lymphangiognesis but not angiogenesis (Marconcini *et al* ., 1999). VEGF-D has also been shown to be mitogenic for microvascular endothelial cells (Achen *et al* ., 1998). In adult tissues VEGF-D mRNA is most abundant in lung, heart, skeletal muscle, colon and small intestine (Yamada *et al* ., 1997). In addition, in invasive carcinomas, the expression of VEGF-C was significantly correlated with lymphatic involvement, lymph node metastasis and tumour size, but not with venous involvement or liver metastasis (Kazama S et al 2004).In contrast, other study has shown that VEGF-C expression correlated with the depth of tumour invasion, lymphatic involvement, venous involvement, lymph node metastasis, and liver metastasis. VEGF-D expression correlated with the depth of tumour invasion, lymph node

metastasis, and liver metastasis (Onogawa S et al 2004). The survival time of patients with both VEGF-C- and VEGF-D-positive tumours was significantly shorter than that of patients with both VEGF-C- and VEGF-D-negative tumours.

At present, little is known whether factors such as hypoxia, growth factors, cytokines and hormones regulate expression of VEGF-C and VEGF-D (Bellomo*et al* ., 2000). It has been recently shown that IL-β could up-regulate VEGF-C (Akagi *et al* ., 1999). Although the regulation of VEGF-C and VEGF-D by other cytokines is still not well established, it is known that cross talk and interactions do exist between them.

1.4.4. VEGF receptors.

The VEGF receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR /Flk-1) and VEGFR-3 (FLT-4) form a subfamily within the platelet derived growth factor (PDGF) receptor class. All three consist of seven immuno-globulin-homology Ig domains, a transmembrane sequence and an intracellular portion containing a split kinase domain (Shibuya M 1995). The ~180-kDa glycoprotein VEGFR-1 has been implicated in upregulated endothelial expression of tissue factor, urokinase-type plasminogen activator and plasminogen activator inhibitor 1 (Clauss *et al* ., 1996, Landgren *et al* ., 1998).

In other cell types, VEGFR-1 has different roles, such as tissue factor induction and chemotaxis in monocytes, and enhancing matrix metalloproteinase expression by vascular smooth muscle cells (Clauss *et al* ., 1996, Barleon *et al* ., 1996). VEGFR-2 is a 200-230-kDa and shares 85% sequence identity with the previously discovered mouse fetal liver kinase 1 (Flk-1) (Matthews *et al* ., 1991, Millauer *et al* ., 1993). VEGFR-3 (Flt-4) is also a member of the *flt* subfamily of receptor tyrosine kinases, but its expression is restricted mainly to the lymphatic endothelium of adult tissues (Kaipainen *et al* ., 1995, Pajusola *et al* ., 1992).

During embryogenesis, the VEGFRs are expressed in vascular endothelial cells from the stage of blood island formation onwards. In adult tissues, VEGFR-1 and VEGFR-2 localize to vascular endothelial cells, whereas VEGFR-3 is expressed mainly in the lymphatic endothelium (Klagsbrun *et al* ., 1996). As already mentioned, the ligand specificities of the receptors differ: VEGFR-1 binds VEGF-A, VEGF-B and PlGF (a remote member of the VEGF family), VEGFR-2 binds VEGF-A, VEGF-C, VEGF-D, whereas VEGFR-3 binds VEGF-C and VEGF-D as showed above (Figure 1.6).

Ligand binding induces receptor dimerization and subsequent auto transphosphorylation. The second Ig domain of VEGFR-1 has been shown to be critical for specific binding of VEGF (Davis-Smyth *et al* ., 1996, Barleon *et al* ., 1997). Expression of VEGFR-3 in the colorectal cancer cells was associated with significantly poorer overall survival, but not with lymph node metastasis or depth of tumour invasion (Witte D et al 2002), and these results suggests that VEGFs promote cancer growth not only by stimulating angiogenesis, but also by acting on receptors present on the cancer cells themselves. VEGF expression and vessel counts may aid in predicting patients at risk for metastasis from colon cancer, the mutation of p53 and activation of the Ras/MAPK pathway might be play a role in the induction of VEGF expression in human colorectal cancer (Cassano A et al 2002).

1.5. Microvessel density (MVD).

Microvessel density assessment is the most commonly used technique to quantify intratumoural angiogenesis in solid tumours. It was first developed by Weidner *et al.* in 1991 and uses panendothelial immunohistochemical staining of blood microvessels mainly with Factor VIII related antigen (von Willebrand's factor), CD31, PECAM-1, or CD34. The first step in Weidner's approach is the identification by light microscopy of the area of highest neovessel density (the so called "hot spot"), by scanning the whole tumoural section at low

power. The importance of angiogenesis in tumour progression has been highlighted by several studies showing that the angiogenic potential of tumours assessed by tumour microvessel density (MVD) directly correlates with poor prognosis (Weidner 1995, Weidner *et al .,* 1991, Folkman *et al .,* 1992b, Weidner 1993, Fox 1997, Marinho *et al .,* 1997).

Moreover, MVD may act as a biological marker in differentiating malignant from benign lesions. A good correlation between MVD, distant metastasis and survival in node-negative and node-positive breast cancer patients has been demonstrated (Weidner *et al .,* 1997). Strong correlation was found between VEGF expression and increased tumour microvasculature, malignancy and metastasis in oesophageal carcinoma and gastric carcinoma (Jin-Rong *et al .,*2003). Clinical applications of quantification of the vascularization of primary tumours using specific endothelial cell markers and the assessment of MVD have been used to assess prognosis in a variety of solid tumours (Weidner *et al .,* 1991, Bosari *et al .,* 1992).

1.6. Vascular vessels as targets of anti-cancer therapy.

Angiogenesis research is currently being translated to the clinic. The importance of neovascular development to support the growth of a tumour mass and the formation of metastases has led to great efforts in developing therapeutic tools to block this process (Carmeliet *et al .,* 2000). Inhibitors of angiogenesis have antineoplastic activity in experimental models, and on the basis of promising preclinical results, many of these compounds have progressed to clinical studies (Brower 1999, Giavazzi *et al .,* 1999, Taraboletti *et al .,* 2001). Some forms of chemotherapeutic drugs have been reported to have anti-angiogenic activity (Miller *et al .,* 2001, Schirner M 2000)

A number of concepts have been assembled from inhibition of angiogenesis by the following observations: 1) mechanical separation of tumour cells from their nearest vascular

bed (Gimbrone *et al .*, 1972), 2) blockade the paracrine action of tumour-derived angiogenic factors on vasulature(Kim *et al .*, 1993) 3) administration of angiogenesis inhibitors(Boehm *et al .*, 1997), 4) blockade of endothelial receptors for angiogenic factors (Millauer *et al .*, 1994), 5) endogenous production of angiogenesis inhibitors from tumour cells (Bouck N 1990, Cao *et al .*, 1998) and 6) demonstration of the preangiogenic phenotype in spontaneous tumours (Hanahan 1996).

Thus tumour cells can `feed' (induce) new blood vessels by producing VEGF which, in turn, can nourish the tumour cells, an insidious and self-perpetuating paracrine loop. The possibility of therapeutic disruption of this loop has stimulated intense interest in the biotechnology and pharmaceutical industries, as well as academic centres, using agents such as antibodies (Witte *et al .*, 1998, Zhu *et al .*, 1998, Schlaeppi *et al .*, 1999), VEGF-toxin conjugates (Ramakrishnan *et al .*, 1996) , aptamers (Zhu *et al .*, 1998) and small molecule VEGF receptor antagonists (Shawver *et al .*, 1997), among others.

Much attention over the years has been devoted to the notion that the tumour blood supply can be targeted with antiangiogenic agents. This has culminated in the recent approval of a monoclonal antibody (Bevacizumab/ Avastin) directed against the vascular endothelial growth factor (VEGF) ligand (which is essential for endothelial cell proliferation) for the treatment of colon cancer, when used in conjunction with chemotherapy. The ability of the antibodies to prolong survival of human colorectal cancer patients has established the validity of the anti-angiogenic approach.(Hurwitz *et al.*, 2004). Furthermore, the clinical success of the small molecule kinase inhibitor imatinib mesylate (Gleevec) in chronic myeloid leukaemia (CML) and gastrointestinal stromal tumours (GIST) has established a paradigm for the treatment of tumours whose growth is acutely dependent on specific kinase targets (Sawyers 2003).

In summary, antiangiogenic therapy is based on the following principles: endothelial cells are a readily accessible cellular target; damage to a few endothelial cells is likely to

cause damage to many tumour cells; antiangiogenic treatment can be effective on different tumour types, provided the target mechanisms and molecules are expressed; as endothelial cells are genetically stable cells, acquired resistance to therapy is not expected (Boehm *et al* ., 1997); antiangiogenic treatment might sensitize tumours to chemotherapy and radiation (Mauceri *et al* ., 1998); and antiangiogenic compounds are expected not to be highly toxic the dose producing the active concentration, rather than the maximum tolerated dose, should be used.

1.7 Colorectal cancer and angiogenesis

Tumour angiogenesis is a critical step in the metastatic cascade of colorectal cancer. Metastasis of colorectal cancer may result from passive entry into the circulation, secondary to an angiogenic process occurring within the tumour. The survival of colorectal tumours and thus their metastases are dependent on the balance of endogenous angiogenic and antiangiogenic factors such that the outcome favours increased angiogenesis. Several growth factors have been identified that regulate angiogenesis in colon cancer; the most important of these is vascular endothelial growth factor VEGF (Ellis 2003). The adverse impact of tumour angiogenesis in the context of colorectal cancer on relapse and prognosis has been evaluated in numerous retrospective studies. The need for dependable prognostic markers in colorectal cancer, both in the advanced as well the as adjuvant setting, is greater than ever.

Most of these studies have tended to be relatively small in terms of patient numbers and sometimes have reached conflicting conclusions. VEGFs mRNA are markedly increased in colorectal tumour cell lines, which can secrete a VEGF-like protein into the medium (Senger *et al* ., 1986, Rosenthal *et al* ., 1990). The expression of VEGF and its receptors correlates with the degree of vascularization of tumours as detected by *in situ* hybridization and immunohistochemistry (Shweiki *et al* ., 1992, Brown *et al* ., 1993, Brown *et al* ., 1995, Hatva *et al* ., 1995, Plate *et al* ., 1992, Takahashi *et al* ., 1995, Warren *et al* ., 1995, Hatva *et*

al ., 1996, Jain *et al .*, 1998). VEGF and its receptor have been used as prognostic indicators of an increased metastatic risk in colorectal cancer (Weidner N 1995).

VEGF expressed was assessed as a biological marker of primary colorectal cancer that may improve clinical staging and provide useful information for the application of novel therapeutic strategies (Nanni et al 2002). VEGF expression was observed in all surgical specimens, including normal mucosa, primary colon cancers, and metastatic tumours, as well as in all human colorectal cancer cell lines used in one study (Ellis *et al .*, 2000). The study reported a direct correlation between VEGF expression and the development of metastatic disease in patients who had not received adjuvant chemotherapy, over a five year followup period. VEGF expression at the invasive edge and in the tumour also correlated with vessel count. More over, patients with low VEGF expression had a significantly better survival than patients with high VEGF expression (Ellis *et al .*, 2000).

The expression pattern of VEGF189 mRNA isoforms is correlated with liver metastasis (M1), not metastasis (M0) stage, and poor prognosis in colon cancer (Tokunaga *et al .*, 1998). Preoperative serum VEGF concentrations was significantly higher than those of healthy controls, and reflect disease progression, depth of invasion, liver metastasis, lymph node metastasis and lymphatic invasion.

Expression of VEGF and KDR (VEGF-R2) was found to be higher in metastatic than in non-metastatic neoplasms and directly correlated with the extent of neovascularization and the degree of proliferation. However, expression of other angiogenic factors (bFGF, flt-1, bek, and flg) did not differ among tumour types. Vessel counts were greater in metastatic tumours than in non-metastatic tumours (Takahashi *et al .*, 1995). These findings support the hypothesis that VEGF is an important angiogenic factor in primary and metastatic human colon cancer and that VEGF expression and vessel counts may aid in predicting patients at risk for metastasis from colon cancer. VEGF level was found significantly higher in colorectal cancer-M1 samples compared with colorectal cancer-M0 samples (Barozzi*et al .*,

2002). VEGF expression increased in human colonic angiodysplasia (Junquera *et al .*, 1999). Consequently, detection of VEGF could serve as a clinically useful marker for colorectal cancer progression and metastasis independent of other markers (Tokunaga *et al .*, 1998, Takeda *et al .*, 2000, Kumar *et al .*, 1998).

Colonic adenomas had a statistically higher level of VEGF mRNA expression than normal tissues. The expression was further increased during the development of adenocarcinomas (Wong *et al .*, 1999). This finding supports the hypothesis that activation of VEGF is a key molecular mechanism for the discrete induction of angiogenesis in the pre-malignant phase of colorectal tumour development. In a separate study using immunohistochemistry it was revealed that VEGF staining was significantly higher in patients with colorectal cancer with liver metastasis and lymph node metastasis (Xie *et al .*, 1999). Other work has shown a positive VEGF staining in 46% of metastatic samples. It is interesting to note that abdominal metastatic tumours (68%) had more VEGF positive staining than the liver metastatic lesions (Cascinu *et al .*, 2000). These studies indicate that the VEGF expression may be different between colon cancer metastatic sites.

Xie et al showed that VEGF-A mRNA was higher in tumours with liver metastasis than in those without liver involvement, and was higher in tumours with venous invasion than in those without(Xie *et al .*, 1999). VEGF-A mRNA was the most abundant in colorectal tissue, followed by VEGF-B, VEGF-C, and VEGF-D. VEGF-A and VEGF-B mRNAs were significantly more abundant in adenomas compared with normal tissues, while VEGF-A and VEGF-C were significantly increased in carcinomas compared with normal tissues (Hanrahan *et al .*, 2003). Moreover, a significantly greater amount of VEGF-C mRNA was present in carcinomas compared with adenomas, whereas there was a significant reduction of VEGF-B in carcinomas compared with adenomas, and VEGF-D mRNA was significantly more abundant in normal tissues than in adenomas and carcinomas.

Expression of PD-ECGF, VEGF-A, and VEGF-C was correlated significantly with metastatic potential of colorectal cancer and prognosis in relation to microvessels density (Kaio *et al* ., 2003). VEGF-C expression at the deepest site of tumour invasion has been shown to be a useful predictor of poor prognosis in advanced colorectal carcinoma. Expression of VEGFR-3 in the colorectal cancer cells was associated with significantly poorer overall survival, but not with lymph node metastasis or depth of tumour invasion (Witte *et al* ., 2002). These results suggest that VEGFs promote cancer growth not only by stimulating angiogenesis, but also by acting on receptors present on the cancer cells themselves. VEGF-R2 (KDR) was higher in metastatic than in nonmetastatic neoplasms and was directly correlated with the extent of neovascularization and the degree of proliferation of cancer cells, whereas expression of VEGF-R1 (flt-1) did not differ among tumour types, vessel counts were greater in metastatic tumours than in nonmetastatic tumours (Takahashi *et al* ., 1995). These findings support the hypothesis that VEGF is an important angiogenic factor in primary and metastatic human colon cancer. VEGF expression and vessel counts may aid risk assessment of metastasis from colon cancer. A number of inconsistencies have arisen from previous studies. Firstly, conclusions from early reports are not consistent as already discussed. Secondly, no reports have investigated the entire VEGF family and their receptors, which make it difficult to compare the expression pattern between different members. Thirdly, virtually all the studies have used qualitative approaches (conventional RT-PCR for example).

1.8 Metastasis of colorectal cancer

Most colon cancers arise from adenomatous polyps. About 5% of adenomatous polyps are estimated to become malignant, a process taking approximately 10 years to develop (Figure 1.7). Cancer can grow inward toward the hollow part of the colon or rectum, and/or outward through the walls of the organs. Advanced disease can cause perforation of

the bowel, leading to infection. Metastasis of the disease may occur to the lymph nodes, liver, lung, peritoneum, ovaries, and brain. Most colorectal cancers, except signet-ring cell carcinoma, invade the proper muscular layer when they grow larger than 20mm.

The metastatic spread of tumour cells is responsible for the majority of cancer deaths. Tumour cell dissemination occurs via a number of routes, including local tissue invasion, haematogeneous and / or lymphatic spread as well as direct seeding of surfaces or body cavities. The pathogenesis of cancer metastasis in general is a cascade of multi-step and complex process. In order for the cancer cell to establish metastasis, it has to succeed to overcome several barriers such as basement membranes, surrounding stroma and enter the circulation, followed by colonisation of the surrounding tissues. More than 90% of malignancies are carcinomas i.e. of epithelial cell origin. Epithelial cells are surrounded by stromal tissues, which consist of endothelial cells, lymphocytes, fibroblasts and fat cells. The epithelial cell – stromal interaction is known to impact on the epithelial cancer cell metastasis. The apparently normal stroma surrounding the cancer plays an important role in the growth and metastasis of cancer. Some "normal" stromal cells such as fibroblasts are morphologically and phenotypically different from fibroblasts in other normal tissues.

The surrounding stroma can secrete growth factors to promote cancer growth and can sometimes help cancer spread and progression. Hepatocyte Growth Factor / Scatter Factor (HGF/SF) for example, is a cytokine that is synthesized and secreted by the mesenchymal stroma. It helps dissociation (Stoker *et al* ., 1987), motility of epithelial cells (Li *et al* ., 1994) as well as invasion through the extracellular matrix (Rosen *et al* ., 1994). Keratinocyte growth factor (KGF) and other factors are also involved in epithelial cell / stromal interaction.

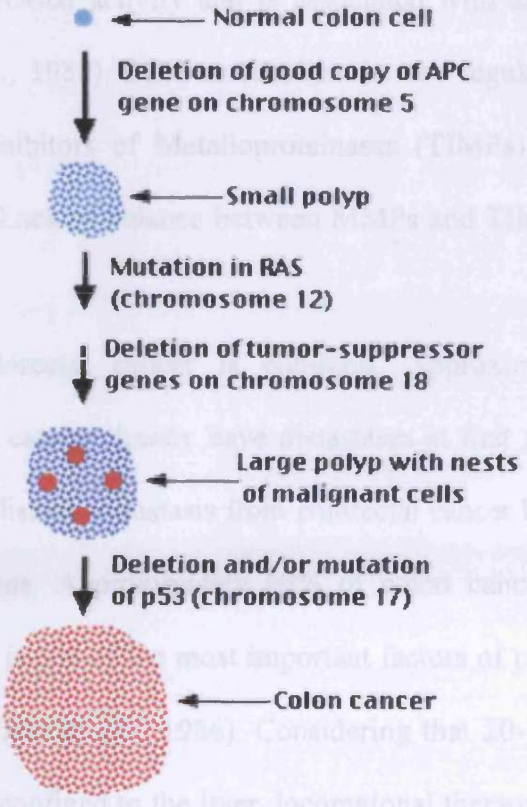

Figure 1.7: The mechanism process of development of colon cancer.

In order for cancer cells to leave the primary tumour and disseminate, there must be a reduction in their normal adhesion mechanisms, both cell-cell adhesion and cell-matrix adhesion (Jiang *et al* ., 2000). Degradation of the ECM is an essential step in the metastasis cascade. This degradation is achieved by several groups of proteases and collagenases. An important group of proteases called Matrix Metalloproteinase (MMPs) (Stetler-Stevenson*et al* ., 1993), can have increased activity and is associated with an increase in cancer cell invasiveness (Liotta *et al* ., 1980). ECM metabolism is also regulated by another group of proteins called Tissue Inhibitors of Metalloproteinases (TIMPs). They are inhibitors of MMPs (Woessner 1991). Lack of balance between MMPs and TIMPs activities can lead to cancer metastasis.

Metastasis of colorectal cancer is common. Approximately 30% of patients diagnosed with colorectal cancer already have metastases at first presentation. The liver is the most common site of distant metastasis from colorectal cancer because of both anatomic and biological determinants. Approximately 60% of colon cancer patients will develop hepatic metastases, which is one of the most important factors of poor prognosis in patients with colorectal cancer (Wiess *et al* ., 1986). Considering that 20–33% of all colon cancer patients die of metastases confined to the liver, locoregional therapy is a reasonable strategy (Wiess *et al* ., 1986, Gilbert *et al* ., 1976). Up to 45% of colorectal carcinoma (CRC) patients will develop local recurrence or metastasis following curative resection. The latter may be the result of shedding of cancer cells from the primary carcinoma prior to or during surgery. This has important implications in assessing disseminated tumour cells in patients.

Commonly used methods and molecular markers of assessing disseminated colorectal cancer cells include; immunohistochemical approaches with anti-(pan)-cytokeratin antibodies (Lassmann *et al* ., 2004, Noura *et al* ., 2002, Bosch *et al* ., 2003), detection of genetic alterations in tumour suppressor (Klump *et al* ., 2004, Nakamori *et al* ., 1997) or

oncogenes (Klump *et al* ., 2004, Linnemann *et al* ., 2004, Yun *et al* ., 2000), as well as qualitative and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assays, which are increasingly popular.

1.9 TUMOUR ENDOTHELIAL MARKERS (TEMs).

1.9.1 The Biology of Tumour Endothelial Markers (TEMs).

Despite the overwhelming interest and extensive efforts in searching for events/ markers that are specific to endothelial cells and to tumour associated endothelial cells, during the past decades, success has been relatively recent, when tumour endothelial markers 1-9 (TEM1-9) were identified (Croix *et al* ., 2000). Using endothelial cells isolated from normal colon tissues and from colon cancer tissues, the authors have conducted microarray analysis and compared the differentially expressed genes in the two cell population. TEMs are considered to be one of several genes elevated in human tumour endothelium and expressed at level at least 20-fold higher in endothelial cells *in vivo* compared with nonendothelial cells (Carson-Walter *et al* ., 2001). TEMs were found structurally and functionally conserved in mouse and human tumour endothelium.

TEMs associated with the cell surface membrane are of particular interest. TEM-1, TEM-5, TEM-7, and TEM-8 contain putative transmembrane domains (Carson-Walter *et al* ., 2001). TEM-5 appears to be a seven-pass transmembrane receptor, whereas TEM-1, TEM-7, and TEM-8 span the membrane once. TEMs are predicted to contain hydrophobic transmembrane domains. TEM-1 was predicted to encode a type I transmembrane protein of 757 amino acids (Figure 1.8). The majority (685 amino acids) of the sequence was predicted to be extracellular, with only a short COOH-terminal cytoplasmic tail (Carson-Walter *et al* ., 2001).

TEM-5 is predicted to encode a seven-pass transmembrane protein of 1331 amino acids. The hydrophobic domains lie with in a 300-amino acid region that shares homology

with seven-pass transmembrane proteins. Similar to TEM-1, TEM-7 was found to encode a type I transmembrane protein with a large extracellular domain, a hydrophobic transmembrane domain, and a short cytoplasmic tail. The extracellular region of TEM-7 contains a plexin-like domain and has weak homology to the ECM protein nidogen. The function of these domains, which are usually found in secreted and extracellular matrix molecules, is unknown. TEM-3 and TEM-7 represent alternative transcripts of the same gene, with the differences simply attributable to the use of alternative polyadenylation sites.

TEM-7 transcript appears to be at least threefold more highly expressed than the other cell-surface TEMs. TEM-7R encodes a protein with 57% amino acid identity to TEM-7 (Carson-Walter *et al*., 2001). Together, these two molecules appear to form a distinct new family of cell-surface proteins. TEM-7R and its mouse counterpart, mTEM-7R, were both found to be selectively expressed on tumour endothelium in humans and mice. These results suggest that both TEM-7 and TEM-7R may be important for angiogenesis in humans, whereas mTEM-7R may play a dominant role in mice. It is also possible that mTEM-7 functions in a different cell type in mice, perhaps in the Purkinje cells of the brain where it's mRNA was found to be expressed (Carson-Walter *et al*., 2001).

Both TEM-7 and TEM-7R contain a plexin-like domain, as well as a weak nidogen-like domain. Although no clear function has been ascribed to these domains, they are often involved in mediating protein-protein interactions. The mouse TEM shows a high level of identity with their respective human TEMs, ranging from 77 to 96% (Carson-Walter *et al*., 2001).

Other endothelial markers identified by SAGE, such as TEM-2, TEM-4, and PRL-3, although not cell-surface receptors, could also prove to be useful targets therapeutically because they encode a GTPase, a guanine-nucleotide exchange factor, and a phosphatase,

respectively. Finally, TEM-8 has been found predominantly expressed in cancer tissues compared to normal tissues, with the following sections discussing TEM-8 in greater detail.

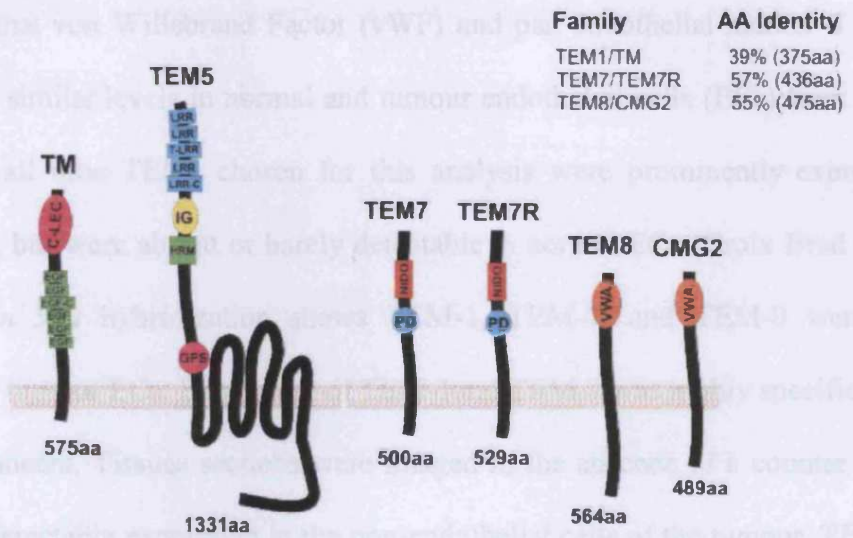

Figure 1.8: The amino acids sequences of TEMs and their identity.

1.9.2 The Role of TEMs in Colorectal Cancer.

Reports on TEMs in colorectal cancer and, indeed, in any tumour types are limited. RT-PCR analysis was used to evaluate the expression of the TEMs transcripts in purified endothelial cells (ECs) derived from colorectal normal and tumour tissues of two patients. It was shown that von Willebrand Factor (vWF) and pan endothelial marker 6 (PEM6) were expressed at similar levels in normal and tumour endothelial cells (ECs) from both patients. In contrast, all nine TEMs chosen for this analysis were prominently expressed only in tumour ECs, but were absent or barely detectable in normal ECs (Croix Brad *et al* ., 2000). Moreover, *in situ* hybridization shows TEM-1, TEM-7, and TEM-9 were specifically expressed in tumour ECs. Expression of TEM-1and TEM-7 was highly specific to the ECs in colorectal cancers. Tissues sections were imaged in the absence of a counter stain to show the lack of detectable expression in the non-endothelial cells of the tumour. TEM-7 was also expressed in ECs from a metastastic liver lesion arising from a primary colorectal cancer.

Such results also show that these transcripts were not simply expressed differentially in the ECs of the original patient, but were characteristic of colorectal cancer. Croix B St et al have investigated whether TEMs transcripts were expressed in angiogenic states other than that associated with tumourigenesis. As assessed by *in situ* hybridization, these transcripts were generally expressed both in the corpus luteum and in the granulation tissue of healing wounds. One possible exception is TEM-8, which was not detected in the corpus luteum. In all tissues examined, expression of the genes was either absent or confined to the EC compartment.

The *in situ* hybridization analysis of human colorectal cancer demonstrated that all four cells surface TEMs (TEM-1, TEM-5, TEM-7 and TEM-8) and TEM-7R were expressed clearly in the endothelial cells of the tumour stroma but not in the endothelial cells of normal colonic tissue. Interestingly, all TEMs demonstrated local regions of intense staining throughout the stromal compartment.

The microcapillaries were likely to account for much of the staining, as vascular casting techniques have demonstrated the presence of a virtually continuous layer of anastomatizing vessels throughout the lamina propria in advanced colorectal cancers. However, the possibility that other stromal cells (*e.g.*, fibroblasts) also express these TEMs cannot be ruled out, although staining was not observed in normal colonic mucosa (Carson-Walter *et al .*, 2001).

1.9.3 TEM-8.

TEM-8 is a type I transmembrane protein, 564 amino acids in length. The 220-amino acid cytoplasmic tail of TEM-8 is much larger than that of the other cell surface TEMs, In the extracellular region, TEM-8 was found to contain a vWF A domain which has a metal ion-dependent adhesion motif. The vWF A domain is also known as an I-domain when present in integrins. The TEM-8 domain is most similar to that of αD integrin, which has been shown to interact with vascular cell adhesion molecule via its I-domain during leukocyte trafficking (Van der Vieren *et al .*, 1999). Both TEM-8 and CMG2 (capillary morphogenic group-2) contain a domain that is homologous to the I domains of integrins, which comprise a Rossmann-like α/β-fold with a metal-ion-dependent adhesion site (MIDAS) motif on their upper surface (Lee *et al .*, 1995). The TEM8-interacting region on collagen alpha 3(VI) was mapped to its COOH-terminal C5 domain may play an important biological role in tumour angiogenesis (Nanda *et al .*, 2004).

TEM-8 and capillary morphogenesis protein 2(CMG2), have been identified as receptor for anthrax toxin. Both bind to the protective antigen portion (PA) of the toxin with high affinity and are capable of mediating toxicity (Wigelsworth *et al .*, 2004). CMG2 and TEM-8 share 60% identity in its I domains as shown in figure 1.9. Homology modelling based on the CMG2 structure shows that this ridge is well conserved in TEM-8 and its murine counterparts, implying that they will bind PA in a similar fashion; however, the

structure and sequence of the ridge are very different in integrins, explaining their weak binding.

Like TEM-8, CMG2 is widely expressed in the body and possesses an I-domain which is highly homologous to that of TEM8. CMG2 binds collagen IV and laminin and thus is also implicated in binding to extracellular matrix proteins. In CMG2 the binding of PA was found to be similar to that of TEM-8, dependent on the I-domain and its ion binding motif, although the cation specificity was slightly different between CMG2 and TEM-8. The crystal structure of the I-domain of CMG2 has recently been solved (Lacy *et al .*, 2002). The structure shows a typical I-domain fold, with a close homology to the open or high affinity conformation of the αM integrin I-domain. Coordination of the metal ion was structurally similar in the two molecules (Figure 1.10) (Santelli *et al .*, 2004).

TEM-8 is the most highly conserved cell surface TEM, with 96% amino acid identity between the human and mouse proteins. The large cytoplasmic tail of both the human and mouse TEM-8 proteins share at least seven potential phosphorylation sites, supporting the hypothesis that TEM-8 is involved in transmitting signals into the cell. The expression pattern of TEM-8 was especially intriguing in that it is the only human TEM characterized thus far that shows no detectable mRNA expression in either the corpus luteum or healing wounds, suggesting that this gene may be highly specific to tumour angiogenesis and not required for "normal" adult angiogenesis. ATR/TEM8 was found highly expressed in epithelial cells lining Bacillus anthracis' three sites of entry (Lung,Skin and Gastrointestinal tract) , and currently three isoforms of ATR/TEM-8 that have been identified as a result of alternative mRNA splicing (Bonuccelli et al., 2005, Scobie et al., 2005).

However, despite some early observations of the expression of pattern of TEM-8 in colon cancer, the regulation of expression of TEM-8 is largely unknown. Furthermore, it is unclear how the TEM-8 structure is related to the angiogenic effects of TEM-8.

A

```
                 **** ********    * ******* ***  * **  * *******    *
CMG2      44     DLYFVLDKSGSVANNWIEIYNFVQQLAERFVSPEMRLSFIVFSSQATIIL
ATR       44     DLYFILDKSGSVLHHWNEIYYFVEQLAHKFISPQLRMSFIVFSTRGTTLM

                  ** **   *  *** *  * * * * ** ***    * ***       *  * *
CMG2      94     PLTGDRGKISKGLEDLKRVSPVGETYIHEGLKLANEQIQKAG--GLKTSS
ATR       94     KLTEDREQIRQGLEELQKVLPGGDTYMHEGFERASEQIYYENRQGYRTAS

                  ******** *       * * **  ** *** ******* **    ** ****
CMG2     142     IIIALTDGKLDGLVPSYAEKEAKISRSLGASVYCVGVLDFEQAQLERIAD
ATR      144     VIIALTDGELHEDLFFYSEREANRSRDLGAIVYCVGVKDFNETQLARIAD

                  **  **** ***** ** ***
CMG2     192     SKEQVFPVKGGFQALKGIINSIL
ATR      194     SKDHVFPVNDGFQALQGIIHSIL
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B

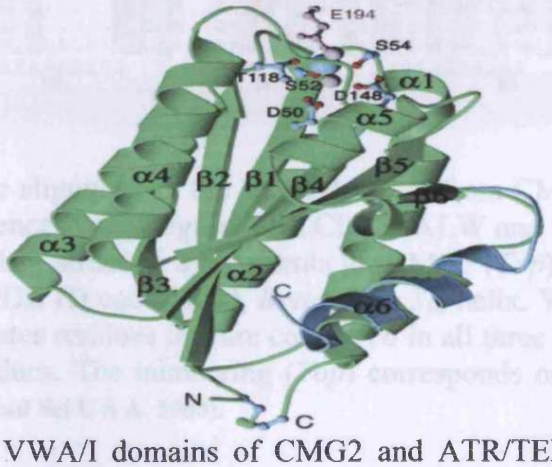


Figure 1.9.(A) The VWA/I domains of CMG2 and ATR/TEM8 are highly related. The aligned amino acid sequences of the VWA/I domains of each protein are shown. Identical residues are indicated by an asterisk, and the five MIDAS motif residues of each protein are indicated by shading. Putative N-linked glycosylation sites found exclusively in ATR/TEM8 are indicated by underlined text.**(B)** Structure of the CMG2 VWA domain. A ribbon diagram of the S38 structure indicates secondary structure elements. Highlighted amino acid residues include the N- and C-terminal cysteines (C39 and C218, respectively) that form a disulfide bond (the sulfur atoms are depicted in yellow) and the conserved amino acids of the MIDAS motif. The Mg^{2+} ion is shown as a large blue sphere with two bound water molecules depicted as beige spheres. The small red spheres correspond to oxygen atoms within the MIDAS amino acids. **(Lacy et al , Proc Natl Acad Sci U S A, 2004)**

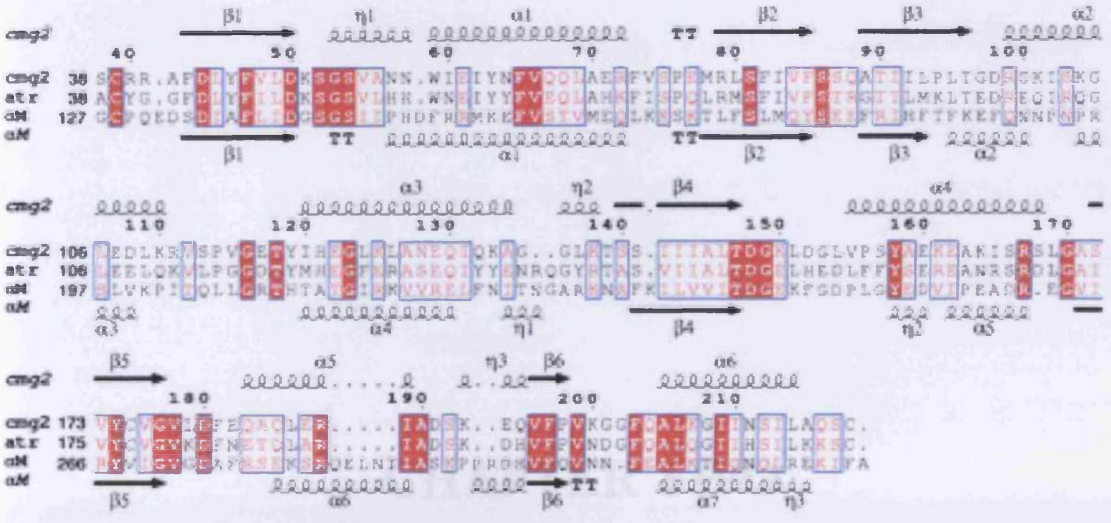


Figure 1.10. Sequence alignment of the VWA domains from CMG2, ATR/TEM8, and the αM integrin. The sequences were aligned with CLUSTALW and were displayed in ESPRIPT along with the secondary structure assignments for CMG2 (*Top*) and the open conformation of the αM integrin (PDB ID code 1IDO, *Bottom*). η, 3_{10}-helix. White lettering boxed with a red background indicates residues that are conserved in all three sequences, and red lettering indicates similar residues. The numbering (*Top*) corresponds only to the CMG2 sequence **(Lacy et al, Proc Natl Acad Sci U S A, 2004).**

CHAPTER TWO

AIMS OF THE THESIS

To-date the role and factors that regulate the tumour endothelial marker TEM-8 in angiogenesis are far from clear and well understood and have not been explored before. Therefore the aim of this thesis was to explore the effects of TEM-8 in tumour angiogenesis and its upregulation by cytokines in endothelial cells.

The main aims of this thesis were therefore:

- To determine, qualitatively and quantitatively the expression of angiogenic factors (VEGF-A, B, C and D), and their receptors (VEGF-R1, R2 and R3), and tumour –endothelial specific markers (TEMs) in colorectal cancer.
- To investigate how tumour-specific angiogenesis such as TEM-8 is regulated, and its effect on angiogenesis.
- To investigate the role of TEM-8 in angiogenesis using an in vitro model, by knocked out TEM-8 from endothelial cells.
- To assess the effects of TEM-8 on endothelial cells growth and migration.
- Investigation of the domain requirement of TEM8 in micro-tubule formation. This will involve the construction of expression cassette that express various part of the TEM-8

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 GENERAL MATERIALS

3.1.1 Cell lines

The following cell lines have been used during this study:

HECV	Human endothelial cell line
HT55	Colorectal cancer cell line
HT115	Colorectal cancer cell line
HRT18	Colorectal cancer cell line
MRC-5	Fibroblast cell line
CHO	Chain's Hammersted ovary
PT67	Fibroblast cell line

The above cells were obtained from The European collection of animal cell culture (ECACC). Human umbilical vein endothelial cells (HUVEC) were obtained from TCS, Biologicals (TCS, Biologicals Ltd, Buckingham, UK). HECV cell line was obtained from Interlab Cell Line Collection (ICLC), Naples, Italy, and CHO cell line from ATCC's Cell Biology Collection Middlesex, UK.

3.1.2 Antibodies

Primary antibodies

The following is a list of primary antibodies used during the study.

From Santa Cruz Biotechnology, California, USA:

Anti- TEM-8	Generated in our Labratory
Anti-Actin	Mouse monoclonal
Anti-Factor VIII	Rabbit polyclonal

Secondary antibodies

PY-99 antibodies Mouse monoclonal

Peroxidase conjugated secondary antibodies to rabbit IgG were obtained from Sigma (Poole, Dorset, UK). A/G protein agarose beads used during immunoprecipitation were obtained from Santa-Cruz Biotechnology (Santa-Cruz, California, USA).

3.1.3 General solutions

The following list of solutions was used routinely during this study. All reagents were supplied by Sigma, Poole, Dorset, and will be in more detaled in Appendix:

Tris-Boric acid-EDTA (TBE)

A stock solution of TBE was made at 10X normal concentration and contained 0.89M Tris, pH 8.3, 0.89M Boric acid and 31mM EDTA. The solution was made by dissolving 108g Tris, 55g Boric acid and 9.3g EDTA in 1 litre dH_2O.

Tris Buffered Saline (TBS)

A 10x stock solution was made up by dissolving 24.22g Tris and 80.06g NaCl in 1 litre dH_2O and adjusting the pH to 7.4 with concentrated HCl. This solution contained 200mM Tris and 1.37M NaCl.

Balanced Salt Solution (BSS)

A 5 litre stock solution of 1xBSS consisted of 137mM NaCl, 2.6mM KCl, 1.7mM Na_2HPO_4 and 8.0mM KH_2PO_4 and was made by dissolving 40g NaCl, 1g KCL, 5.75g Na_2HPO_4 and 1g KH_2PO_4 in dH_2O and adjusting the pH to 7.4 with NaOH.

Tris-EDTA (TE) buffer

A 1 litre stock solution at 1x concentration contained 10mM Tris, pH 7.6 and 1mM EDTA and was made by dissolving 1.211g Tris and 0.292g EDTA in 1 litre dH_2O.

3.1.4 Other reagents and chemicals

- IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12, interferon-γ (IFN-γ), HGF/SF, tumour necrosis factor alpha (TNF-α) and VEGF-D were purchased from National Biology Standard Bureau - NBSB (Salisbury, England, UK) and Chemicon International (Temecula, California).
- MTT reagent used to assess cell growth was obtained from Sigma (Pool, Dorset, UK).
- PicoGreen® dsDNA quantitation Kit was obtained from Eugene (Oregon, USA)
- The chemiluminescent substrate kit KPL (Kirkegaard and Perry Laboratories, Maryland, USA) LumiGLO™ was distributed by Insight Biotechnology (Middlesex, UK).

3.2 GENERAL METHODS

3.2.1 Cell culture and storage

3.2.1.1 Preparation of Growth Medium and Maintenance of Cells

Cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) (pH 7.3) containing; 2mML-gluamine, 15mMHEPES and 4.5mMNaHCO3, DMEM was then supplemented with 10% heat inactivated foetal calf serum (PAA Laboratories, Austria), 50 units/ml of benzylpenicillin (Britannia, Pharmaceuticals, Ltd) and 50 μg/ml of streptomycin (Gibco BRC, Paisley, Scotland). The cell lines were cultured in monolayers

either 25cm² or 80cm² culture flasks (Cell Star, Germany) at cell densities of $1x10^5$ cells/ml. Culture flasks were loosely capped and placed in an incubator at 37°c with a 98% humidification (water tray in the incubator) with 5% CO_2 in air. The flasks were then left until sub-confluent (2-3 days) for experimental work or fully confluent (7 days) for subculture.

3.2.1.2 Trypsinization and Counting of cell lines

- All handling of cells performed under sterile conditions using class II hoods and autoclaved instruments to keep conditions sterile as much as possible.
- The cell lines were removed from the incubator and the medium was removed, by aspiration.
- The flasks were rinsed once with 5ml of HBSS (Hanks Balanced Salt Solution) (137mM NaCL; 8mM Na_2HPO_4; 3mM KCl; 1.5mM KH_2PO_4) buffer to remove all possible traces of serum, which would inhibit the enzymatic action of trypsin.
- 1-2ml of trypsin / EDTA (trypsin 0.01% (w/v) and EDTA 0.05% (w/v) in HBSS buffer) was added to the flask and it was incubated for 5 minutes at 37°c to allow for cell detachment.
- Once the cells had detached from the surface of the flask, the effect of trypsin was neutralised by the addition of 5ml of DMEM and placed in a universal container.
- The cells were centrifuged at 1600 rpm for 5 minutes.
- The excess medium aspirated and the pellet re-suspended in 5mls of DMEM.
- The cells were then re-cultured in flasks, counted (see below) for immediate experimental work or stored by freezing in liquid nitrogen (see below).

- Cell counts were performed using an improved Neubauer haemocytometer counting chamber with an inverted microscope (Reichert, Austria) at 10x10 magnification.

3.2.1.3 Storage of cell lines by freezing in nitrogen

The cell lines were stored in liquid nitrogen by resuspending at a cell density of $1x10^6$ cells/ml in DMEM containing 10% (v/v) dimethylsulphoxide (DMSO; Fisons, UK). 1ml aliquots of cell suspension were transferred into cryopreserved tubes, transferred to –80°C for 24 hours before storage in liquid nitrogen (-196°C) until required.

3.2.1.4 Resuscitation of cell lines

After removal from liquid nitrogen, the cells were allowed to thaw rapidly to 37°c and the cell suspension was transferred to a universal container, with 2ml of DMEM. The cells were then incubated at 37°c for 10 minutes, centrifuged (MSE) at 1600 g for 5 minutes and the excess medium was removed. The cell pellet was re-suspended in DMEM and washed twice to remove any possible trace of DMSO. After the final wash, the cell pellet was re-suspended in 5ml of DMEM and the cell suspension transferred to a $25cm^2$ tissue culture flask. The cells were incubated at 37°C, 98% humidification and 5% CO_2 in air.

3.2.2 Generation of cell line DNA templates for application in PCR

3.2.2.1 Total RNA isolation using the guanidine thiocyanate method

RNA is susceptible to degradation by RNases and therefore special care must be taken to minimise this during its isolation. All methods involving RNA isolation rely on the use of strong denaturants to inhibit the action of endogenous RNases for intact RNA isolation. The use of guanidine, thiocyanate and chlorine are amongst the most effective protein

denaturants and inhibitors of ribonucleases. The guanidine thiocyanate method described by Chomczynski and Sacchi (1987) involves a rapid procedure for combining acid guanidine thiocyanate – phenol-chloroform, in a single step RNA extraction. Using this method, the extraction of RNA is set under acidic conditions, so that the DNA is selectively partitioned into the organic phase whilst the RNA remains in the aqueous phase. The quality and concentration of the RNA isolated can then be detected using a spectrophotometer at a wavelength of A_{260nm}/A_{280nm}.

Cell lines were grown until 80-90% confluent in $25cm^2$ tissue culture. The culture medium was aspirated and the monolayer of cells was detached from the surface of the flask by using 1ml of RNA isolation reagent (Advanced Biotechnologies Ltd, UK). 1ml aliquots of resultant homogenate were transferred to polypropylene tubes and incubated on ice for 5 minutes to permit the complete dissociation of nucleoprotein complexes. One fifth of the volume of chloroform (200µl per sample) was added to the homogenate, followed by vigorous shaking for 15 seconds, incubation on ice for 5 minutes and centrifugation at 15000 rpm for 15 minutes at 4^0C. The homogenate will be separated into two distinct phases: the lower organic phase containing DNA and protein and the upper aqueous phase containing RNA. The majority of the aqueous phase (40 – 50% of the total volume of the homogenate) was carefully aspirated (without disturbing the interphase) and transferred to a polypropylene tube. An equal volume (about 500 - 600µl per sample) of isopropanol (Sigma) was added to the aqueous phase and incubated on ice for 10 minutes.

This was followed by centrifugation at 12000 rpm for 10 minutes and the supernatant fraction was subsequently removed. The resultant RNA pellets were washed once using 1ml of 75% ethanol in diethyl pyrocarbonate (DEPC; Sigma) treated H_2O at 12000 rpm for 5 minutes. The RNA pellets were briefly dried at 55°c for 2 - 5 minutes in order to evaporate the remaining ethanol and dissolved in 50 - 75µl of DEPC treated water by vortexing for 1 minute. The purity of the resultant RNA was determined by measuring

its absorbance at wavelength A_{260nm}/A_{280nm} (WPA UV 1101, Biotech Photometer). A_{260nm}/A_{280nm} ratio gives an estimate of the purity of the RNA. Pure RNA solutions have an optical density ratio of 2.0. Optical density values less than 1.5 indicate ethanol or protein contamination. The samples were either stored at -80°C until used later or ready for reverse transcriptase (RT-PCR), (see below).

3.2.2.2. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR is a simple, versatile and sensitive technique which has greatly enhanced the study of genes and how they are controlled. It provides an alternative and more sensitive approach for the analysis of mRNA, compared to other procedures such as Northern blots and RNA dot blots. This technique has the advantage over more traditional methods in that it requires a small amount of RNA and yet is more sensitive and rapid. In this study R*everse*-iT™ First Strand Synthesis Kit was used. According to the manufacturer's instructions the following components were mixed together in an eppendorff:

RNA template (volume depends on concentration)

PCR H_2O (volume depends on RNA concentration)

[RNA template and PCR H_2O mix provides 13 µl volume)

1µl of anchored oligo dT at a concentration of 0.5µg/µl.

4µl of 5 x 1st strand synthesis buffer

2µl of 5mM dNTP mix – 5mM each

The resultant total volume in each eppendorf is 20µl. The samples were heated at 47°c for 50 – 60 minutes followed by incubation at 75°c for 10 minutes in order to inactivate any RTase. The cDNA samples were then diluted to 1:2 by adding 20µl of PCR H_2O. The samples are ready for amplification using PCR or they were stored at -20°c until required.

3.2.2.3. Polymerase chain reaction (PCR)

The polymerase chain reaction is a very useful technique for enzymatically amplifying a nucleic acid target sequence. It was discovered by Karey Mullis in 1983, for which he was awarded the Nobel prize in 1994. Two short synthetic oligonucleotides that hybridise to complementary strands flanking the target sequence are used as primers for DNA synthesis; the primers are aligned with 3' ends directed towards each other. Thus repeating cycles of denaturation of template, annealing of primers and extension by DNA polymerase results in amplification. A key development in PCR technology was the use of Taq polymerase, a thermostable enzyme from *Thermus aquaticus*, a bacterium native to hot springs. The use of this enzyme means that fresh polymerase does not need to be added after each denaturation step, facilitating automation and recycling costs.

Amplification of cDNA templates previously prepared was performed using PCR. The following method briefly outlines the PCR procedure used for cDNA amplification in this study:

1µl of 1:2 diluted cDNA template

1µl of forward primer (at a working concentration of 10 picomoles)

1µl of reverse primer (at a working concentration of 10 picomoles)

The above components were added to the pre-aliquoted (23µl) PCR Ready-Load Master Mix™ (0.625U of *Taq* DNA Polymerase from *Thermus aquaticus*; 75mM Tris-HCl (pH 8.8 at 25°C); 20mM $(NH_4)_2SO_4$; 1.5mM $MgCl_2$; 0.01% (v/v) Tween 20; 0.2mM from each of the following: dATP, dCTP, dGTP and dTTP, supplied by Advanced Technologies Ltd, UK) in a thin walled PCR tube to make the volume to 26µl. alternatively, the reaction volume can vary (see separate experimental chapters), for example 12µl reaction (6µl Master Mix, 3µl PCR H_2O, 2µl of primers (F & R) & 1µl cDNA) or 1 16µl reaction (8µl

Master Mix, 5µl PCR H_2O, 2µl of primers (F & R) & 1µl cDNA. PCR reactions were performed using a Gene Amp PCR thermal cycler.

PCR conditions were variable between reactions (see relevant experimental chapters), but generally consisted of 35-40 cycles of denaturation (94^0C), annealing (55^0C) and extension (72^0C), with a final extension phase at 72^0C for 10 minutes.

3.2.2.4. Agarose gel electrophoresis

The generated PCR products were separated on either a 0.8% or 2% gel depending on the size of the PCR product. 0.8g or 2g was dissolved in 100ml of TBE (Tris Borate Electrophoresis Buffer) buffer (0.45M Tris-HCl; 0.44M boric acid; 12.5mM EDTA). A low percentage gel (large pore size) would be used if one were to separate PCR products with a high number of base pairs and conversely, high percentage gel (small pore size) would be use to separate PCR products with a low number of base pairs (<500 bp). In the present study, 20mls of agarose gel solution at the appropriate concentration was poured into a universal container and cooled down before casting the gel in the mould in the electrophoresis tank. A comb was then inserted into the gel mould and the gel was allowed to set at room temperature for about 30-40 minutes.

Once the gel had set, TBE buffer was carefully poured into the electrophoresis tank until it reached a level of about 5mm from the surface of the gel. The PCR products were loaded into the wells (5µl per well depending on the comb type).

A 1 kb pair ladder was prepared according to the manufacturers instructions (Pharmacia Biotech, USA) and delivered into the first well (5µl per well) in the same manner as for the PCR products.

A power pack (Gibco BRL, Life Technologies Inc) was connected to the electrophoresis apparatus and the gel was run at a constant voltage of 100 Volts. Electrophoresis was continued for about 35 – 55 minutes or until the samples had migrated

about two-thirds down the agarose gel (depending on the PCR product size). The PCR products were then stained using ethidium bromide (10mg/ml) for 5 minutes with continuous agitation to ensure even staining of the agarose gel. This was sometimes followed by de-staining in tap water for about 1-2 hours, if preferred, to reduce the background staining on the agarose gel from the fluorescent dye. PCR products were then visualised on the agarose gel using an UV transilluminator (UVP Inc) and a digital camera. Images were printed using a thermal printer paper (Uvi Doc system, Uvitee, Cambridge, England) was taken of the gel as a permanent record.

3.2.3. Real time quantitative RT- PCR

Quantitative real-time polymerase chain reaction (PCR) is a relatively new technology that provides a broad dynamic range for detecting specific gene sequences with excellent sensitivity and precision. DNA and RNA can be quantified using this method without laborious post-PCR processing. It is based on the detection of a fluorescent signal produced proportionally during amplification of a PCR product.

In TaqMan Q PCR system the chemistry is the key to detect the target gen (Figure. 3.1). A probe (TaqMan) is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labelled at the 5' end with a reporter fluorochrome (usually 6-carboxyfluorescein 6-FAM) and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine) added at any T position or at the 3' end.

The probe is designed to have a higher T_m than the primers, and during the extension phase, the probe must be 100% hybridized for success of the assay. As long as both fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter.

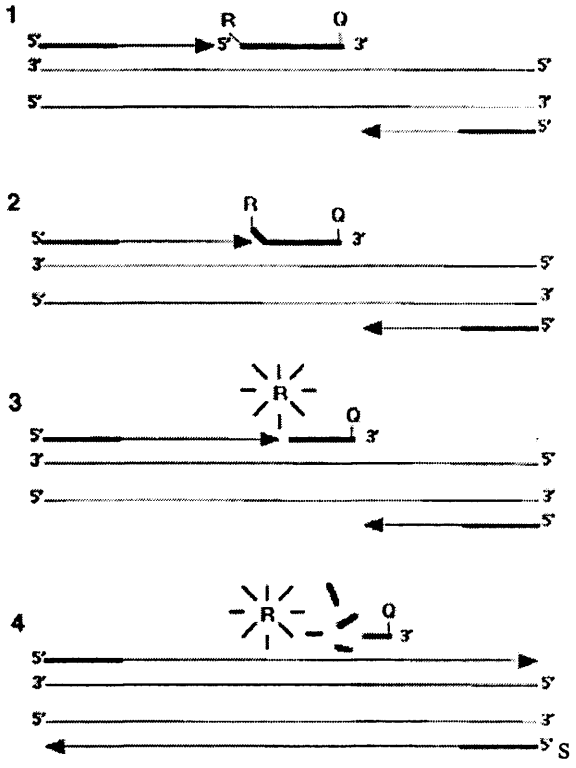


Figure 3.1. Fluorogenic 5' nuclease chemistry. **(1)** Forward and reverse primers are extended with *Taq* polymerase as in a traditional PCR reaction. A probe with two fluorescent dyes attached anneals to the gene sequence between the two primers. **(2)** As the polymerase extends the primer, the probe is displaced. **(3)** An inherent nuclease activity in the polymerase cleaves the reporter dye from the probe. **(4)** After release of the reporter dye from the quencher, a fluorescent signal is generated.

However, as *Taq* polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of *Taq* degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle.

Using Amplifluor™ technology, we developed a rapid and extremely sensitive assay for cytokine mRNA quantification (Figure 3.2). The high signal-to-noise ratio of the assay that is achieved by the unique structure of Amplifluor primers allows for closed-tube fluorescence detection and quantification during PCR amplification (real time) or at endpoint. Elimination of laborious post-PCR sample processing enables high-throughput analysis and greatly reduces the risk of carryover contamination. The Amplifluor primer system is a molecular switch for detecting DNA amplification by energy transfer between fluorophore and quencher(Nazarenko IA 1997). The OFF-to-ON transition occurs when the conformation of the Amplifluor primer changes from "closed" intramolecular stem-loop structure to an "open" extended structure. This structural change is achieved when the Amplifluor primers are incorporated into a double-stranded DNA molecule by primer-mediated DNA amplification such as PCR. The unique features of the Amplifluor system, direct incorporation into amplification products and low fluorescence background observed with unincorporated primers, allow closed-tube quantification by endpoint or real-time analysis.

The system used was iCycler iQ™ detection system consists of a 96-well thermal cycler connected to a laser and charge-coupled device (CCD) optics system. An optical fibre inserted through a lens is positioned over each well, and laser light is directed through the fibre to excite the fluorochrome in the PCR solution. Emissions are sent through the fibre to the CCD camera, where they are analysed by the software's algorithms. Collected data are subsequently sent to the computer. Emissions are measured every 15 seconds.

Conditions for real-time QPCR were as follows: Amplifluor: 95°C for 15s, 55°C for 60s and 72°C for 20s.Taqman: 95°C for 15s, 54°C for 20s and 60°C for 60s. The information is analysed by the computer and two graphs are plotted to show the threshold cycle against the log starting quantity, copy number to show the correlation coefficient and the slope. In addition a table is generated to show the number of copies in each well of the plate. More specific conditions will be explained separately in the relevant experimental chapters.

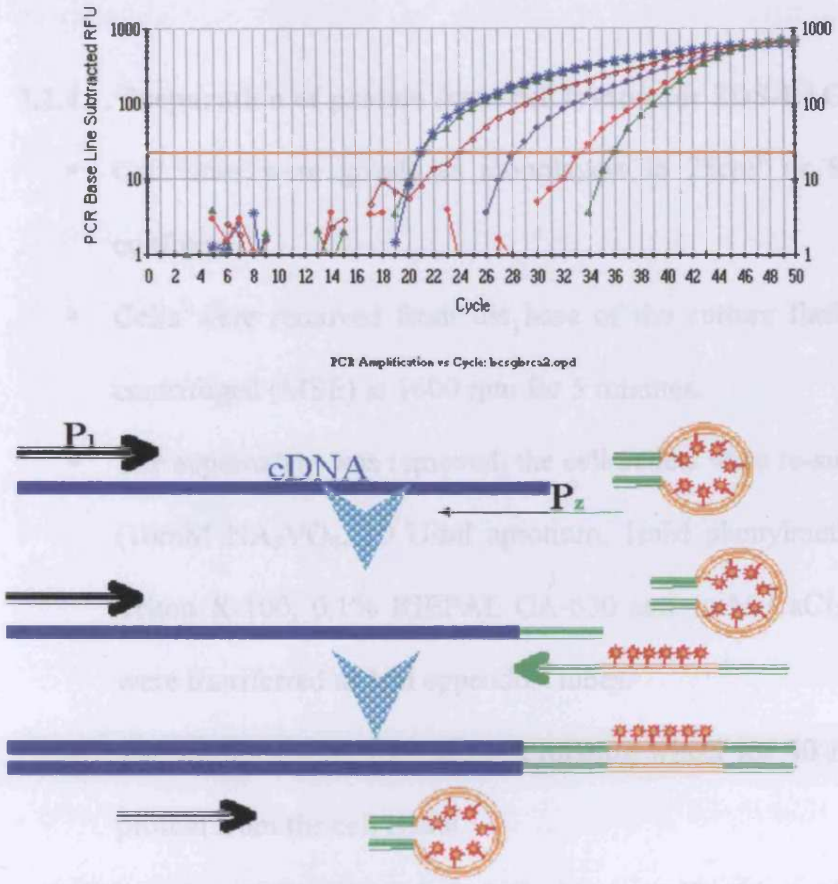


Figure 3.2. Quantitative PCR analysis using the Amplifluor system. The analysis is comprised of four main components, in addition to the routine enzymes and chemicals, the target specific forward primer (P1), target specific reverse primer (Pz) with a unique Z sequence (green part of Pz), which is complementary to the stem of a universal probe (green stem in the orange probe). The probe is conjugated with the fluorophone, FAM.

3.2.4. Western Blotting

These techniques are comprised of a series of complex steps that detect the presence, or absence, of a specific protein in the cell lysates. Detailed description is explained below.

3.2.4.1. Preparation of protein from cell lysates for SDS-PAGE

- Cell lines were grown as monolayers in $25cm^2$ or 80cm2 culture flasks until confluent.
- Cells were removed from the base of the culture flask using a cell scraper and centrifuged (MSE) at 1600 rpm for 5 minutes.
- The supernatant was removed, the cell pellets were re-suspended in cell lysis buffer (10mM NA_3VO_4, 10 U/ml aprotinin, 1mM phenylmethylsulfonyl fluoride, 1.5% Triton X-100, 0.1% IGEPAL CA-630 and 4mM $CaCl_2$) and the resultant lysates were transferred to 1ml eppendorf tubes.
- The samples were rotated on a rotating wheel for 40 minutes in order to extract protein from the cell lysate.
- Protein was subsequently collected by centrifugation at 16,000 rpm for 10 minutes in order to remove cellular debris.
- The samples were optionally stored at -20^0C to continue later.

3.2.4.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

The method used in this study was the one based on the method used by Laemmli in 1970 (Laemmli 1970). The technique works on the basis that when a protein sample is heated in the presence of a reducing agent such as 2-Mercaptoethanol (2-ME; Sigma) and sample buffer containing an ionic detergent SDS, the protein becomes denatured with subsequent protein coating from SDS. This coating gives the protein a high negative charge, which is proportional to the length of the polypeptide chain.

The samples are then loaded onto a polyacrylamide gel and a high voltage is applied, this causes the protein components to migrate in the direction of the positively charged anode. The ionic detergent SDS binds to proteins in proportion to their molecular weights and this binding is independent of their sequence. Furthermore, the resultant protein-SDS complexes are then separated on the basis of their molecular mass, through the molecular sieving properties of the gel. The size of the separated proteins can then be determined by comparing the gel mobility of a particular band, with that of a known protein standard (Ladder). Alternatively, a plot of molecular weight from known protein standards can be plotted against their mobility, in order to determine the size of unknown protein bands. Following electrophoresis, bands on the membrane may be visualised by staining with Coomassie Blue, or the proteins are electroblotted onto nitrocellulose membranes for subsequent probing with relevant antibodies.

3.2.4.3 Gel preparation

Polyacrylamide gels are formed by polymerising acrylamide with a cross-linking agent (methylene-bis-acrylamide) in the presence of a catalyst / chain initiator mixture(30% acrylamide mix, crosslinker ratio 29:1; Bio-Rad), producing a cross-linked matrix with a particular pore size. The most commonly used catalyst is TEMED (N,N,N,'N'-tetramethylethylenediamine; Sigma) and the initiator is the persulfate ion (S_2O8^-), obtained in the form of ammonium persulfate (Sigma). The rate at which gels set may be controlled by varying the concentrations of persulphate and TEMED within the acrylamide mixture. The porosity of the gel is determined by the relative proportion of the acrylamide monomer to cross-linking agent.

Gels are usually referred to in terms of the total percentage of acrylamide, monomer and bis present. Most protein separations are carried out using gels within the range of 5-15%. The appropriate choice of acrylamide concentration is based upon the

separation range required for the protein. Therefore, a low percentage gel (large pore size) would be used if one were to separate proteins of a high molecular weight and conversely, a high percentage gel (small pore size) would be used to separate low molecular weight proteins. Table 3.1 outlines the ingredients, which were used for preparing resolving and stacking gels used in this study.

SDS-PAGE was carried out by assembling the gel (Mini Protean II gel system) apparatus according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond Ca, USA). A brief description of the procedure is outlined below:

- Using 1ml transfer pipette the resolving gel mixture was added to the gel system, by carefully running the solution down one side between the glass plates. This solution was added until it reached a position of 1.5cm away from the lower end of the gel plate.
- To ensure that the gel set with a smooth surface a 0.1% solution of SDS was run down the side between the glass plates until a layer of about 2mm formed on top of the gel solution (the great difference in densities between the SDS solution and the gel, resulted in the SDS solution resting on the surface of the gel mixture without causing serious mixing of the solutions), the gel was allowed to set at room temperature for about 30 –50 minutes. When the gel had set a very clear refractive index change was observed between the polymerised gel and the overlaying SDS solution.
- The SDS solution was removed with some filter paper and the gel rinsed once using distilled water. The stacking gel was then prepared as outlined in Table 3.1 as well as in appendix.
- The stacking gel was added to the gel cassette in the same manner as the resolving gel until it reached the top end of the gel plate.

- A well forming comb was gently inserted between the glass plates until it reached within 1mm from the top of the resolving gel and left to set at room temperature. The refractive changes around the comb indicated that the gel had set.
- The comb was carefully removed from the stacking gel and the gel cassette was assembled in the electrophoresis tank as outlined in the manufacturer's instructions (Bio-Rad Laboratories, Richmond, USA).
- The central reservoir was then filled with the running buffer (25mM Tris-HCl; 3.5mM SDS; 192mM glycine) so that it flooded over and filled the wells.
- The standard protein (ladder) or high molecular weight markers (Sigma) were prepared according to the manufacturer's instructions (detection range 29-205kDa , Sigma,SDS 6H) and loaded (10µl/well) first.
- The protein samples prepared previously were loaded into the wells (10-15µl per well). The protein samples were pre-mixed with a dense loading buffer (10% glycerol; 5% 2-mercaptoethanol; 3% SDS; 80mM Tris-HCl (pH 6.8); 0.012% bromophenol blue), which ensured that the sample settled at the bottom of the well.
- The power pack was connected to the apparatus and the gel was run at a constant current of 15mA per gel. Electrophoresis was continued until the samples had reached the bottom of the gel (as indicated by the bromophenol blue present in the loading buffer).
- Following SDS-PAGE the gel was removed from the gel cassette, rinsed in transfer buffer (25mM Tris-HCl; 192mM glycine; 20% (v/v) methanol) to facilitate the removal of electrophoresis buffer, salts and detergents. The gel was then left to equilibrate in transfer buffer for 20 minutes.

Table 3.1. SDS-PAGE resolving and stacking gel components. The volumes shown are for 10ml (resolving gels) and 5ml (stacking gels) respectively.

Stock Solutions	***15ml Resolving Gel (8%)***	***15ml Resolving Gel (10%)***	***15ml Resolving Gel (12%)***	***5ml Stacking Gel***
DH_2O	6.9ml	5.9ml	4.9ml	3.4ml
30% acrylamide mix	4.0ml	5.0ml	6.0ml	0.83ml
1.5M Tris (pH 8.8)	3.8ml	3.8ml	3.8ml	N/A
1.0M Tris (pH 6.8)	N/A	N/A	N/A	0.63ml
10% SDS	0.15ml	0.15ml	0.15ml	0.05ml
10% ammonium persulfate	0.15ml	0.15ml	0.15ml	0.05ml
TEMED	0.009ml	0.006ml	0.006ml	0.005ml

Electroblotting: Proteins are transferred onto an inert membrane support (e.g. nitrocellulose or nylon) here we used nitrocellulose membrane (Hybond C super-Amerham Biosciences), and once attached, the protein of interest can be detected by a specific monoclonal or polyclonal antibody against it. A nitrocellulose membrane (Amersham International Plc) was cut to the dimensions of the gel (9 x 6cm) and immersed in transfer buffer for about 10-20 minutes to ensure proper binding of the protein to the membrane. Similarly, filter paper was cut to the dimensions of the gel (9 x 6cm) and soaked in transfer buffer for 10-20 minutes. Two pieces of wetted filter paper were placed on the bottom electrode (cathode) and on top of this was placed one piece of pre-wetted nitrocellulose membrane and this was then covered with two further pieces of pre-wetted filter paper forming a sandwich (illustrated in Figure 3.3). The surface of this sandwich was carefully smoothed out to remove the formation of air bubbles, which may interfere with protein transfer. The top electrode was placed on top (anode) of this sandwich and a current was applied. The proteins were then transferred for 40 minutes using the following settings: 5 Volts, 500mA and 8 Watts.

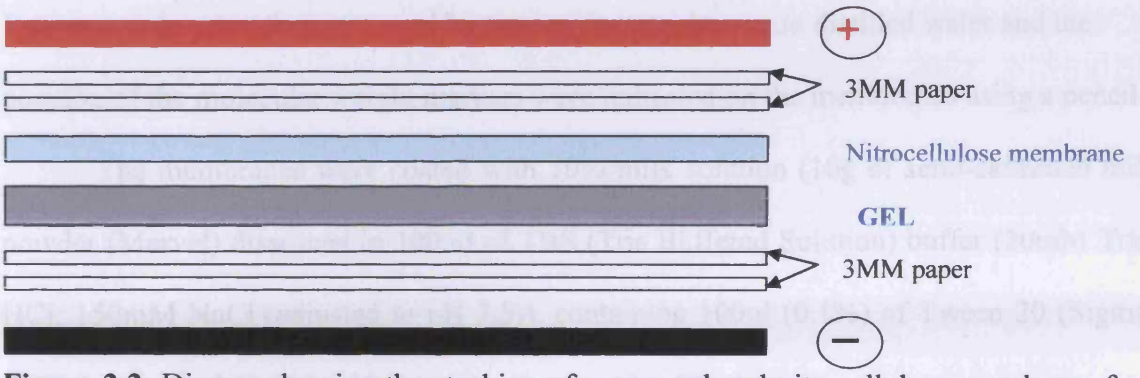


Figure 3.3. Digram showing the stacking of paper, gel and nitrocellulose membrane for electroblotting.

Following electroblotting, the nitrocellulose membranes were removed and stained in Ponceau S (40ml of 2% w/v Ponceau S concentrate; containing 30% w/v trichloroacetic acid and 30% w/v sulphosalicyclic acid (Sigma) was diluted with 360ml of distilled water to provide a 1:10 working solution) for one minute at room temperature. The staining of proteins on nitrocellulose membranes using Ponceau S has two functions; firstly, it verifies that the proteins have been transferred to the membranes and secondly, it aids in the visualisation of the molecular weight markers. In addition the Ponceau S stain is a reversible protein stain and does not interfere with subsequent immunoprobing steps. Excess staining was then removed by rinsing the membranes in distilled water and the position of the molecular weight markers were indicated on the membranes using a pencil.

The membranes were coated with 10% milk solution (10g of semi-skimmed milk powder (Marvel) dissolved in 100ml of TBS (Tris Buffered Solution) buffer (20mM Tris-HCl; 150mM NaCl (adjusted to pH 7.5)), containing 100µl (0.1%) of Tween 20 (Sigma) detergent to block non-specific protein sites for 40 – 60 minutes.

The membranes were transferred to 50ml polypropylene tubes (Falcon) and incubated in 5ml of 3% milk solution in TBS and 0.1% Tween 20 containing the relevant primary antibodies in the appropriate concentrations for 1 hour at room temperature on a rotating wheel to allow for an even antibody coating on the membrane.

Excess primary antibody was removed by washing the membranes three times, for 10 minutes per wash, using 5 – 10ml of 3% milk with 1x TBS and 0.1% Teen 20 solution per wash.

The secondary horseradish peroxidase-conjugated antibodies were prepared in a similar manner to that of the primary antibodies. The membranes were incubated with 5ml (for each membrane) of secondary peroxidase-conjugated antibody at room temperature on a rotating wheel for 1 hour.

Excess secondary peroxidase-conjugated antibody was removed by washing the membranes in the same manner described above for the primary antibody.

This was followed by two 15-minute washes in 5 – 10ml of TTBS buffer (Tween 20 Tris Buffered Solution) containing 100ml of TBS buffer and 200µl (0.2%) and Tween 20 (Sigma) detergent. Finally the membranes were washed with 10 ml of TBS buffer only.

Detection using chemiluminescence:

In the presence of hydrogen peroxide (H_2O_2) and the chemiluminescent substrate luminol, horseradish peroxidase oxidises the luminol with concomitant production of light, the intensity of which is increased 1000 fold by the presence of a chemical enhancer such as phenol. The light emission can be detected by exposing the nitrocellulose membrane to a photographic film

10ml of KPL chemiluminescent reagent A was mixed with 10ml of KPL chemiluminescent reagent B in a universal container and poured into a weighing boat. The nitrocellulose membranes were removed from the TBS solution and excess buffer were allowed to drain onto a tissue. The membranes were immersed in the KPL chemiluminescent solution for about 1 minute and excess solution was allowed to drain off the membranes using tissue paper.

The membranes were covered in Saran Wrap, placed in an autoradiography film cassette and transferred into a dark room. Using safe darkroom lighting conditions, a piece of autoradiography film (Kodak XOMAT-AR) was placed on top of the membranes and the cassette closed. Exposure of the film to the membranes was conducted for 1 and 5 minutes in the first instance, in order to ascertain the strength of the signal. The exposure times for subsequent membranes was adjusted to give the best possible resolution of the antibody signal on the film. Following exposure, the film was developed using an automated film developer (X-ograph Imaging Systems, Malmesbury, Wiltshire, UK).

After all the films had been developed, the membranes were stained using Amido black stain (100mg of Naphthalene black dissolved in 100ml of 10% v/v acetic acid and 25% v/v propan-2-ol) by immersing them for 15 seconds. The membranes were then placed in an Amido black destain (10% v/v acetic acid and 25% v/v propan-2-ol) solution in order to bring up the protein bands on the membrane. The Amido black stain provided a permanent record of the membrane, which can then be compared to corresponding autoradiography film, to aid in the orientation of the membrane.

3.2.4.4.Immunoprecipitation

Immunoprecipitation provides a powerful technique for the detection and quantitation of specific target antigens within cellular lysates. In addition, the detection limits using this technique are capable of detecting small amounts of protein (around 100pg) providing a sensitive method. Furthermore, immunoprecipitation proved to be invaluable with the analysis of intracellular phosphorylation events, occurring following extracellular stimulation. The process of immunoprecipitation involves cell lysis, followed by the addition of a specific antibody directed against the target protein present within the cell lysate sample. The resultant antigen-antibody complexes are then collected by the addition of staphylococcal protein A, which is covalently attached to sepharose or agarose. These immune complexes are precipitated by centrifugation, separated by SDS-PAGE and analysed by immunoprobing. A brief description of the immunoprecipitation method used in this study is outlined in the following paragraph.

- The primary antibody was added to each sample at the appropriate concentrations, in order to precipitate out the desired protein for subsequent detection by immunoprobing.
- The samples were rotated on a rotating wheel for 1 hour.

- Conjugated A/G protein agarose beads at the appropriate concentrations were then added.
- The samples were rotated on a rotating wheel for one hour to allow to the antibody – antigen complex to get attached to the beads.
- Centrifugation at 7000 – 8000 rpm for 5 minutes, to remove cellular debris and unbound protein complexes from the supernatant fraction.
- The protein pellets were washed twice using 500µl of lysis buffer (10mM NA_3VO_4, 10 U/ml aprotinin, 1mM phenylmethylsulfonyl fluoride, 1.5% Triton X-100, 0.1% IGEPAL CA-630 and 4mM $CaCl_2$).
- The samples were then re-suspended in 40-60µl of sample buffer (10% glycerol; 5% 2-mercaptoethanol; 3% SDS; 80mM Tris-HCI (pH 6.8); 0.012% bromophenol blue).
- The samples were denatured under reducing conditions by boiling at 100°c for 5 minutes.
- The samples were then stored at -20°c until ready for use for SDS-PAGE.

3.2.5 Cell growth assays

To determine the effects of TEM-8 on cellular growth after knockout of TEM-8 from human endothelial cells, growth assays were employed during this study.

3.2.5.1 Crystal violet assay

This is a simple assay useful for obtaining quantitative information about the relative density of cells adhering to multi-well cluster dishes. The dye in this assay, crystal violet, stains DNA. Upon solubilization, the amount of dye taken up by the monolayer can be quantitated in a spectrophotometer or plate reader. The final concentration of crystal violet was 0.5% (W/V) in dH_2O. Cells (wild HECV, control plasmid GFP and knockedout TEM-8 cells) were counted with a haemocytometer counting chamber and a specific number of cells were seeded to each well with culture medium (DMEM) in a 96-well culture plate for 72 hours. The medium was discarded. The cells were then fixed with 100μl of 10% formaldehyde added into each well. 100μl of crystal violet solution was then added into each well. The absorbance of the colourimetric products was then measured at a wavelength of 540nm using a spectrophotometer (Titertek).

3.2.5 Cloning and construction of expression cassects in mammalian cells

The method by which *Taq* polymerase amplified PCR products are inserted into plasmid vectors for constitutive or induced expression in selected mammalian cell lines is described below.

3.2.5.1 Production of PCR products

The design of primer sets is critical for correct expression of PCR products in a vector. Primer sets are designed to amplify up the desired target region DNA of interest and may require modifications to create suitable PCR products. Depending on the vector, the products to be cloned may have to incorporate a Kozak codon and / or a stop codon for proper translational termination. Some vectors require PCR to produce amplified products that can be cloned directly in frame so as to ensure the correct codon arrangement.

Following PCR, the PCR products should reveal a single discrete band and if so may be used directly in the cloning reaction. Alternatively, if multiple bands and / or smearing are produced, the PCR reaction may require modification to optimise conditions, before progressing to the cloning reaction.

The cloning reaction involves the ligation of the PCR products (insert) into the reading frame of the plasmid vector. This ligation requires the proper vector-insert molar ratio (1:1-1:3 dependant on vector), and may occur spontaneously if the PCR primers were designed to allow PCR products straight insertion into the reading frame of the vector, which has already been activated for ligation.

3.2.5.2 TOPO cloning

Spontaneous ligation between vector and appropriate insert occurs when approximately 10 ng of insert (0.5-0.2 µl of a typical PCR sample) is mixed gently with the vector at correct ratio (dependant upon vector), and the reaction is made up to 5 µl with sterile water, then left for five minutes at room temperature. Following ligation, the cloning reaction should be transformed immediately into E. coli, otherwise the transformation efficiency may decrease.

3.2.5.3 Construction of non-TA based expression vectors

Alliteratively, for vector and inserts that will not TA ligate into a vector, a digestion of the DNA strands may be required, with specific restriction enzymes prior to a ligation reaction. This ligation requires the presence of an enzyme known as T4 DNA ligase (Promega Corporation, Madison, USA), which catalyses the joining of two strands of DNA between the 5'- phosphate and 3'-hydroxyl groups of adjacent nucleotides resulting in either a cohesive-ended or blunt-ended configuration. This ligation reaction requires incubation at room temperature for three hours or as appropriate depending on the stickyness of the ligation end, followed by precipitation of DNA prior to transformation into E. coli.

3.2.5.4Transformation into E.coli

The cloning reaction was transformed into the competent E. coli cells (JM109, Promega U.K. Ltd.) as follows: a 100 µl aliquot of the chemically competent bacteria (stored at -70°C) was thawed by immersion in an ice-bath for 5 minutes. Following this, the cloning

reaction was added and gently mixed with a pipette, this suspension was then placed on ice for 30 minutes. Cells were then heat shocked, in a water bath, at exactly 42°C for 2 minutes, then immediately placed back on ice for 2 minutes, following which, 900 µl of SOC medium or LB medium (as appropriate) was added. These cells were then incubated for 60 minutes at 37°C with shaking at approximately 225 rpm. The resultant transformation mix was then plated out (at two different volumes) onto pre-warmed LB-agar plates containing the appropriate antibiotic (approx. 50 µg/ml ampicillin for the current study) and incubated at 37°C overnight.

LB (Luria-Bertani) medium and agar plates (1 litre)

Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water. Then adjust the pH to 7.0 with NaOH, and made up to 1 litre. Autoclave the solution, then allow to cool, and add the appropriate antibiotic, this can then be stored at room temperature. To create LB plates, add 15g of agar to the one litre of LB medium before autoclaving. Autoclave, then when cooling add the desired antibiotic and pour into plates. Allow to set, then store inverted at 4°C.

3.2.5.5 Selection and analysis of colonies

Following overnight incubation, the plates should reveal a large number of bacterial colonies containing the plasmid vector. The plasmid vector encodes a gene to enable resistance to specific antibiotics and when transformed into the E. coli, confers this resistance to these cells. Thus, selection occurs in the presence of the antibiotic, as cells without the plasmid vector will not survive. The next step is to establish which of these surviving cells containing the plasmid vector also has the insert incorporated into the vector, arranged in the correct position and direction. Some cloning reactions, particularly during blunt end ligations, result in the PCR products being inserted into the plasmid back

to front. Screening of the colonies is required to determine which of the colonies grown on the plate contains the vector plus insert in the correct arrangement, prior to amplification.

Screening involves conventional PCR, of approximately 10 colonies or as appropriate, to amplify the target sequence of insert DNA at the correct position within the plasmid vector. Individual colonies are examined by using a pipette tip touched against a labeled colony then placed into the PCR reaction cocktail ready for specific amplification of the desired sequence. This is achieved through use of the forward primer for the plasmid and the reverse primer specific for the inserted PCR products. This ensures that amplified products, at the expected size, are that of the plasmid and insert in the correct position. These colonies are then ready for amplification.

3.2.5.6. Amplification and purification of plasmid DNA

Following identification of positive colonies with the appropriate vector and insert positioning, a single positive colony is transferred, aseptically, to inoculate 2ml of LB medium, containing 100 μg/ml of an appropriate antibiotic, and incubated until culture grows to mid-log phase at 37°C in a rotary shaker. At which stage, the resultant culture was then added into 100 ml LB medium (plus antibiotics such as ampicillin), and incubated overnight at 37°C under rotation. The resulting amplification of recombinant plasmids within E.coli, then requires plasmid DNA extraction via a plasmid DNA purification kit (Filter Maxi System, QIAGEN, West Sussex, UK), as described below.

This method uses QIAGEN filter cartridges to recover plasmid DNA from the 100 ml of bacterial cells. To harvest the culture, centrifuge the cells at 6000g 4C° for 15 minutes, then remove the medium so that only the cell pellet remains. This pellet is then resuspended in 10 ml of resuspension buffer, which contains RNase inhibitors (available in the pack). To lyse the cells, 10 ml of cell lysis buffer is added and the solution is mixed

gently to avoid shearing of genomic DNA. Following a 5-minute incubation at room temperature, 10 ml of neutralization buffer is added and mixed, and then transferred to the QIAGEN filter cartridge. After 10 minutes at room temperature, the cell lysate is filtered into the barrel of the QIAGEN tip, which is then allowed to enter the resin by gravity flow. The plasmid DNA binds to the anion-exchange resin.

To remove all contaminants from the plasmid preparation, the resin is washed through with wash buffer. RNA, proteins, dyes, and low molecular weight impurities are removed by this medium-salt wash. The DNA was eluted from the resin through the addition of a high salt elution buffer, and was collected in a 50 ml universal tube. Plasmid DNA was then concentrated and desalted by isopropanol (10.5ml) precipitation, followed by a series of centrifugation and washing steps. The pellet was then dried and resuspended in a suitable volume of water. The yield of DNA was determined through quantitation. Following which, small amount of plasmid DNA was run on agarose gel (0.8%) to check both plasmid purity and size. After that, the DNA inserts were isolated by restriction digestion, if required.

3.2.5.7. Restriction enzyme digestion

Restriction enzymes cut specific DNA sites, dependant on enzyme, when a particular DNA sequence is recognized. Therefore, specific restriction enzymes can be used to cut open the plasmid DNA at specific and desired sites, which result in the release of the fragment of insert DNA. Briefly, to 1-2 μg of purified plasmid, the following were added: 2 μl of 10x appropriate restriction buffer, 10-20 units of each type of appropriate restriction enzyme, and made up to a volume of 20 μl with sterile water. This reaction was then incubated overnight in a water bath at 37^0C. After digestion was completed, 2 μl of the reaction was run on agarose gel (0.8%) to determine the size of the restriction products. After confirmation of successful digestion of the plasmid, the total digest reaction was separated

on a large agarose gel (0.8%) and the DNA bands, corresponding to the insert DNA, excised from the gel. The insert DNA was then isolated and purified from the agarose using a DNA column purification system (DNA mini-prep system, Promega, UK). The resultant DNA was re-suspended in 20 µl sterile water and its concentration was determined by a spectrophotometer. The DNA was then stored at -20°C until needed.

3.2.5.8. DNA sequencing

The sequencing of a DNA fragment detects and produces the specific nucleotide sequence of the DNA sample under examination, for identification and analysis purposes. This sensitive technique allows the identification and confirmation of gene and protein expression in all manner of DNA samples. DNA sequencing has been employed to confirm the nature of PCR products, cloned fragments of DNA, and any nucleotide mutations. The sequencing technique used here utilized the "BigDye Terminator Cycle Sequencing Ready Reaction Kit" (PE Applied Biosystems, California, USA).

Each sequencing reaction requires 4.0µl of Terminator ready reaction mix, 200-500ng of DNA, 0.5 µl of specific sequencing primer and deionised water to the volume of 20 µl. These reagents are added to thin walled PCR tube and undergo PCR for 25 cycles (96^0C for 10 seconds; 50^0C for 5 seconds; 60^0C for 4 minutes). Prior to sequencing, the products are precipitated, washed and dried as previously described. The nucleotide sequence was then determined using an automated DNA sequencer (ABI PRISM, Version 3). Resulting nucleotide sequences were submitted to the Gene Bank database for alignment and analysis. Confirmation of gene presence in a DNA sample is enabled through this method.

3.2.5.9. Transfection of plasmid DNA, via electroporation, into mammalian cells

Once the plasmid DNA has been isolated, purified and quantitated it is ready to be added to cultured mammalian cells. The method employed during this study utilized electroporation of cultured cells to allow plasmid DNA to be incorporated into the cells. This electroporation technique used the Easy Jet Plus system (Flowgen, Staffordshire, UK), which passed a voltage of 310 volts across the cells to produce small perforations in the cell wall integrity, thus allowing passage of plasmid DNA across cell membranes to be integrated into the cells. This procedure is described here: 3 μg of plasmid DNA was added to resuspend (~ $1x10^6$) cells and mixed. The mixture was left to stand at room temperature for 2-5 minutes. The mixture was then transferred into an electroporation cuvette (Euro Gentech, Southampton, UK) ready for electroporation. The cuvette was loaded into the electroporator and a pulse of electricity (310 volts) was passed through the cuvette. The mixture was then immediately transferred into 10 ml of prewarmed culture medium (must be within 30 seconds). This reaction was then cultured under the usual incubation conditions.

3.2.5.10. Establishing a stable expression mammalian cell line

To create a stable cell line that expresses the gene of interest, the culture must first be selected to yield only a population of cells expressing the plasmid-insert construct, then induced to promote expression of the DNA fragment initially inserted into the plasmid vector.

The above electroporated cells grow in culture flasks until they reach semi-confluence. During this stage a selection process is used to remove cells that do not contain the plasmid insert. Selection of plasmid positive cells relies on the presence of an additional drug (antibiotic) resistance gene in the plasmid. Plasmids used in this study have dual resistance genes, i.e. one for prokaryotic selection such as ampicillin resistance and

one for mammalian selection for example neomycin resistance. For the latter, modified antibiotics that are capable of entering mammalian cells are used, such as G418, Zeocin or Hygromycin B. Thus antibiotics (dependant upon plasmid) are added to cultured cells at a concentration of around 100 μg/ml to kill any plasmid absent cells. This antibiotic selection period may continue for a number of weeks, at which point the cells remaining should all contain the plasmid and the inserted DNA fragment. Cells are routinely tested for the presence of the plasmid and insert, using RT-PCR.

Once the plasmid has been incorporated into the mammalian cells, the inserted DNA fragment may be expressed consecutively, or may require an inducing agent to stimulate and promote the expression of the desired gene, depending on the expression vector used. The regulated expression system used required the addition of inducing agents, such as doxycycline (Clontech Laboratories, Califbmia, USA). Following induction, this cell line may then be employed in a series of in vitro studies, to examine for the presence and influence of the expressed gene.

3.2.6 Immunohistochemical staining

Immunohistochemistry was performed on either frozen or fresh tumour sections. Fresh sections were formalin - fixed paraffin wax embedded using the labelled streptavidin method. Fresh 4μm thick sections were de-waxed in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked using 15 minutes incubation in 3% hydrogen peroxide in dH2O. Antigen retrieval was achieved by microwaving in 0.01M citrate buffer pH6. Frozen sections were cut and fixed in 50% methanol and 50% acetone for 15 minutes. The sections were then dried in air for 10 minutes and stored at -20^0C in foil-wrapped slide trays. The following steps were then applied

- Specimens placed in PBS (Optimax wash buffer) for 5 minutes.
- A specific dilution (1:50) of primary antibody added for 1 hour.
- Wash 4 times with PBS.
- Uuniversal multi-link biotinylated secondary antibody (raised in horse) added using various dilutions (1:100) and incubated for 30 minutes.
- Wash 4 times with PBS.
- Slides placed in avidin biotin complex (ABC – Vector Labs) for 30 minutes.
- The bound antibody was detected with diaminobenzidine tetrahydrochloride - DAB (Sigma). DAB was added for 5 minutes (Slides covered for the reaction to take place in the dark). DAB (3,3'-diaminobenzidine) is a substrate for peroxidase and in the presence of H_2O_2 a brown coloured precipitate is formed indicating the presence of a specific protein when used in conjunction with specific antibodies.
- Wash with H_2O for 5 minutes.
- Slides placed in Mayer's haematoxylin for 1 minute.
- Wash with H_2O for 10 minutes (nuclei become blue).
- Dehydration in methanol (3 times).
- Clear in 2 changes of xylene.
- Mount under cover slip and read the slides

Negative controls (using PBS buffer instead of the primary antibody) and positive controls were used in this study. The concentrations of antibodies were different for each staining, details of which will be explained separately in the relevant experimental chapters. Immunostaining was scored semi-quantatively in accordance with the proportion of positive tumour / normal cells.

CHAPTER FOUR

THE EXPRESSION LEVELS OF VEGF-A, B, C, D AND THEIR RECEPTORS (FLT-1, KDR AND FLT-4); AND ITS CORRELATION WITH PROGNOSIS IN PATIENTS WITH COLORECTAL CANCER.

4.1 INTRODUCTION

Colorectal carcinoma is one of the world's most common malignancies, and the prognosis of patients with colorectal carcinoma is dependent on the presence of lymph node metastasis (Dukes *et al.*, 1958, Chapuis *et al.*, 1985, Fielding *et al.*, 1986). Angiogenesis is an essential step in tumour growth and metastasis (Senger *et al.*, 1983). As explained in chapter one, during the angiogenic process, endothelial cells migrate, proliferate and form tubules, by producing MMPs which degrade the basement membrane and extracellular matrix, thus permitting migration of endothelial cells. Supported by surrounding matrix and stromal cells, endothelial cells will divide and organize into hollow tubules that evolve gradually into a mature network of blood vessels. After reaching 2-3 mm^3, tumours become dependent on the angiogenic process, in order to gain a supply of adequate oxygen and nutrients.

The process of angiogenesis is governed by angiogenic activators (predominant in pathological conditions such as cancer), and angiogenic inhibitors (predominant in physiological states such as wound healing). Vascular endothelial growth factors (VEGFs) are the most potent angiogenic factors and commonly associated with tumour angiogenesis. VEGFs are powerful mitogens and act specifically on endothelial cells, thereby profoundly altering the expression pattern of genes associated with angiogenesis (Senger *et al.*, 1983, Dvorak *et al.*, 1999, Neufeld *et al.*, 1999, Leung 1989, Crystal RG 1999).

VEGFs expression is induced in tumour cells by factors such as PDGF, bFGF, TNFα, TGFβ, IL-1β and IL-6. Activation of Raf, Ras and Src as well as loss of suppressor genes like p53 correlate with increased VEGF secretion from tumour cells (Kieser *et al.*, 1994). VEGF increases microvascular permeability, leading to protein extravasation, fibrin deposition and formation of a matrix within which tumour cells are likely to sequester (Senger *et al.*, 1993). The human VEGF gene is assigned to chromosome 6p12-p21 and is

organised into 8 exons separated by 7 introns (Houck *et al*., 1991, Mattei *et al*., 1996, Tischer *et al*., 1991). The coding region spans approximately 14kb. Alternative splicing from this gene results in production of 5 types of mRNA that encode VEGF variants that differ in their molecular mass and in their biological properties (Neufeld G*et al*., 1999). The VEGF family includes VEGF-A, (Dvorak *et al*., 1995, Ferrara N 1996b), -B (Olofsson B*et al*., 1996, Grimmond S*et al*., 1996), -C (Joukov V*et al*., 1996) and –D (Stacker S A*et al*., 2001) as well as placenta growth factor (PlGF) (Veilkkola T*et al*., 2000). The biological effect of an individual VEGF is mediated through the activation of specific tyrosine kinase receptors expressed mainly on angioblast and endothelial cells (Neufeld G*et al*., 1999). VEGF-A and VEGF-B are known ligands for FLT-1/VEGFR-1, VEGF-A and VEGF-C for KDR/VEGFR-2, and VEGF-C and VEGF-D for FLT-4/VEGFR-3 (Hiratsuks S*et al*., 1998). VEGF gene products are markedly increased in certain human tumours: lung, thyroid, breast, gastrointestinal tract, kidney, bladder, ovary and uterine cervix. Although, a few studies about VEGF and tumorgenesis have been reported, there are few articles describing the relationship between VEGF-B,-C,-D and their receptors in human colon cancer progression. Limited studies have examined the whole family of VEGFs and their respective receptors. Here we analysed the expression of VEGF-A, -B, -C and –D, and their receptors VEGFR-1, R-2 and R-3 in a cohort of patients with colorectal cancer and we also correlated these molecules with progression of colorectal cancer.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Colorectal cancer cell lines (HRT18, HT55 and HT115) and human endothelial cell lines HECV cell line, which possesses both vascular and lymphatic characteristics (Ye*et al* ., 2003), was purchased from Interlab Cell Line Collection (ICLC), Naples, Italy. Cell lines

were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) (pH 7.3) containing; 2mML-gluamine, 15mMHEPES and 4.5mMNaHCO3, DMEM was then supplemented with 10% heat inactivated foetal calf serum (PAA Laboratories, Austria), 50 units/ml of benzylpenicillin (Britannia, Pharmaceuticals, Ltd) and 50 μg/ml of streptomycin (Gibco BRC, Paisley, Scotland).

Colorectal tissues (Normal and Cancer) were collected between 1993-1995 in the department, from patients with colorectal cancer, immediately after surgery (with the local Research Ethic Committee approval) and stored at -20 C° until required. The samples consisted of colon tumour tissues (n=48) and samples as following (16 samples Dukes A, 16 samples Dukes B and 16 samples Dukes C) and normal background tissues (n=48) from the same patients. The nature and histological informations were obtained from the respective histology report using Dukes staging, and further verified in our Department using Hematoxylin and Eosin Stain (Hiscox *et al* 1997). The Dukes stage matches the UICC TNM staging: Dukes A equals to Stage I T1 N0 M0 or T2 N0 M0), Dukes B to Stage II T3 N0 M0 or T4 N0 M0) and Dukes C to Stage III Any T N1 M0 or Any T N2 M0)(Manual. 1997, American Joint Committee on Cancer Staging Colon and rectum 1988, rectum. 1997, Winawer *et al*., 1997)

4.2.2 Methodology

4.2.2.1 Generation of cDNA from colorectal tissues (normal and cancer) and cell lines

RNA extraction and reverse transcription kits and PCR mix were purchased from ABgene (Surrey, England, UK). RNA was extracted from colorectal tissues, cell lines (HRT18, HT55 and HT155) and HECV cultured cells (in RNA extraction buffer) using the standard guanidine isothiocyanate method by following the manufacturer's protocol. The purity and concentration of RNA were determined by spectrophotometer at 260 nm. Reverse

transcription was performed from 1μg of total RNA using oligo dt primer according to the manufacturer's instructions.

4.2.2.2 Conventional RT-PCR

Conventional PCR primers were designed using the Beacon Designer software (Palo Alto, California, US) and synthesized by Life Technologies (Paisley, Scotland, UK) (Table 4.1). The agarose gel extraction kit was purchased from Life Technologies. Conventional PCR to amplify the transcripts of VEGF-A, VEGF-B, VEGF-C, VEGF-D and their receptors VEGFR-1, VEGFR-2 and VEGFR-3 was carried out using cDNA from normal colorectal and colorectal cancer tissues. The reaction conditions were: 94 C° for 5 minutes, 36 cycles at 94 C° for 40 sec, 54 C° for 30 sec, 72 C° for 50 sec followed by extension phase of 10 minutes at 72 C°. B-actin was used as an internal housekeeping gene. The PCR products were separated on 2 % and 0.8% agarose gel and stained with 10μl of ethidium bromide prior to examination and photographing under UV light.

	Sense primer (5' – 3')	Antisense primer (5' – 3')
VEGF	attggaggcttgccttgc	gctctatctttctttggtc
VEGF-B	tggtgtcatggatagatgtgtatac	cttggcaacggaggaagc
VEGF-C	ggcttctcctggtgacatctg	ttgcttgggacacattgacattc
VEGF-D	cgatcatctcagtccacattg	cttctggcaggcagcaggtctc
VEGF-R1	gaacgagaaggacggactc	tggtggaactgctgatgg
VEGF-R2	gcctctgtgggtttgcctagtg	ccctctctcctctcccgactttgttg
VEGF-R3	ctgtgcctgcgactgtg	cagcgtggacaggttgag

Table 4.1. Primer sequences for conventional PCR.

4.2.2.3 Real-time quantitative polymerase chain reaction (QPCR).

We employed the iCycler IQ system (BioRad, Camberley, UK) to quantify the levels of respective transcripts of the angiogenic factors and tumour endothelial markers in colorectal specimens, as have been recently reported (Jiang *et al*., 2003a, Jiang *et al*., 2003b) (shown as copies/μl from internal standard). All colorectal cDNA samples were simultaneously examined for each of the VEGFs, VEGF-Receptors along with an appropriate set of plasmid standards and negative controls. Primer sets and probes used in this technique are given in table 4.2.

The detection of VEGF-B, VEGF-C, VEGF-D and VEGFR-1 employed a universal probe system (UniPrimer™) (Intergen, Oxford, England). The UniPrimer system used two primers in conjunction with a universal probe (UniPrimer™), which recognised a specific sequence (z sequence), which had been incorporated into the primers (table 4.2). VEGF-A, VEGF-R2 and VEGF-R3 detection used the Taqman system, which employs a pair of primers and a FAM-labelled probe that recognises the specific product. A hot-start quantitation master mix (Abgene, Surrey, England) was used for the reactions. PCR conditions for the Amplifluor system were as follows: 95°C for 12 minutes, followed by 50 cycles at 95°C for 15s, 55°C for 60s and 72°C for 20s, and detection was at its annealing step.The condition for its TaqMan system was 95°C for 9 minutes, 95°C for 15s and 60°C for 90s, as well documented in section 3.2.2.

Statistical Analysis

Conventional RT- PCR results were analysed by using chi square (χ^2) test. Quantitative data were analysed using student's *t* test.

UniPrimer systems	Sense primer (5' – 3')	Z primer (5' – 3')
VEGF-B	cacagtcaagggcaccac	actgaacctgaccgtacagatgtgtatactcgagct ac
VEGF-C	cctgagtccctggtctct	actgaacctgaccgtacagcttctcctggtgacatc
VEGF-D	gctccagtaatgaacatgg	actgaacctgaccgtacaatctgatgttacgatcgtt
VEGF-R1	ttaaaaggcagcac	actgaacctgaccgtacaatctgctgttcagatcgtt

TaqMan systems	Sense primer (5'–3')	probe	Antisense primer(5'–3')
VEGF-A TAQ	tacctccaccatgccaagtg	Fam-tcccaggctgcacccatggc	atgattctgccctcctccttc
VEGF-R2. TAP	tgtggctctgcgtggaga	Fam-cgggccgcctctgcgggttt	gggcagatcaagagaaacac tagg
VEGF-R3 TAQ	acggcctggtgagtggc	Fam-ccatgacccccccgaccttga	cgtttgactcctccgtgatg

Table 4.2 .Primer sequences for quantitative PCR.

4.3 RESULTS

4.3.1 Expression of VEGFs (VEGF-A,-B,-C and –D) in colorectal cancer cell lines.

VEGF-A and VEGF-D were expressed in HT115 and HRT18, VEGF-C gene was expressed in HT55 and HRT18. VEGF-B expression was in the three cell lines used (HT115, HT55 and HRT18) (Figure 4.1).

4.3.2. Expression of VEGFs (VEGF-A,-B,-C and –D) and their receptors in colorectal cancer tissues.

VEGF-B was expressed in 91.7 % of colon cancer tissues, compared with in 49 % of normal tissues (p=0.001). Similarly, expression of VEGF-C was significantly higher in cancer tissues compared with normal (78.9 % vs 46.4 %, p=0.02). However, the expression of VEGF-A and VEGF-D was found similar between tumour tissues and normal tissues (p=0.15 and p=0.13, respectively) (Figure 4.2 & Table 4.3).

VEGFR-1, R-2 and R-3 were found expressed in HECV cells. VEGF-R3 (Flt-4) expression was detected at a similar rate in colonic tumours and in normal tissue (p=0.14). In contrast, expression of VEGF-R1 (Flt-1) and VEGF-R2 (KDR) were greater in colonic cancer than normal (p=0.01 and P=0.005, respectively) (Figure 4.3& Table 4.3).

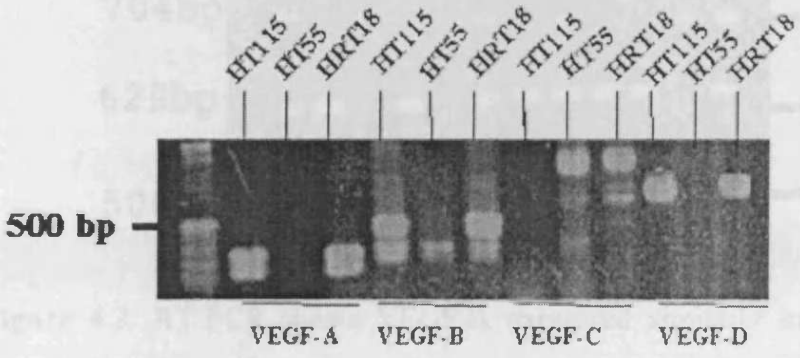


Figure4.1. RT-PCR shows the expression of VEGF-A, -B, -C, and -D in different colorectal cancer cell lines.

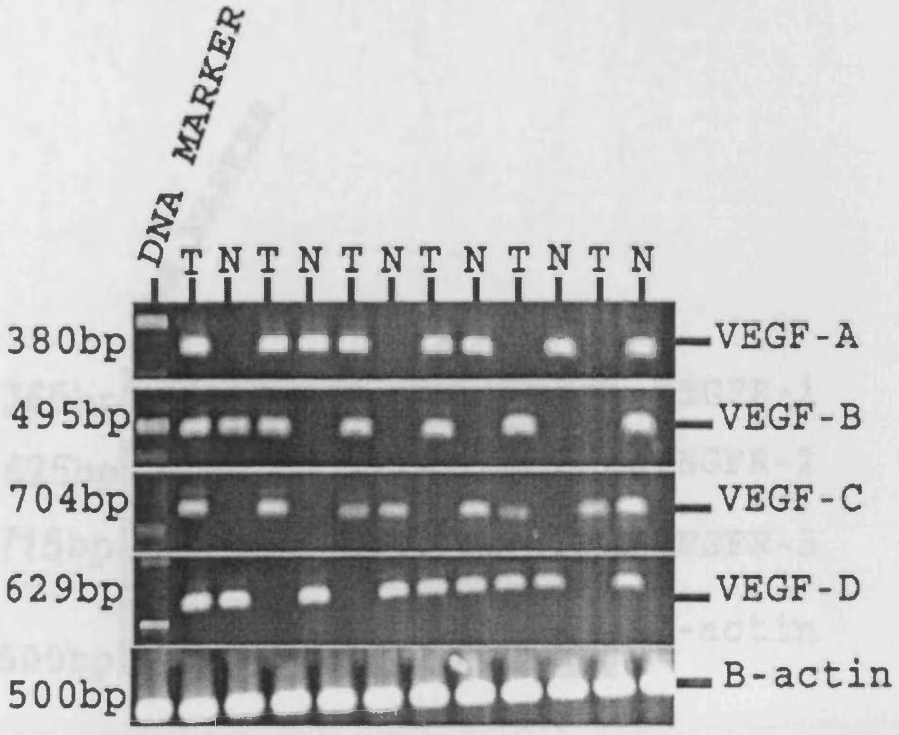


Figure 4.2. RT-PCR shows VEGF-A expressed similarly in colon cancer and normal mucosa. VEGF-B was expressed higher in colon cancer than normal mucosa. VEGF-C was higher in colon cancer, whereas, VEGF-D no difference shown in the expression levels in tumours and normal tissues.

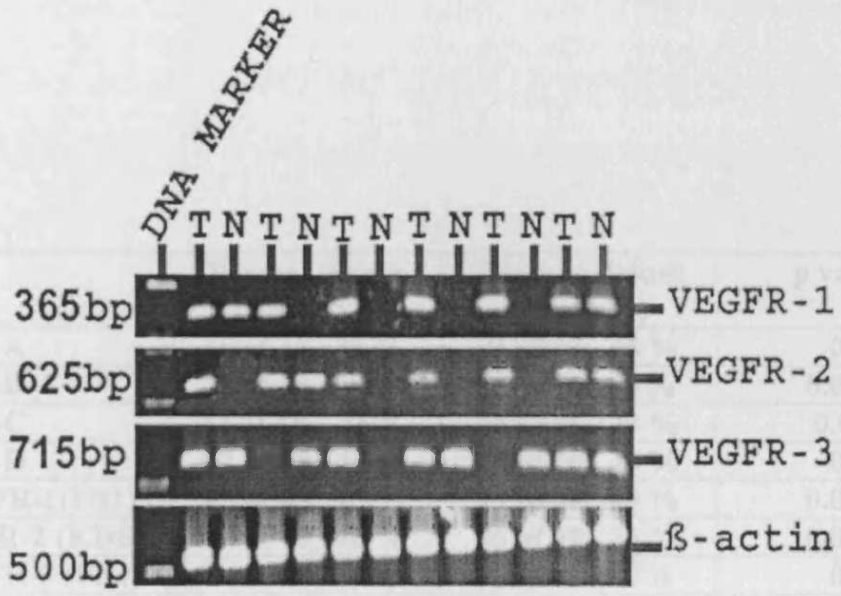


Figure 4.3. RT-PCR shows Flt-1(VEGF-R1) expressed greater in colon cancer than in normal mucosa.KDR (VEGF-R2) expressed much higher in colon cancer than in normal. Flt-4 (VEGF-R3) expressed almost equally in colon cancer and in normal mucosa.

	Normal tissues (n and %)	Tumour tissues (n and %)	p value*
VEGF-A	**18 of 48, 38 %**	**21 of 48, 45 %**	**0.1**
VEGF-B	**24 of 48, 49 %**	**43 of 48, 91%**	**0.001***
VEGF-C	**22 of 48, 46 %**	**38 of 48, 79 %**	**0.02***
VEGF-D	**22 of 48, 46 %**	**23 of 48, 48 %**	**0.1**
VEGFR-1(Flt1)	**6 of 48, 13 %**	**19 of 48, 40 %**	**0.019***
VEGFR-2 (KDR)	**19 of 48, 40 %**	**36 of 48, 76 %**	**0.005***
VEGFR-3 (Flt4)	**16 of 48, 33 %**	**23 of 48, 47%**	**0.1**

Table 4.3. Expression of VEGFs and their respective receptors in colon tissues (Number of expressed samples and percentage positive), using conventional PCR.
*** chi square test ($\chi 2$ test).**

4.3.3. VEGFs and VEGFRs transcript levels in different Dukes Stages.

We went on to analyse, quantitatively, transcript levels in tumour tissues in relation to Dukes staging. The number of transcripts of VEGF-A and VEGF-D was higher in Dukes A compared with B and C. The level of VEGF-B and VEGFR-2 was significantly higher in advanced Dukes C tumour compared to Dukes A ($p=0.02$). The highest level of VEGF-C was found in Dukes B and C ($p=0.04$) compared to Dukes A tumour (Figure 4.4).

Similar to VEGF-C, the level of VEGFR-1 was found to be similar in Dukes B and C and was higher than that in Dukes A tumours. Dukes C had a higher level of expression of VEGFR-2 than Dukes A and Dukes B. The number of copies VEGFR-3 was found almost the same in three Dukes Stages (A, B and C) (Figure 4.5).

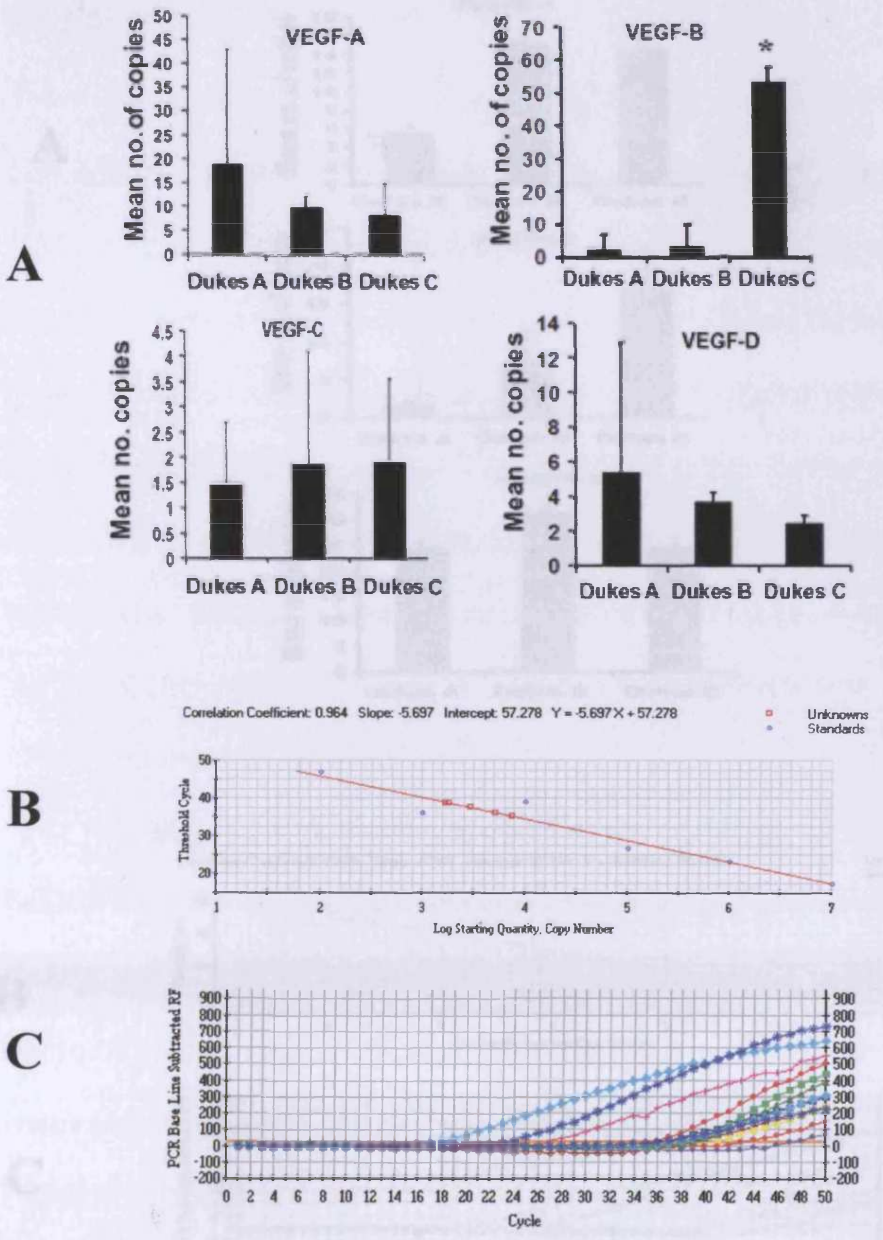


Figure 4.4. Levels of expression of VEGFs in colon tissues in tumours with different Dukes stages, using quantitative PCR (Mean copy number /ng mRNA),VEGF-A E+05 copies ,VEGF-B E+09 copies, VEGF-C E+02 copies and VEGF-D E-5 copies. VEGF-B is significantly higher in Dukes C compared to Dukes A tumour (* p=.02 vs Dukes A) using student's t test (A) . The correlation coefficient (B), shows a slope of 0.964 comparing VEGFs levels with those of a standard. (C) shows the amplification of copy numbers in relation to the cycle number.

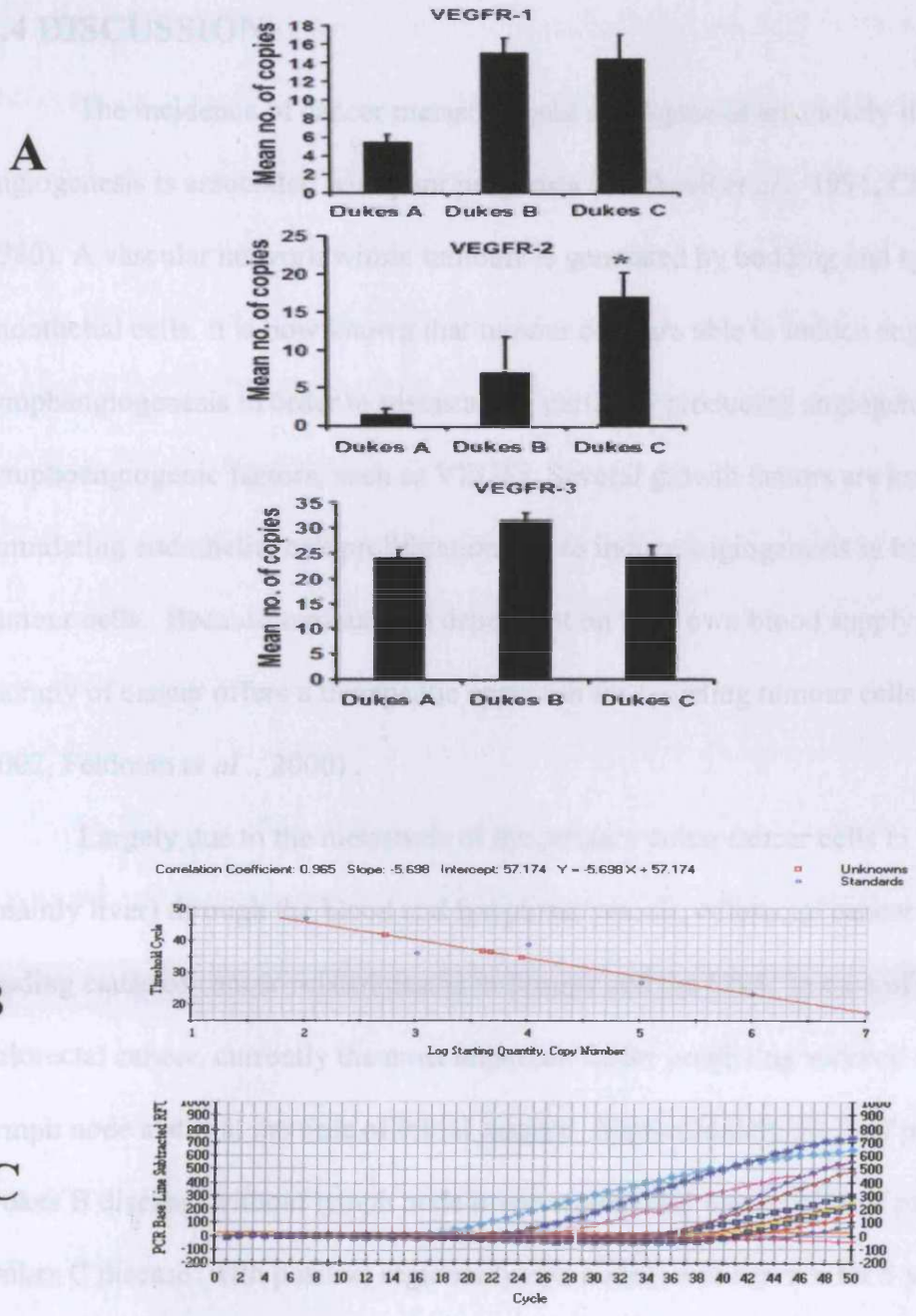


Figure 4.5. Real Time quantitative RT-PCR. (Mean copies/ng mRNA) VEGFR-1 E+04 copies, VEGFR-2 E+05 copies and VEGFR-3 E+02 copies . Levels of expression of VEGF receptors in colon tissues in tumours with different Dukes stages. Dukes C tumour expressed greatest level of VEGFR-2 (* p=0.04vs Dukes A) in contrast to early stage Dukes A tumour using student's t test (A). The correlation coefficient (B), shows a slope of 0.965 comparing VEGF receptor levels with those of a standard. (C) Shows the amplification of copy numbers in relation to the cycle number.

4.4 DISCUSSION

The incidence of cancer metastasis and angiogenesis are closely linked, and tumour angiogenesis is associated with poor prognosis (Bricknell *et al*., 1991, Chodac *et al*., 1980). A vascular network within tumours is generated by budding and sprouting from endothelial cells. It is now known that tumour cells are able to induce angiogenesis and lymphangiogenesis in order to metastasize, partly by producing angiogeneic and lymphoangiogenic factors, such as VEGFs. Several growth factors are known to be stimulating endothelial cell proliferation and to induce angiogenesis in both normal and tumour cells. Because tumours are dependent on their own blood supply, anti-angiogenic therapy of cancer offers a therapeutic approach for targeting tumour cells (Papetti *et al*., 2002, Feldman *et al*., 2000) .

Largely due to the metastasis of the primary colon cancer cells to the other organs (mainly liver) through the blood and lymphatic vessels, colorectal cancer is the second – leading cause of cancer-related deaths in Europe and the USA. In case of manifest colorectal cancer, currently the most important factor predicting survival is the regional lymph node status at the time of initial surgery. Approximately 50 % of patients with Dukes B disease (without lymph node involvement) and nearly 20% of patients with Dukes C disease (with positive regional lymph nodes) will survive for 5 years after curative resection. There are some limited studies on the role of VEGF expression in predicting the prognosis of the patients with cancer, especially colorectal cancer (Kang *et al*., 1997 ., Werther *et al*., 2000). However , this remains highly controversial (Lee *et al*., 2000), Khorana *et al*., 2003).

In the current study, we investigated the expressions of other VEGFs (VEGF-B, C and VEGF-D) in addition to VEGF-A and their receptors (VEGF-Rs) in colorectal cancer as well as investigating the transcript expression level of these angiogenic factors in colorectal cancer tissues and correlated that to the tumour progression. The results have

shown that VEGF-B, VEGF-C and their receptors Flt-1 and KDR mRNA were expressed at a much higher level in colorectal cancer than in normal mucosa. On the other hand, VEGF-A, VEGF-D and Flt-4 expression were not significantly different between colorectal cancer and normal tissues.

These results are in contrast with other studies, which showed that, the detectable level of VEGF-A189 subtype gene in most of colorectal cancer (Tokunaga *et al*., 1998). However, our results are in agreement with Andre's (Andre *et al*., 2000) who reported a higher-level expression of VEGF-C in colorectal cancer. Our finding is also in agreement with Petri's (Petri *et al*., 1998) who indicated that VEGF-B and VEGF-C are expressed in most human tumours. The different findings might be due to the different technical approaches used. These results show that some of these molecules (VEGFs and VEGF-Rs) are higher in normal than cancer tissues and suggest that these molecules could have important role in physiological angiogenesis rather than tumorigenesis, and other molecules which have increased expression in cancer might have a trophic role on tumour cells.

In addition, VEGF-B and VEGF-R2 levels were found significantly higher in colorectal cancer with regional lymph node involvement (Dukes C). Levels of VEGF-C and VEGF-R1 were similar Dukes B and Dukes C, and demonstrated the possible prognostic value of these molecules in colorectal cancers.

This is the first study using Q-RT-PCR for measuring the transcript levels of other vascular endothelial growth factors (VEGF-B, C and D) and their receptors together, in colon cancer, and normal background tissues respectively. In conclusion, we identified several components of the angiogenic cascade during the progression of colon cancer. Our data indicate that the persistent expression or elevation of these angiogenic factors and their receptors may be critical for the development of all colorectal cancers. We conclude that VEGF-B and VEGF-R2 levels are associated with either nodal involvement, and/or

disease progression, and may have a prognostic significance in colorectal cancer development. These angiogeneic factors other than VEGF-A may have potential prognostic value in colorectal cancer and provide a therapeutic approach. The Avastatin/ Bevacizumab (recombinant humanized monoclonal antibody to VEGF-A) had been approved as front-line therapy for metastatic colorectal cancer, further study on VEGFs and their receptors may provide new targets for anti-angiogenic treatment.

CHAPTER FIVE

PROGNOSTIC VALUE OF TUMOUR ENDOTHELIAL MARKERS (TEMs) IN PATIENTSWITH COLORECTAL CANCER.

5.1 INTRODUCTION

Patients with colorectal carcinoma are dependent on the presence of vascular and lymph node metastasis for their tumour prognosis (Dukes CE and HJR. 1958, Chapuis *et al*., 1985, Fielding *et al*., 1986). Fortunately, colon cancer is highly treatable if it is diagnosed in the early stages. Unfortunately, it is often not symptomatic until it has progressed into a more advanced stage. Actually the greatest risk factor for colon cancer is age. Rates of colorectal cancer rise from 10 per 100,000 at age 40-45 years to 300 per 100,000 at age 75-80 years. Cancer that does begin in the colon or rectum is assigned a "stage" by physicians. The earlier the stage of the cancer, the greater the chance of survival.

Angiogenesis, defined as the sprouting of new capillaries from pre-existing vessels characterized by expansion of the endothelium by proliferation, migration and remodelling, is a normal physiologic process that plays an important role in embryogenesis, pregnancy, and tissue repair(Kerbel *et al*., 2002).

Angiogenesis is a dynamic multistep process, which involves retraction of pericytes from the abluminal surface of the capillary, release of proteases from the activated endothelial cells, degradation of the ECM surrounding the pre-existing vessels, endothelial cell migration toward an angiogenic stimulus and their proliferation, formation of tube-like structures, fusion of the formed vessels and initiation of blood flow.

Recently, circulating endothelial cells have also been shown to contribute to new vessel formation by a process referred to as post-natal vasculogenesis (Lyden *et al*., 2001). Targeting endothelial tumour lining blood vessels as an anticancer strategy has become a hot research topic. Firstly, endothelial cells are directly exposed to circulating blood, facilitating drug delivery and enabling the use of high molecular weight therapeutics. Secondly, a significant bystander effect can be expected because each capillary is thought

to support hundreds of tumour cells. Thirdly, targeting the genetically stable endothelial cells may reduce the likelihood of developing resistant disease(Kerbel 1991). Finally, this type of therapy should be applicable to a wide variety of tumour types.

Tumour endothelial markers (TEM-1-9) were recently identified as novel endothelial cell surface markers that appear to be specific to tumour endothelial cells and are potentially involved in tumour angiogenesis(Croix *et al* ., 2000). TEMs were to be found structurally and functionally conserved in mouse and human tumour endothelial cells,TEMs are considered to be one of several genes elevated in human tumour endothelium and expressed at levels at least 20-fold higher in endothelial cells in vivo compared with nonendothelial cells (Carson-Walter *et al* ., 2001).TEMs located on the cell-surface are of particular interest. These TEMs are likely to be the most accessible to pharmacological agents and may be involved in signalling pathways that regulate angiogenesis(Nanda *et al* ., 2004).

An index involving the key factors of angiogenesis may be clinically important for evaluating the prognosis as well as the determining the patients to benefit from anti-angiogenic therapies.In this study we analysed the expression of TEMs (1, 2, 6, 7,7R and 8) in a cohort of colorectal cancer tissues and correlated these molecules with progression of colorectal cancer.

5.2 MATERIALS AND METHODS

5.2.1 Materials

5.2.1.1 Colorectal tissues (cancer &normal) collection.

Colorectal tissues were collected from patients with colorectal cancer immediately after surgery and stored at -20 °C until required. The samples consisted of colon tumour tissues (n=48) and normal background tissues (n=48) from the same patients. Histological

information was obtained from respective histology reports as mentioned in previo chapter.

5.2.2 Methodology

5.2.2.1 RNA Extraction

RNA extraction, reverse transcription Kits and PCR mix were purchased fro ABgene (Surrey, UK). Total RNA was isolated using the standard guanidi isothiocyanate method by following the manufacturer's protocol. The purity a concentration of RNA were determined by spectrophotometer at 260 nm. Rever transcription was performed and cDNA samples were synthesized in 20 µl reacti mixtures.

5.2.2.2 Conventional RT-PCR

Conventional PCR primers were designed using the Beacon Designer softwa (California, US) and synthesized by Life Technologies (Paisley,UK). The agarose g extraction kit was purchased from Life Technologies. Primer sequences are given in Tab 5.1. Conventional PCR to amplify the transcripts of TEMs (TEM1-8) was carried out usi colorectal cancer and normal colorectal tissues. The reaction conditions were: 94 °C for minutes, 36 cycles at 94 °C for 40 sec,54 °C for 30 sec,72 °C for 50 sec followed l extension phase of 10 minutes at 72 °C. β-actin was used as an internal housekeepi gene. The PCR products were separated on a 2 % and 0.8% agarose gel and stained wi 10-µl ethidium bromide prior to examination and photographing under UV light.

5.2.2.3 Real-time quantitative polymearase chain reaction (QPCR).

We employed the iCycler IQ system (BioRad, Camberley, UK), to quantify the level (shows as copies/μl from internal standard) of tumour endothelial markers in the colorectal specimens as we have recently reported (Jiang *et al* ., 2003a, Jiang *et al* ., 2003b). All colorectal cDNA samples were simultaneously examined for each of the TEMs (TEM-1 -8), along with the appropriate set of plasmid standards and negative controls. Primer sets and probes used in this technique are given in table 5.2.

The detection of tumour endothelial markers (TEMs) employed a universal probe system (UniPrimer™) (Intergen, Oxford, England). The UniPrimer system used two primers in conjunction with a universal probe (UniPrimer™), which recognised a specific sequence (z sequence), which had been incorporated into the primers (Table 5.2). A hot-start quantitation master mix (Abgene, Surrey, England) was used for the reactions. PCR Conditions for real-time QPCR were as follows: 95°C for 12 minutes, followed by 50 cycles at 95°C for 15s, 55°C for 60s and 72°C for 20s.

Statistical Analysis

Conventional RT- PCR results were analysed by using χ^2 test. Quantitative data were analysed using student *t* test.

Table 5.1. Primer sequences for conventional PCR

Primers	Sense primer (5' – 3')	Antisenes primer (5' – 3')
TEM-1	gtggcttcgagtgttattg	gaagagctccggatatttg
TEM-2	agccatgatgaagactttgt	cttgaggtcactgttgacg
TEM-6	acccgtgacgtcattttc	tgtacttgcttcgagcatc
TEM-7	ggagcaggtcacgatgag	gtgaaactgcccttgtctt
TEM-7R	cttgattggcagtatggagt	gagatgtacatggtcccact
TEM-8	catttcaagttgtcgtgaga	gacgcatattgttgttgaga

Table 5.2. Primer sequences for quantitative PCR.

UniPrimer systems	Sense primer (5' – 3')	Z primer (5' – 3')
TEM-1	cttgcccactgggatgat	actgaacgtgaccgtacaacctatgaatcctc tgatgg
TEM-2	agtctcaccttgagtgtggt	actgaacctgaccgtacactcctccacagca tctctta
TEM-6	acccgtgaggtcattttc	actgaacctgaccgtacattcaaccttcccat agtcag
TEM-7	agaacgaccacatcaccttt	actgaacctgaccgtacatggagagagttgg agtcaa
TEM-7R	cttgattggcagtatggagt	actgaacctgaccgtacagtctaccgccttg agaaag
TEM-8	acagggtcctctgcagctt	actgaacctgaccgtacactttcatgccaact tgttt

5.3 RESULTS

5.3.1 Expression of tumour specific endothelial markers TEMs (TEM1-8) in colorectal cancer tissues.

TEM-1, TEM-7, TEM-7R and TEM-8 were found to be over –expressed in colon cancer tissues compared with normal tissues ($p=0.01$, $p=0.04$, $p=0.03$ and $p=0.001$, respectively). Conversely, no differences were found between TEM-2 and TEM-6 expression in tumour and normal tissues ($p=0.61$ and $p=0.56$) (Figure 5.1 & Table 5.3).

5.3.2 . The level of expression of TEMs transcripts in different Dukes stages.

We went on to analyse, quantitatively, levels of transcript of tumour tissues in relation to Dukes staging. The number of copies of TEM-1 transcript was highest in Dukes B, while the transcript copies of TEM-2 was significantly higher in Dukes A compared to Dukes C ($p < 0.05$). The level of expression of TEM-7 and TEM7-R was found higher in Dukes C compared to that in Dukes A tumours. However, there was no statistical significance in the trend. TEM-8 expression was significantly higher in Dukes C compared to that in Dukes A tumours ($p=0.016$) (Figure 5.2).

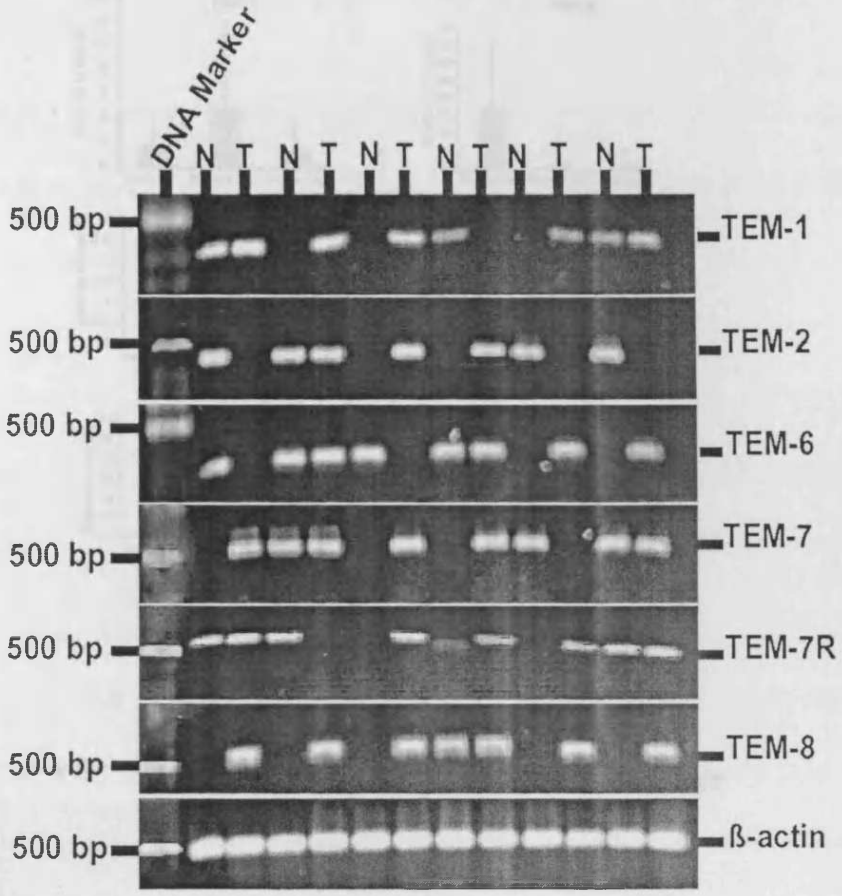


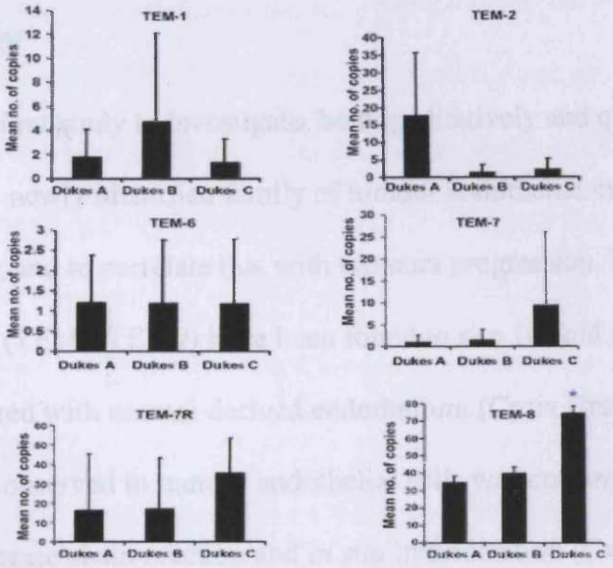
Figure 5.1. RT-PCR shows the high expression of TEM-1, TEM-7 and TEM-7R in colorectal cancer (* p=0.01, p=0.04 and p=0.03). TEM-8 was over-expressed in colorectal cancer compared to normal colon (*p=0.001). No significant difference was observed between expression of TEM-2 and TEM-6 in normal and cancer colorectal tissues.

	Normal tissues (n and %)	**Tumour tissues (n and %)**	**p value ***
TEM-1	18 of 48, 38 %	46 of 48,95.5 %	0.01*
TEM-2	22 of 48, 45 %	28 of 48,58.3 %	0.61
TEM-6	17 of 48, 35%	27 of 48,56 %	0.56
TEM-7	7 of 48, 15 %	37 of 48,77.5 %	0.04*
TEM-7R	6 of 48,12.5 %	38 of 48,79.5 %	0.03*
TEM-8	1of 48, 2 %	46 of 48,95 %	0.001*

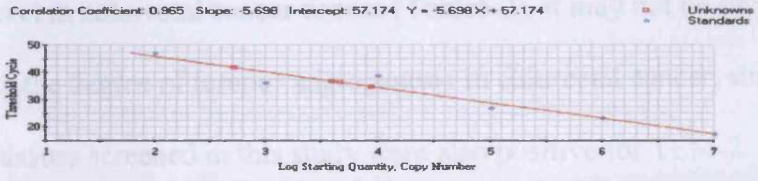
*** chi square test (χ2 test).**

Table5.3. Expression of TEMs in colon tissues (percentage positive), using conventional PCR.

A



B



C

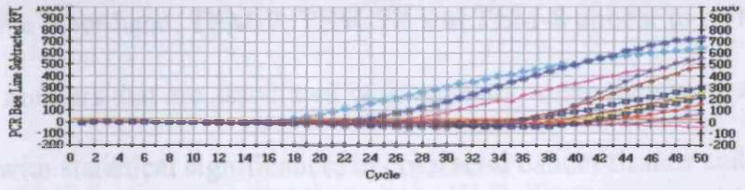


Figure 5.2. Real Time quantitative RT-PCR. (Mean copies/ng mRNA) TEM-1 E+06 copies,TEM-2 E+04 copies, TEM-6 E+03 copies,TEM-7 E+05,TEM-7R E+06 and TEM-8 E+06 .Levels of expression of TEMs in colon tissues in tumours with different Dukes stages. The number of transcripts of TEM-1 was higher in Dukes A and TEM-2 mRNA copies higher in Dukes B tumour .TEM-6 shows no differenc in level of expression in all stages (Dukes A,B and C). Dukes C tumour expressed greatest level of TEM-8 (* p=.001vs Dukes A).Both TEM-7 and TEM-7R shows higher level of expression in Dukes C but no significant ($p>0.05$) (A). The correlation coefficient (B) shows a slope of 0.965 comparing TEMs levels with those of a standard. (C) Shows the amplification of copy numbers in relation to the cycle number.

5.4 DISCUSSION

This was the first study to investigate, both qualitatively and quantitatively, transcript levels of a newly identified family of tumour endothelial markers (TEMs) in colon cancer tissues; and to correlate that with tumours progression. Previously tumour endothelial markers (TEM1-TEM9) have been found to rise 10-fold in tumour-derived endothelium compared with normal-derived endothelium (Croix Brad *et al*., 2000). The elevated expression observed in tumour endothelial cells was confirmed by reverse transcriptase polymerase chain reaction and *in situ* hybridization (Carson-Walter *et al*., 2001). This study has shown that TEMs are elevated in colorectal cancer tissues compare with normal background tissues. Although in the current study TEM-2 is expressed at a very high level in colorectal cancer tissues (Table5.2), it may not be such a good indicato for assessing the degree of tumour angiogenesis in colorectal cancer, since almost over h the normal tissues screened in this study were also positive for TEM-2.

On the other hand, TEM-7, TEM-7R and TEM-8 appear to be more superior endothelial markers that are associated with tumours, since they were also highly expressed, with statistical significance, in colorectal cancer tissues and detected in less th half of the samples of normal background tissues. Our study has clearly demonstrated tha TEM-1, -7,-7R and TEM-8 were over-expressed in colorectal cancer than in normal tissues. Although TEMs are initially thought to be specific only to endothelium in tumou tissues(Croix Brad et al , 2000) , our result has shown that certain TEMs do exist in norm colon mucosa, notably TEM-1 ,TEM-2 and TEM-6. This raises some questions as to the specificity of these markers when used in assessing tumour induced angiogenesis. However, the increased expression of the TEMs in cancer tissues, as reported here, is supported by a recent study(Carson-Walter et al ., 2001), which reached a similar conclusion using different approaches, i.e. in situ hybridization.

Our results raise two questions; first, the potential surrogate nature of TEM-7, TEM-7R and TEM-8 endothelial markers that are unique to tumour associated endothelial cells. Second, the potential histological function of these markers. These results may be due to the unique structures of TEM-7R and TEM-8, which may make them more important in tumour angiogenesis. For example, the extracellular portion of TEM-8 has been shown to contain a vWF like A domain containing a metal ion dependent adhesion site (MIDAS) (Colombatti. *et al .,* 1991, Lee *et al .,* 1995). Interestingly, the vWF like A domain of TEM-8 has also been termed as an I-domain when present within integrins and it also bears a close resemblance to a D integrin(Van der Vieren *et al .,* 1999, Dickeson *e al .,* 1998). vWF has been shown to be an important endothelial marker in angiogenesis, whereas, integrins are cell adhesion molecules which facilitate cell-matrix adhesion. Down regulation in the assembly of integrins mediates adhesion complexes that have bee shown to result in a gain in the invasive potential of a number of cancer cell types(Hood *e al .,* 2002).

In the current study, TEM-8 was the only endothelial marker to be significantly elevated in colorectal cancer tissues with nodal involvement (Dukes C). Interestingly, TEM-2 and TEM-6, which have been found to be highly expressed in normal colorectal tissues together with colorectal cancer, are raised in patients who had early stage of cance (Dukes A). Identifying suitable markers with clear prognostic indices in the first instance may offer earlier detection and better management of colorectal cancer.

TEM-8 appears to be unique among the cell surface TEMs in that its expression has not been detected during other forms of physiological angiogenesis in the adult, although expression has been observed in endothelial cells of the developing mouse embryo, (Croix *et al .,* 2000, Carson-Walter *et al .,* 2001). In addition to our findings, TEM-8 is over -expressed in colorectal cancer and its significantly raised in patients with advanced colorectal cancer. These results together with the recent success of

Avastin, a humanized monoclonal antibody against vascular endothelial growth factor (VEGF), in prolonging the lives of patients with metastatic colon cancer, establishes the validity of the anti-angiogenic approach (McCarthy 2003), making TEM-8 a particularly attractive candidate for antiangiogeneic targeting for colorectal cancer.

This is the first study to show a quantitative method using Q-RT-PCR for measuring the transcript levels of tumour endothelial markers in colorectal cancer, and normal background tissues respectively. In the current study, the levels of tumour endothelial markers TEM-1, TEM-7, TEM-7R and TEM-8 were found to be elevated in colorectal cancer tissues. TEM-8 was found to be significantly raised in cancer patients with most aggressive tumours i.e., muscular wall penetrating cancer and nodal involvemen (Dukes C) but no other tumour endothelial markers show that.

We conclude that levels of TEM-1, TEM-2 and TEM-7R are higher in tumour that invade through the muscularis propria into subserosa, or into pericolic or periectal tissues (Dukes A and Dukes B), and that TEM-8 is associated with either nodal involvement, and/or disease progression (Dukes C), and may have a prognostic significance in colorecta cancer development.

CHAPTER SIX

TUMOUR ENDOTHELIAL MARKER 8 (TEM-8) IN HUMAN COLON CANCER AND ITS ASSOCIATION WITH TUMOUR PROGRESSION.

6.1 INTRODUCTION

Tumour stage remains the most important clinical/ pathological prognostic factor in patients with colorectal carcinoma (CRC). Although there have been a lot of reports showing the significance of many other prognostic parameters including histological grade, age, serum CEA levels, gender, location of the primary tumour, histological subtypes and flow cytometric DNA analysis, none of those has been widely used in clinical practice (Skibber *et al*., 2001, McLeod *et al*., 1999, Akbulut *et al*., 1998). Recently, angiogenesis has gained an increasing interest as a prognostic factor in a variety of solid tumours. The immunohistochemical analysis of newly formed vessels is the most widely used index of angiogenesis in solid tumours (Barbareschi *et al*., 1995, Szabo *et al*., 1998).

Traditionally, angiogenesis in tumours has been assessed by several endothelial markers, including factor VIII (von Willebrand factor) (Teo *et al*., 2002), PECAM-1 or CD31 (Solovey *et al*., 2001), pan endothelial marker or CD34, VE-cadherin(Croix *et al*., 2000), P1H12 (Solovey *et al*., 2001), VEGFR2 (Carson-Walter *et al*., 2001) and Tie receptors (Croix *et al*., 2000). These markers have been shown to be expressed in endothelial cells from both normal and tumour tissues.

Recently it has been reported that certain markers exist that can distinguish between normal endothelial cells and tumour derived endothelial cells (Croix *et al*., 2000). Comparison of non-tumour and tumour-derived endothelial cells, using microarray technology, identified a new panel of markers named tumour endothelial markers, (TEMs) (Croix *et al*., 2000). TEMs are uniquely expressed in tumour associated endothelial cells. Reports have indicated that TEMs are expressed at higher levels in tumour endothelial cells compared to endothelial cells from normal tissues (Carson-Walter *et al*., 2001). Recently it has been reported that the expression pattern of TEMs varies in human breast cancer (Davies *et al*., 2003).

We demonstrated that, although most of the TEMs can be detected in normal background tissues, they are at a higher level in tumour tissues. Amongst the nine TEMs examined in our studies, we have found that TEM-8 was consistently expressed at very low levels or below detection in normal tissues; however tumour tissues revealed a significant increase in TEM-8 expression. Data presented in chapter five has shown that this is the case for TEM-8 in colon cancer.

The current chapter examines the level of tumour endothelial marker 8 (TEM-8) in colon cancer tissues and normal background tissues at mRNA level, and at the protein level using a specific antibody to human TEM-8 developed in our laboratory.

6.2 MATERIALS AND METHODS

6.2.1 Materials and tissues collection.

As mentioned in previous chapters, colorectal tissues were collected from patients with colorectal cancer immediately after surgery and stored in the deep freezer until use. The samples consisted of colon tumour tissue (n=48) and normal background tissue (n=48) from the same patients.

The human endothelial cell line (HECV) was obtained from Interlab Cell Line Collection (ICLI), Naples, Italy. Human fibroblast cell line (MRC5), colorectal cancer cell line HRT18, breast cancer cell lines MCF-7, MDA-MB 231, ZR751, the prostate cancer cell line DU-145, human epithelial cell line ECV304, human bladder cancer cell line EJ138, and pancreatic cancer cell line PANC-1 were obtained from European Collection of Animals Cultured Cells (ECACC) (Salisbury, England). Cells cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) (pH 7.3) containing; 2mMl L-glutamine, 15mMhepes and 4.5mMl $NaHCO_3$, DMEM were supplemented with 10% heat inactivated foetal calf serum (PAA Laboratories, Yeovil, England, UK), 50units/ml ofBenzopenicillin (Britannia Pharmaceuticals Ltd) and 50µg/ml of streptomycin (Gibco BRC, Paisley,

Scotland). RNA extraction, reverse transcription kits and PCR master mix were purchased from Abgene (Surrey, England, UK).

6.2.2 Methodology

6.2.2.1 RNA Extraction.

Total RNA was isolated from frozen-sectioned colorectal tissues (normal and cancer) as explained in previous chapters. Conventional PCR primers were designed using Beacon Designer software (Palo Alto, California, USA), to allow amplification of regions that have no overlap with known genes and span at least one intron. Primers were synthesized by Life Technologies (Paisley, Scotland, UK) (Table 6.1).

6.2.2.2 Conventional RT-PCR.

Conventional PCR to amplify the transcripts of TEM-8 was carried out using cDNA from normal colorectal and colorectal cancer tissues. The reaction conditions were: 94 °C for 40 seconds, 54°C for 30 seconds, 72°C for 50 seconds and a final extension phase of 10 minutes for 38 cycles. B-actin (Table 6.1) was used as an internal housekeeping gene. The PCR products were separated on a 0.8 % agarose gel and stained with ethidium bromide prior to examination under UV light.

	Sense primer (5' 3')	Antisense primer (5'– 3')
TEM-8 for RT-PCR	catttcaagttgtcgtgaga	gacgcatattgttgttgaga
ß-actin	atgatatcgccgcgctcg	cgctcgtgtaggatcttca
UniPrimer systems	**Sense primer (5' – 3')**	**Z primer (5' – 3')**
TEM-8 for Q-PCR	acagggtcctctgcagctt	actgaacctgaccgtacactttcatgccaacttgttt

Table 6.1: Primers for conventional (top) and quantitative (bottom) RT-PCR

6.2.2.3 Real-time quantitative polymerase chain reaction (QPCR).

We employed the iCycler IQ system (BioRad, Camberley, UK), to quantify the transcript level (shows as copies/μl calculated from internal standard) of the tumour endothelial marker 8 in the colorectal specimens as we previously reported (Jiang *et al .*, 2003b). All colorectal cDNA samples were simultaneously examined for TEM-8 along with an appropriate set of plasmid standards and negative controls.

TEM-8 was analysed using the Amplifluor system as we previous reported (Jiang *et al .*, 2003), which was used in conjunction with a universal probe (UniPrimer™). This probe recognised a specific sequence (z sequence), which had been incorporated into the primers (Table 6.1). Conditions for real-time Q RT-PCR were as follows: 95°C for 15s, 54°C for 20s and 60°C for 60s.

6.2.2.4 Generation of anti-TEM8 antibody

Briefly, a 15 amino acid peptide matching the N-terminus of human TEM-8 was synthesised (Sigma-Genosys, Cambridgeshire, UK), purified, conjugated to KLH (Keyhole Limpet Hemocyanin), and injected into rabbits with complete Freund's solution following a standard procedure. Antisera were obtained after Series injection and booster injections. Immunoglobulin was purified using a protein-A Sepharose affinity column (Sigma, Poole, Dorset, England). Briefly, the rabbit serum underwent delipidation by mixing 1,1,2-trichloro-trifluoroethane with the rabbit serum in a 3:2 mixture and underwent agitation for 30min. Then the mix was spun down at 5000 rpm for 10 min and the top layer kept for purification. Delipidated antisera was diluted with PBS buffer and loaded onto the Sepharose column (equilibriated with 10 column volumes of 10 mM PBS; pH to 7.4). The column was then washed with 7 column volumes of PBS. Elution of the bound antibodies was achieved with 5 column volumes of elution buffer (11.1g Glycine-HCl brought to volume of 1 liter with dH2O then pH to 2.7). One-ml fractions were collected into tubes

containing neutralization buffer (12.1 g Tris, 4.2 ml HCl, brought to 100 ml and pH to 8.0), and mixed immediately. Purified HAI-1 and HAI-2 antibodies were then stored in 50% glycerol at 20°C.

The specificity of the antibodies was verified using the TEM-8 peptide and other synthetic peptides irrelevant to TEM-8, as well as cell lysates. Human endothelial cells (HECV), a human fibroblast cell line (MRC5), colorectal cancer cell line HRT18, breast cancer cell lines MCF-7, MDA-MB 231, ZR751, the prostate cancer cell line DU-145, human epithelial cell line ECV304, human bladder cancer cell line EJ138, and pancreatic cancer cell line PANC-1 were examined using slot blot and Western blotting analysis to confirm the presence of TEM-8 protien. The TEM-8 peptide and control peptides were blotted onto a nitrocellulose membrane, at four different concentrations, to confirm the specificity of the antibody. This TEM-8 antibody was used to examine the expression of TEM-8 at protein level in colorectal cancer, using Western Blotting as described below or immunohistchemistry.

6.2.2.5 Western Blotting.

From sections of colorectal tissues (normal and tumour) were homogenised and lysed in HCMF buffer containing 0.8% NaCl, 0.4mg/ml KCl 0.09mg/ml, $Na_2 HPO_4, 7H_2O$, 1mg/ml glucose, 2.4mg/ml HEPES, 0.5%SDS, 0.5% Triton X-100, 2 mM $CaCl_2$, 100µg/ml phenylmethylsulfonyl fluoride, 1mg/ml leupeptin, 1mg/ml aprotinin, and 10mM sodium orthovanadate for 40 minutes. The lysates were then quantified and diluted to the same concentration with HCMF buffers, prior to boiling in sample buffer at 100°C for 5 minutes (Jiang *et al*., 1995). Equal concentrations of protein from each sample were loaded onto a 10% polyacrylamide gel. Following electrophoresis, proteins were blotted onto nitrocellulose sheets and blocked in 10% skimmed milk in TBS and 0.01 % Tween 20 for 30 min, before probing with primary antibody serially diluted between 1:50, and 1:240

(1:60), anti-TEM-8 for one hour. This was followed by 3 washes with 3% milk in TBS and 0.01% Tween 20, (10 minutes each wash). Peroxidase-conjugated secondary species-specific antibodies (1:1000) were added for another hour followed by 2 washes with 3% milk, 2 washes with Tween 20 in TBS and a final wash with TBS only. Protein products were visualized using a chemiluminescence system (KPL, Insight Biotechnology, and Surrey, England).

6.2.2.6 Immunohistochemistry

This is as we recently reported (Jiang *et al*., 2003b). Briefly, frozen tissues (normal and Cancer) were sectioned using a cryostat, air dried and fixed in 50% methanol and 50% acetone for 15 minutes. The sections were then air dried once more for 10 minutes and stored at -20^0C in foil-wrapped slide trays. Immediately before staining, specimens were placed in a optimax wash buffer for 5 minutes. The slides were incubated with primary rabbit polyclonal antibodies against TEM-8 (over a range of dilutions) or anti-vonWillebrand Factor at 1:50 dilution for 1 hour. After 4 washes with PBS, the slides were placed in universal multi-link biotinylated solution, prepared from a kit (Dako) the slides were washed 4 times after 50 mintues. The secondary antibody (biotinylated) was added (in horse serum/buffer solution) at (1:100) dilution and incubated for 30 minutes. This was followed by 4 washes with PBS. Slides were then placed in avidin biotin complex (ABC – Vector Labs) for 30 minutes. The bound antibody complex was detected using diaminobenzidine tetra hydrochloride (3, 3'-diaminobenzidine) - DAB (Sigma), chromogen for 5 minutes. The slides were washed with H_2O for 5 minutes and placed in Mayer's haematoxylin for 1 minute, followed by differentiation in H_2O for 10 minutes. This was followed by dehydration in methanol (3 times) and clearing in 2 changes of xylene before mounting under cover slip and examined on the microscope. Negative controls (using PBS buffer instead of the primary antibody).

The number of micro-vessels stained by immunohistochemistry using anti-TEM-8 and anti-factor VIII in each slide (each sample) were a counted, and statistically analysed using a Student's t-test by independent researches as we reported (Martin *et al*., 2003).

Statistical Analysis:

Statistical analysis was performed using a Student's t-test.

6.3 RESULTS

6.3.1 Expression of TEM-8 protein in connection with the expression of TEM-8 mRNA, in colorectal cancer tissues.

With the anti-TEM-8 antibody rasied, western blotting was carried out to analyze TEM-8 protein in colon tissues (Normal & Tumour) (Figure 6.1 A).This is compared with the matched PCR results (Figure 6.1 B). TEM-8 mRNA was detected in majority of tumour tissue but only in a small number of normal colon tissues (P=0.001). This suggests an over- expression of TEM-8 in colon cancer tissues. This TEM-8 over- expression was confirmed at the protein level as revealed by western blotting showing elevated levels of TEM-8 in cancer tissues compared to normal colon tissues.

6.3.2 Analysis of TEM-8 transcript by real time quantitative PCR (Q-PCR).

As showed in chapter five (General study of all TEMs), Q-PCR revealed an elevated numbers of copies of the TEM-8 transcript in colon cancer tissues from patients with poor prognosis (Dukes C), compared with patients showing early stage disease (Dukes A) (p=0.01 vs. Dukes A), and there was no significant change between Dukes B and A (p=0.2 vs. Dukes A) (Figure 6.2).

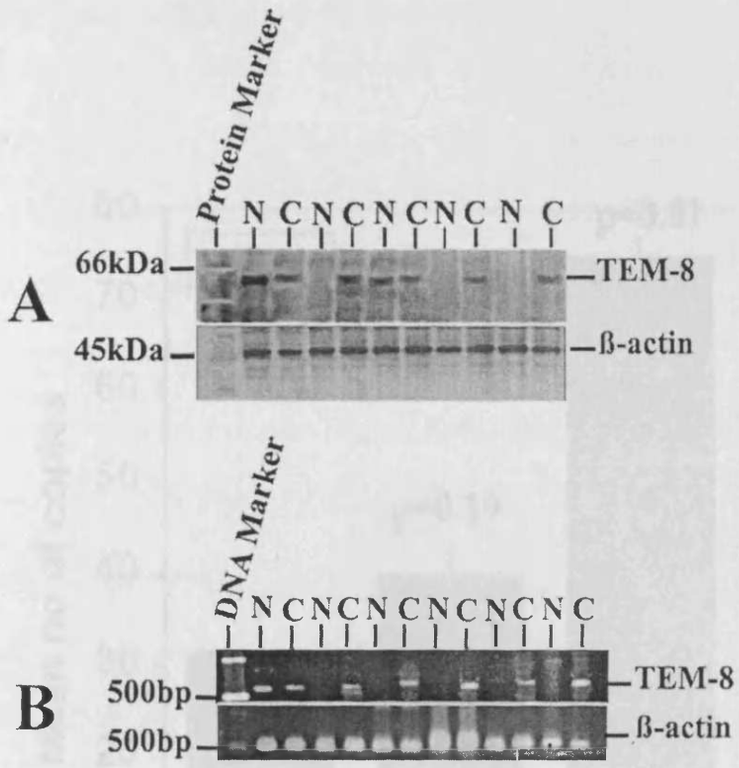


Figure 6.1. (A) Western Blotting showing the high expression of TEM-8 in colon cancer tissues compared to normal sample at protein level. The β-actin transcript was present in all 6 matched colon cancer samples. (B) The detection of representative tumour endothelial marker 8 (TEM-8) in normal colon tissues and colon cancer tissues respectively in 6 matched samples using RT-PCR. Colon cancer tissues expressed a higher level of tumour endothelial markers at the mRNA level compared to normal background tissues.

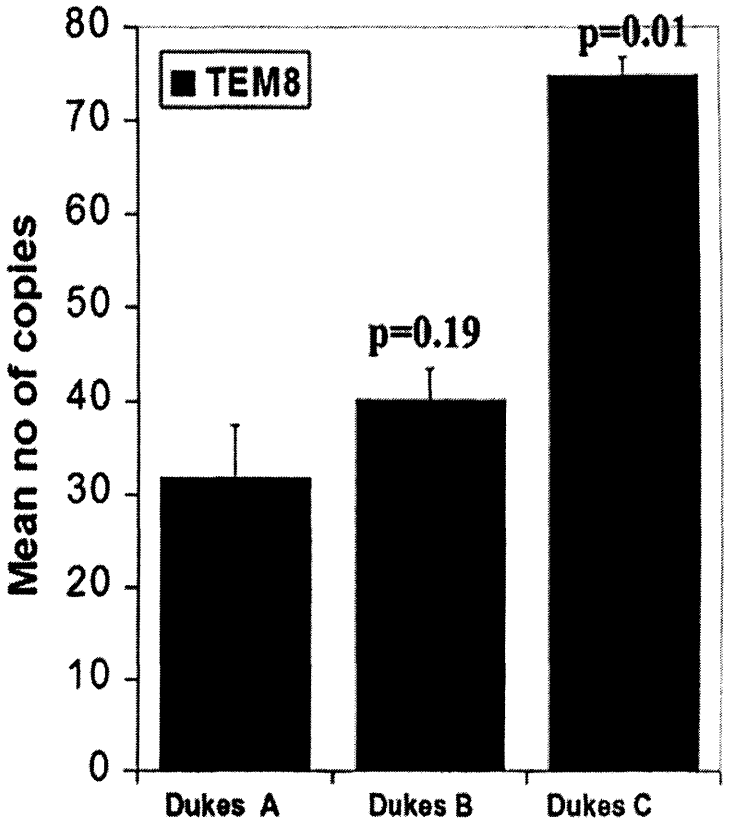


Figure 6.2. The levels of tumour endothelial marker 8 (TEM-8) detected in colon cancer tissues using Q-RT-PCR (transcript copies/ng RNA) and their relation to Dukes staging. The level of TEM-8 transcripts (copies E+06) was higher in advanced stage refers to those patients who had lymph node involvement i.e. Dukes C. Statistical analysis was performed using a Student's t-test (Dukes c $P < 0.01$ vs. Dukes A).

6.3.3 Generation and purification of TEM-8 antibody

For the current study, we first raised polyclonal antibody to human TEM-8. Purified IgG from antisera specifically recognized the respective peptides used to generate antibody. We show that, this antibody is specific against TEM-8, as the TEM-8 antibody identified the TEM-8 peptide,ie,the polyclonal antibody obtained was highly specific to TEM-8, and had no cross reactivity with other peptides, as shown in a slot blot (Figure 6.3 A). In addition, this antibody identified TEM-8 in a variety of normal and cancer cell lines. A specific band at Mr 63,000 was identified, which corresponded to the TEM-8 mature peptide.Western blotting revealed that the antibody recognised a specific band at approximately 63kDa, the predicted size of TEM-8. Endothelial cells (HECV) were shown to express a high level of TEM-8. Interestingly, a number of other cell lines, including MRC5, HRT18, MCF7 and PANC-1, also produced the TEM-8 protein to varying degrees (Figure 6.3B).

6.3.4 Immunohistochemistry.

An immunohistochemical study for TEM-8 and vonWillebrand Factor (factor-VIII) was performed in colon tissues. TEM-8 staining was largely confined to micro-vessels of tumour colon tissues and showed some degree of staining of surrounding epithelial cells. In normal colon tissues, vonWillebrand Factor (factor-VIII) identified micro-vessels in sub-mucosa area. In comparison, TEM-8 stained significantly less number of micro-vessels in normal tissues (4±0.8 for TEM-8 positive vessels, vs. 7±1.6 for factor-VIII positive vessels, p=0.008). Interestingly, TEM-8 antibody identified significantly higher number of TEM-8 positive vessels in colon cancer tissues (13±3.1), compared with that of factor-8 antibody in cancer tissues (6.7±2.5, p=0.02), and compared with that of TEM-8 positive vessels in normal tissues (4±0.8, p=0.002) (figure 6.4 and Table 6.1). This strongly suggests that TEM-8 antibody has identified micro-vessels occurring in tumour tissues which may be associated with tumour-specific angiogenesis. Weak TEM-8 staining was seen in epithelial cells, but strongly in colon cancer cells (figure 6.4).

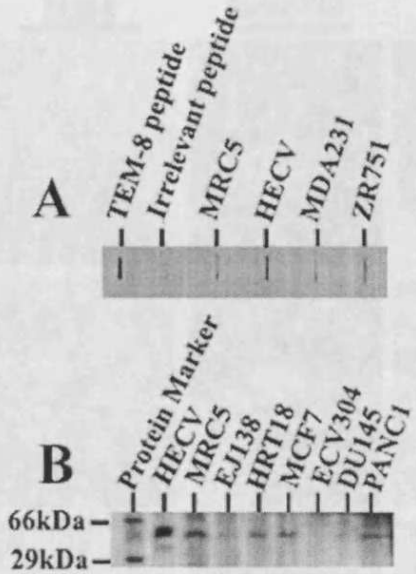


Figure 6.3. A Slot blotting shows the specificity of the anti-TEM-8 antibody and the strong signal of TEM-8 in TEM-8 peptide and protein of HECV cell and no signal in other synthetic irrelevant peptide (negative control). **B:** Western Blotting showing the TEM-8 antibody expressed in human endothelial cell and in different cancer cell lines (HECV, MRC5, HRT18, MCF7 and PANC1)

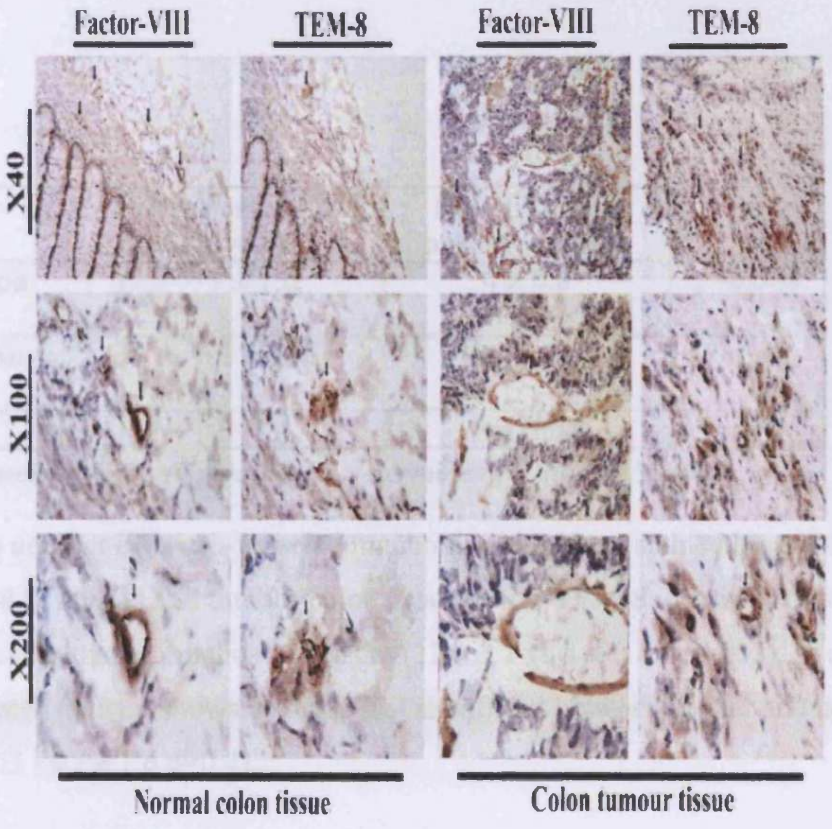


Figure 6.4. Immunohistochemical staining of micro-vessels (Indicated by arrows), using anti- factor -VIII (left two panels) and anti- TEM-8 (right two panels) in normal (panel 1 and 2) and colon cancer (panel 3 and 4) tissues. More micro-vessels identified in colon cancer tissue by anti-TEM-8 compared to the normal colon tissues (Panel 4 in right). Anti Factor VIII stain more micro-vessels in normal colon tissues than the cancer one (Panel 1 in left). Shown at 40X, 100X and 200X magnifications.

	No. Factor-VIII positive vessels.	No. of TEM-8 positive vessels.	P value
Normal colon Tissues	7 ± 1.6	4 ± 0.8	P=0.008
Cancer colon Tissues	6.7 ± 2.5	13 ± 3.1	P=0.02
P value	P=0.4	P=0.002	

Values are mean ± SD and p values as determined by Student's t-*test*

Table 6.2: The number of micro-vessels immunohistochemistry stained by anti-Factor-VIII and anti-TEM-8 in normal and tumour colon tissues. Anti-TEM-8 identified more vessels in cancer colon tissues than normal colon tissues (13 ± 3.1 vs 4 ± 0.8 P=0.002), in contrast anti-Factor-VIII related antigen shows no statistical significant between cancer and normal colon tissues (6.7 ± 2.5 vs 7 ± 1.6 p=0.4).

6.4 DISCUSSION

It has been well established that the incidence of tumour angiogenesis is associated with poor prognosis (Chodac *et al*., 1980, Bricknell *et al*., 1991). A number of clinical studies have shown angiogenesis to have a prognostic significance in colon cancer (Nakasaki *et al*., 2002). Several angiogenic factors have been identified, which play a crucial role in the proliferation and migration of endothelial cells, that leads to increased angiogenesis (Ferrara N and Davis-Smyth T .,1997, Martiny-Baron *et al*., 1995). However, most of the angiogeneic markers are non-specific and can not distinguish between normal and tumour associated endothelium.

TEM-8 is highly conserved and shares 96% amino acid identity between mouse and human(Carson-Walter *et al*., 2001). TEM-8 is a type I transmembrane protein composed 564 amino acids in length, of which 220 amino acids make up the cytoplasmic tail. The large cytoplasmic tail of both the human and mouse TEM-8 proteins share at least seven potential phosphorylation sites, supporting the hypothesis that TEM-8 is involved in transmitting signals into the cell, from the extracellular environment. In the extracellular region, TEM-8 was found to have the von Willebrand factor type A domain containing a metal ion-dependent adhesion motif (I-domain)(Colombatti. A and P. 1991, JO *et al*., 1995). The Von Willebrand factor type A domain is often found in extracellular domains of integrins where they constitute ligand-binding sites, and has been shown to interact with vascular cell adhesion molecules via TEM-8 I-domain during leukocyte trafficking. Therefore, TEM-8 has been postulated to be involved in the interaction of cells with the surrounding extracellular matrix(Van der Vieren *et al*., 1999, JO *et al*., 1995).

This study investigated the expression of the newly identified family of tumour endothelial marker (TEM-8) in colon cancer tissues. Previously, St. Croix *et al* evaluated the expression of tumour endothelial marker transcripts at mRNA level by using RT-PCR in purified cultures of endothelial cells derived from both normal and tumour colon tissues.

They found the level of tumour endothelial markers to be predominantly expressed in tumour endothelium, however, in normal endothelial cells the level of tumour endothelial markers were either absent, or barely detectable. In a later study conducted by Carson-Walter *et al.* this finding of elevated TEM-8 levels in colon cancer was corroborated using *in situ* hybridisation. Our previous work by using RT-PCR (Davies *et al*., 2003) and indeed as presented in chapter five, showed that out of nine tumour endothelial markers examined, only TEM-8 is highly expressed in colon cancer.

In the current study, by using the newly developed antibody to TEM-8, it was found that the TEM-8 expression was significantly elevated in colorectal cancer tissues. In addition, TEM-8 has identified more positive vessels in tumour tissues than normal tissues. This is particularly interesting, as an endothelial marker that is non-specific to tumour associated endothelial cells, Factor-8 (vonWillebrand Factor), failed to reveal more micro-vessels in tumour tissues. This provides further evidence that TEM-8 does indeed recognize micro-vessels (endothelial cells) that are specific to tumour angiogenesis. It has to be pointed out that normal tissues also have TEM-8 positive micro-vessels (figure 6.4). Two possibilities exist. First, the normal tissues used here were from the same patients (distant to tumour lesion). Factors that are generated by tumour or its stroma may diffuse to the adjacent normal cells.As in next chapters, we will illustrate that, IL-1ß is able to up-regulate the expression of TEM-8 in endothelial cells. IL-1ß is known to increase in colon cancer cells and tissues(Riche *et al*., 1995, Strassmann *et al*., 1993, Jarry *et al*., 1999) and may well contribute to the increased expression of TEM-8 in its surrounding tissues. Second, we have reported that normal tissues from other organs including mammary gland and normal endothelial cells (HUVEC) do also express low levels of TEM-8. TEM-8 is the same as the anthrax toxin (protective) antigen receptor(Bradley *et al*., 2003, Bradley *et al*., 2001), which is also expressed at low levels in normal tissues. Taken together, TEM-8 becomes highly

expressed in tumour associated endothelial cells and does constitute a very good marker in identifying micro-vessels associated with tumour. Interestingly, TEM-8 was also significantly raised in patients who had nodal involvement and invasion colorectal cancer (Dukes C).

Our findings suggest TEM-8 to be a crucial marker to identify tumour associated endothelial cells. Presently it is not clear how TEM-8 regulates angiogenesis. The extracellular portion of TEM-8 has been shown to contain a vWF like A domain and has a metal ion dependent adhesion site (MIDAS)(Colombatti. A and P. 1991, Shihu *et al*., 2003). Interestingly, the vWF like A domain of TEM-8 has also been termed as an I-domain, when present within integrins, and it also bears a close resemblance to an αD integrin(Van der Vieren *et al*., 1999, Dickeson SK and Santoro. 1998). vWF has been shown to be an important endothelial marker in angiogenesis, whereas, integrins are cell adhesion molecules which facilitate cell-matrix adhesion. Down regulation in the assembly of integrins mediates adhesion complexes have been shown to enhance the invasive potential of a number of cancer cell types (Hood *et al*., 2002).

Thus, the vWF domain may play an important role here. In addition to endothelial cells, we have also demonstrated that tumour cells including colon cancer (HRT18) also expressed TEM-8. This is interesting as TEM-8 has not been indicated to have a function in cells other than endothelial cells. This indicates that TEM-8 may either act as a receptor for cancer cells, for functions yet to be identified or act as feeding source for endothelial cells, thus contributing to angiogenic process. Future work would have to address this important questions, particularly given the fact that TEM-8 also acts as anthrax toxin receptor(Bradley *et al*., 2001, Bradley *et al*., 2003). This is the first study to show the expression of TEM-8 at protein level in cancer by using western blotting, immunohistochemistry and Q-RT-PCR to quantitatitate TEM-8 transcript levels in colorectal cancer.

The current study demonstrates that the level of tumour endothelial marker 8 (TEM-8) in colorectal cancer tissues was found to be elevated, and also significantly raised in cancer patients with nodal involvement. TEM-8 levels have been shown to be up regulated in tumour development, but the expression in normal endothelial cells had been virtually undetectable, making it an ideal endothelial marker for assessing the level of tumour specific angiogenesis. We conclude that the level of TEM-8 is associated with nodal involvement, and advanced stage of colon cancer, and may have a prognostic significance in colorectal cancer development.

CHAPTER SEVEN

UPREGULATION OF TUMOUR ENDOTHELIAL MARKER-8 (TEM8) BY INTERLEUKIN-1ß AND ITS IMPACT IN IL-1β INDUCED ANGIOGENESIS

7.1 INTRODUCTION

Malignant cells are surrounded by stroma and extracellular matrix which are composed of various cells like macrophages, lymphocytes, neutrophils, fibroblasts, vascular endothelial cells as well as natural killer cells (Leek *et al*., 1994). All these cells including the malignant cells interact with each other via a complex network of extracellular signals, such as cytokines and growth factors and other protein molecules. These interactions are thought to regulate the proliferation and metastatic activity of malignant cells as well as modulate the host immune system towards the tumour cells (Hasday *et al*., 1990).

Cytokines play an essential role in carcinogenesis and subsequent proliferative behaviour. Systemic and local cytokine environment may modulate the immunogenicity of colorectal cancer cells, and affect anti-tumour immune functions of tumour-infiltrating lymphocytes. Cytokines can modulate expression and presentation of tumour antigens, adhesion molecules, and the composition of the cellular infiltrate and functional activity of tumour-infiltrating lymphocytes. They can also affect production of immunosuppressive factors by tumour cells. It is plausible that the local cytokine milieu, acting on the tumour cells or on the adjacent cells, can either block or facilitate tumour growth (Anna *et al*., 2004).

Cytokine regulation of human colorectal cancer is not clearly understood. Sera of patients with colorectal cancer have abnormally high levels of IL-6, IL-4, IL-10, TNF-α and TGF-β1(Berghella *et al*., 1998, Shibata *et al*., 1996, Tsushima *et al*., 1996., Zaloudik *et al*., 1999). TGF-ß and IL-10 have been assumed to play a role in tumour-induced immunosuppression in colorectal carcinoma (CRC) patients. Impaired cytokine production by mitogen-stimulated peripheral blood mononuclear cells from patients with colorectal cancer (IFN-α, IFN-g, IL-1α, IL-2, IL-12, TNF-α) has also been described (Heriot *et al*., 2000, Lahm *et al*., 1998, O'Hara *et al*., 1998).

As many of the pro-inflammatory cytokines have potent tumour-promoting activity by inducing tumour angiogenesis, synthesis of matrix metalloproteinases, or by directly supporting tumour cell growth (K Xie 2001, Rosen *et al .*, 1993, Opdenakker *et al .*, 2001, Kossakowska *et al .*, 2000, Wilson *et al .*, 2002). Cytokine pathways in colon carcinogenesis is now well established (Barnetson *et al .*, 2000). These studies demonstrate the importance of cytokines in regulating colorectal cell function and growth.

As stated in the General Introduction chapter, the last few years have witnessed the identification of specific markers to the vascular endothelium secreted by endothelial cells (ECs) including vWF (Ginsburg *et al .*, 1985), PECAM-1(Scholz *et al .*, 1997) and CD34(Delia *et al .*, 1993) gene products that are involved in regulating the development of the vascular endothelium. Most recently, novel tumour endothelial markers (TEMs) have been shown to be restricted to endothelial vessels in normal and tumour tissues and mostly TEM-8 associated with the tumour endothelium (Croix *et al .*, 2000), and have indeed been documented in chapters five and six.

The regulation of these genes in endothelial cells to date not very clear. Endothelial cells participate in many physiological and pathophysiological processes including angiogenesis during embryo development and tumour growth. Furthermore, specific characteristics of endothelial cells provide the basis for the specialized function of endothelial cells within each organ. The molecular components that characterize the endothelial cell phenotype and regulate endothelial specific gene expression are not yet identified. The effects of factors like hypoxia or cytokine regulation on their expression have not been explored in the vascular endothelium.

It is not clear as to which factors are able to regulate the expression of TEM-8 in human endothelial cells and the role of TEM-8 in tumour associated angiogenesis. Therefore, in this chapter the effects of several cytokines on the expression of these

markers in endothelial cells were studied. Consequently , we examined the effect of IL-1β, a cytokine known to initiate and complete the processes of angiogenesis (Elena *et al* ., 2003), on the expression of TEM-8, at both mRNA and protein levels, using a new antibody raised in our laboratory. In addition, we have assessed the impact of IL-1ß and TEM-8 on angiogenesis *in vitro*. Here, we report, for the first time, the stimulatory effects of IL-1ß on TEM-8 expression in human vascular endothelial cells and the impact on angiogenesis.

7.2MATERIALS AND METHODS

7.2.1 Materials

HECV cell line, which processes both vascular and lymphatic characteristics (Ye *et al* ., 2003), was obtained from Interlab Cell Line Collection (ICLC), Naples, Italy. IL-1β, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12, Interferon-γ (IFN-γ), Tumour growth factor-α (TGF-α), HGF/SF and VEGF-D were purchased from National Biology Standard Bureau - NBSB (Salisbury, England, UK) and Chemicon International (Temecula, California). A rabbit anti-human IL-1R1, monoclonal antibody to human actin, and Peroxidase conjugated secondary antibodies were purchased from Santa-Cruz Biotechnologies (Santa Cruz, California, USA). RNA extraction and reverse transcription kits and PCR mix were purchased from Abgene (Surrey, England, UK). Protein AIG agarose beads were obtained from Santa-Cruz Biotechnologies (Sant Cruz, CA, USA).

7.2.2 Methodology

7.2.2.1 Generation of cDNA from cell lines and RT PCR

HECV cells were cultured in DMEM medium as previously mentioned (Section 3.2.1.1) using 12 x 25cm^2 flasks. The cells were treated with the following cytokines using the concentrations shown (incubated for 24 hours 37°C and 5% CO_2): IL-1β (50U/ml), IL-

2 (60U/ml), IL-4 (100ng/ml), IL-5 (20ng/ml), IL-6 (10000U/ml), IL-7 (40ng/ml), IL-8 (60ng/ml), IL-10 (40ng/ml), IL-11 (400ng/ml), IL-12 (50ng/ml), IFN-γ (1μg/ml), TGF α (10ng/ml), HGF/SF (10ng/ml), VEGF-D (40ng/ml). Na_3VO_4 was used as negative control throughout the experiments. The concentrations used were based on our previous studies in our Laboratories as well as from the literature.

7.2.2.2 RNA extraction and RT-PCR

RNA extraction and reverse transcription kits and PCR mix were purchased from ABgene (Surrey, England, UK). RNA was extracted from HECV cultured cells (in RNA extraction buffer) using the standard guanidine isothiocyanate method by following the manufacturer's protocol. The concentration of RNA was measured with a spectrophotometer. Reverse transcription was performed from 1μg of total RNA using oligo dt primer according to the manufacturer's instructions. Conventional PCR primers were designed using Beacon Designer software (Palo Alto, California), to allow amplification of regions that have no overlap with other known genes and span at least one intron. Primers were synthesized by Life Technologies (Paisley, Scotland, UK) (Table 7.1).

Conventional PCR was performed using cDNA from cells together with the PCR master mix using respective primers. The reaction conditions for TEM-8 in HECV cells treated with 13 different cytokines were: denaturation at 94°C for 5 minutes; 38 cycles at 94°C for 40 seconds, 55°C for 50 seconds, 72°C for 45 seconds; and a final extension phase 72°C for 10 minutes; for TEM-8 in IL-1β time course cell lines were: 94 C° for 5 minutes; 36 cycles at 94 C° for 30 seconds, 54 C° for 40 seconds, and 72 C° for 50 seconds; followed by 72 C° 10 for minutes. The PCR products were separated on a 2% and 0.8% agarose gel and stained with 10μl ethidium bromide (10mg/ml) prior to examination under UV light and a photograph taken.

Table 7.1 Sequence and size of pairs of primers used in the RT-PCR and quantitative RT-PCR.

Primer	*Forward (5' – 3')*	*Reverse (3' – 5')*	*Size*
β-ACTIN	atgatatcgccgcgctcg	cgctcggtgaggatcttca	520bp
TEM-8	catttcaagttgtcgtgaga	gacgcatattgttgttgaga	510bp
UniPrimer systems	Sense primer (5' – 3')	Z primer (5' – 3')	
TEM-8	acagggtcctctgcagctt	actgaacctgaccgtacactttcatgccaacttgttt	

7.2.2.3. Quantitative analysis of gene expression.

Real time quantitative PCR was carried out using the iCycler iQTM system (Bio Rad) to determine the level of expression of TEM-8 (Table 7.1) amplification. The iCycler iQTM system incorporates a gradient thermocycler and a 96-channel optical unit. AmplifluorTM detection system was used in the current study (Intergen, England, UK), which included the use of specific sense primer, a universal FAM-labelled probe and a specific anti-sense primer that incorporates a Z sequence that is complementary to the probe. The internal standards used in the study were specific plasmids generated using a pCR2.1 cloning vector (Invitrogen, Paisley, Scotland, UK) as described in details in chapter 3 (section 3.2.3). Quantitative PCR was carried out in 96-well plate with 10pmol sense primer, 1pmol anti-sense-Z primer (Table 7.1), and 10pmol FAM-probe, using a customer hot-start Q-PCR master mix, with the following conditions: 95°C for 15 minutes, followed by 50 cycles at 95°C for 15 seconds, 55°C for 40 seconds and 72°C for 15 seconds. The copy number of each transcript was calculated from the internal standards and shown here as copies/50ng RNA.

7.2.2.4 Western Blotting and Immunoprecipitation

HEVC cells treated with IL-1β were cultured in DMEM until 75% confluent. Cells were pelleted and lysed in HCMF buffer as previously documented in section 3.2.4. Immunoprecipitation was used to detect the activation of IL-1R in HECV cells. The cells were subject to serum hunger for 2 hours before being exposed to IL-1β, medium alone, or sodium orthovanadate as a positive control (1 mM Na_3VO_4 with 0.03% H_2O_2) for 15 and 45 minutes. Following extraction of protein using the HCMF buffer, equal volumes of the lysate were precipitated with an anti-IL-1R antibody (5µl/ sample)(1:500 concentration) for 1hr, followed by addition of protein A/G agarose beads on a spinning wheel for 1hr. The Ag/Ab complex was pelleted by centrifugation(13,000 rpm for 1min), and washed in HCMF buffer and dissolved in sample buffer (10% glycerol; 5 % 2-mercaptoethanol; 3 %

SDS; 80 mM Tris-HCL (PH 6.8);0. 012 % bromophenol blue) followed by boiling at 100°C for 5 minutes. Proteins were separated on 10 % polyacrylamide gels by electrophoresis at a constant current of 15 mA per gel. Proteins were then blotted onto nitrocellulose membrane using semi-dry immunoblotter at current voltage of 5 volts for 40 mins.Non-specific binding of AB was blocked by incubating membrane in 10% milk and a roller for 40 minutes before probing with a monoclonal anti-phosphotyrosine antibody (PY-99) (1:1000 dilution), and horse radish peroxidase conjugated secondary antibody, respectively. Protein products were visualized with a chemiluminescence system.

7.2.2.5 Endothelial tubule formation assay

This was based on a Matrigel-sandwich tubule forming assay developed in our laboratory(Jiang *et al,* 1999, Cai *et al* ,1999, Martin *et al* ., 1999). Briefly, 200 μg of cold Matrigel solution in 100μl of medium (reconstituted basement membrane, Becton-Dickinson, Bristol, England) was added to a 96 well plate and allowed to air-dry at 37°C. Following rehydration of Matrigel to allow for the formation of a thin bottom layer, HECV cells were seeded onto the Matrigel at 10,000 per well and allowed to attach for up to 2 hours. The medium was carefully removed. The second solution of Matrigel, which was mixed with test agents (IL-1ß or medium), was added to the cells, followed by incubation at 37°C for 3 hours to allow for the second layer of Matrigel to solidify. Medium with matched IL-1β or HGF as a positive control (40ng/ml) was added over the second layer of Matrigel and the cells were then incubated at 37°C for 24 hrs. Endothelial tubules were visualised microscopically and photographed using a digital camera. The length of tubules in a fix-sized frame was quantified using Optimas-6 software, as we have previously described (Jiang *et al*, 1999, Martin *et al* ., 1999).

Statistical Analysis. Statistical analysis was carried out using Two Sample *student's t-test.*

7.3 RESULTS

7.3.1 The effects of cytokines on the expression of Tumour endothelial marker 8 (TEM-8) in human endothelial cells

Amongst the cytokines tested, IL-1β increased the expression of TEM-8 in HECV cells. This effect was consistent with repeating the experiments (Figure 7.1). Although both IL-8 and HGF were also found to increase the expression of TEM-8, their effects were not statistically significant. Therefore, only IL-1β was used in subsequent experiments.

7.3.2 Quantification of the effects of cytokines on Tumour endothelial marker 8 (TEM-8) Expression.

Real time quantitative RT-PCR was used in order to quantify the levels of expression for TEM-8 in HECV cells after treatment with 13 different cytokines. The level of TEM-8 expression was significantly higher in HECV cells that were treated with IL-1β, compared to TEM-8 expression in HECV cells without treatment. Although the level of IL-8, HGF and VEGF-D expression were higher than the control (without treatment with IL-1β), it did not reach statistical significance. The data is also represented graphically in (Figures 7.2a).

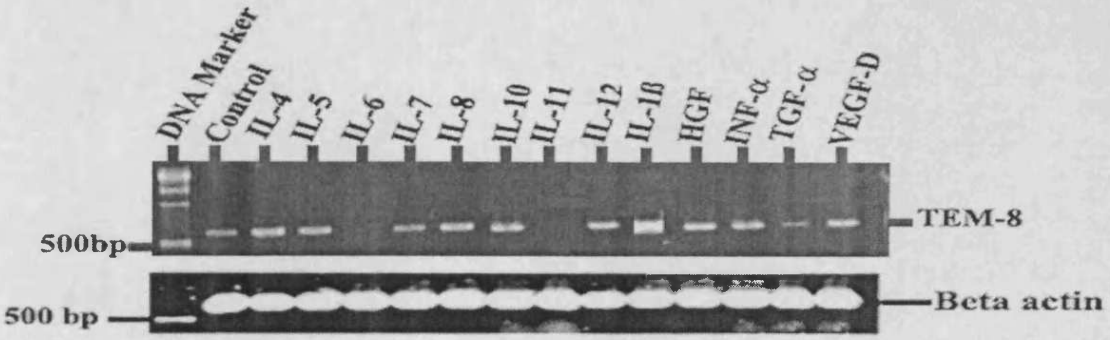
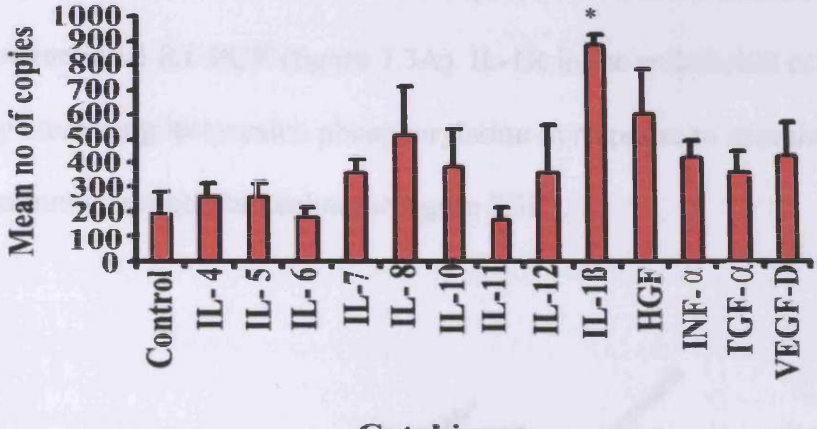
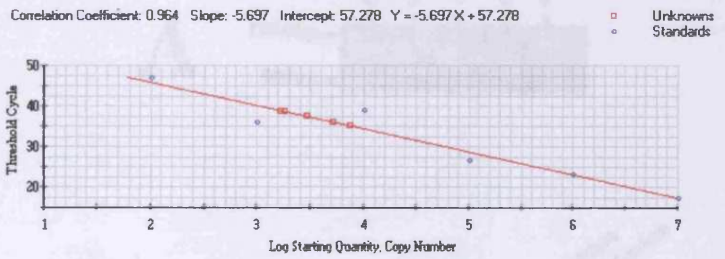


Figure 7.1 IL-1β increases the expression of TEM-8 in HECV cells treated with IL-1β at the mRNA level. Treatment with other cytokines does not have any persistent effect on the expression of these markers. IL-8 and HGF appeared to have increased the expression of TEM-8 in HECV cells, but this was not significant as with TEM-8 (upper panel). The control means HECV cells not treated with any cytokine. β-actin was used as an internal housekeeping gene.

A



B



C

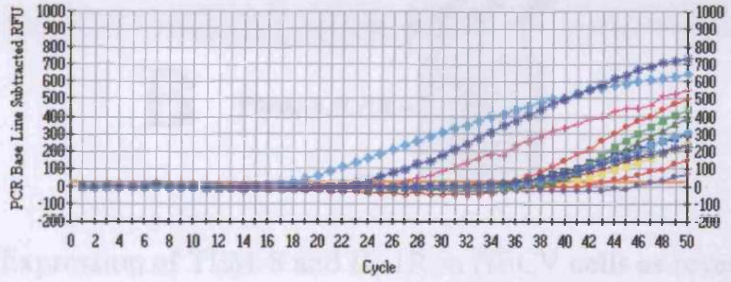


Figure 7.2 Real Time quantitative RT-PCR. Mean copy number / 50ng mRNA TEM-8 is significantly higher in HECV cells treated with IL-1β compared to control (HECV not treated with IL-1β), *$p<0.05$ using student's t test (A). The correlation coefficient (B), shows a slope of 0.964 comparing podoplanin levels with those of a standard. (C) shows the amplification of copy numbers in relation to the cycle number.

7.3.3 Expression of TEM-8 and IL-1R mRNA and protein in Human Endothelial cell (HECV)

HECV cells expressed both TEM-8 and IL-1 receptor (IL-1R) at the mRNA level, as shown by a conventional RT-PCR (figure 7.3A). IL-1R in the endothelial cell was fully functioning by increasing its tyrosine phosphorylation in response to stimulation by IL-1ß by using immunoprecipitation technique figure 7.3B).

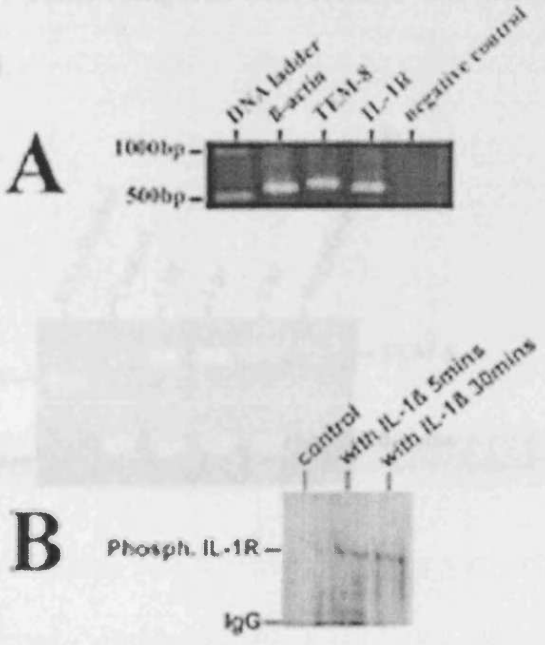


Figure 7. 3. A: Expression of TEM-8 and IL-1R in HECV cells as revealed by RT-PCR. **B:** Activation of IL-1 receptor (IL-1R) by recombinant human IL-1ß, as revealed by Immunoprecipitation and Western blotting.

7.3.4 IL-1ß increased expression of TEM-8 at both mRNA and protein level in human endothelial cells (HECV)

Treatment of HECV cells with IL-1ß increased the level of TEM-8 mRNA as revealed by conventional RT-PCR (figure 7.4A).To determine whether the increase in TEM-8 mRNA expression induced by IL-1β was associated with an increase in TEM-8 at protein level, cells were treated with the cytokine with varying concentration for 8 hours. TEM-8 was detected using Western Blotting. Treatment with IL-1β increased the amount of TEM8 protein in HECV cells, compared with control. The maximum effect occurred after 1 hour (Figure 7.4B).

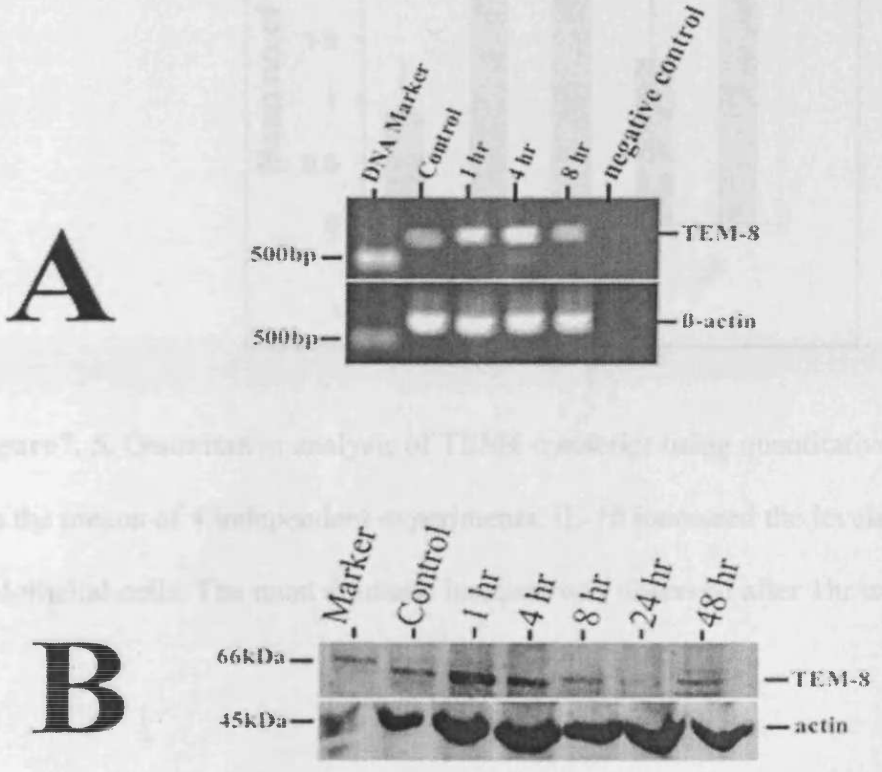


Figure 7.4. Effects of IL-1β on the expression of TEM8 in human endothelial cells (HECV). HECV cells were treated with IL-1β in serum-free medium for the different times incubated. TEM-8 mRNA (**A**) and TEM-8 protein (**B**), were detected using RT-PCR and Western blotting respectively. Expression of TEM8 was increased at both mRNA and protein levels with a maximum effect seen between 1-4 hours.

7.3.5 Levels of TEM-8 transcripts in endothelial cells increased in response to IL-1ß

The level of transcripts of HECV cells treated with IL-1ß were analysed using quantitative RT-PCR. The number of TEM-8 transcripts was increased over time, with a maximum effect seen 1 hour after stimulation with IL-1β (Figure 7.5).

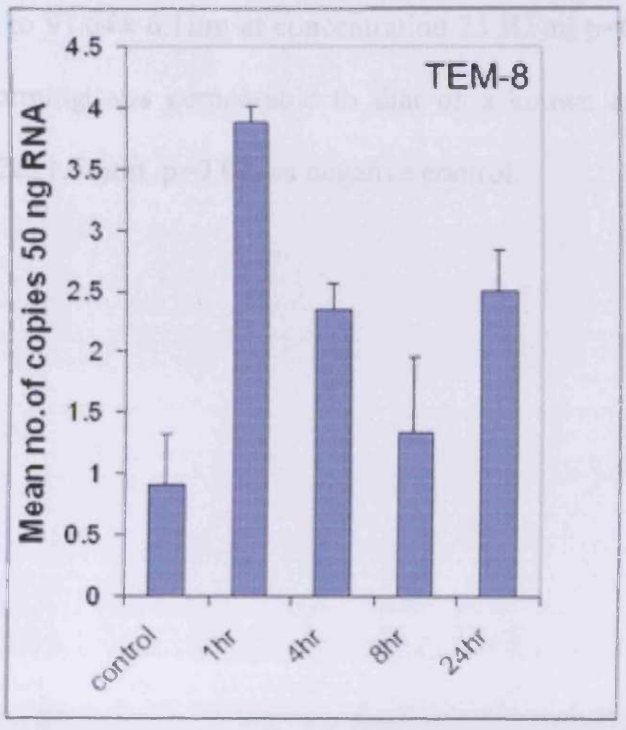
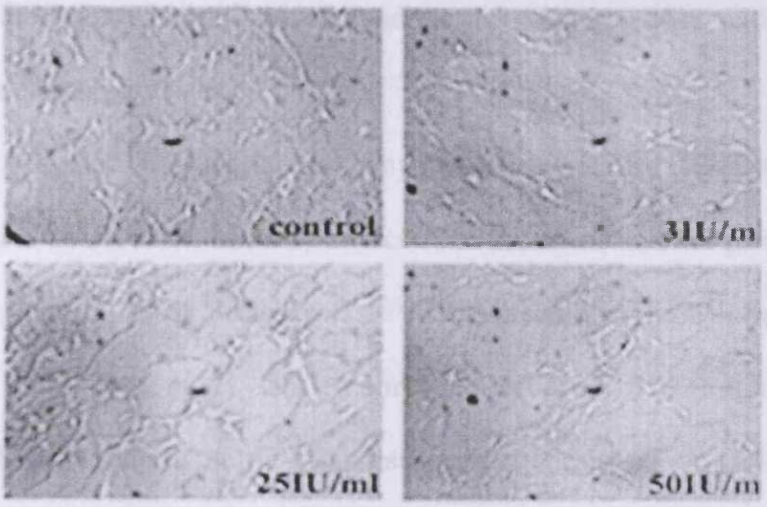


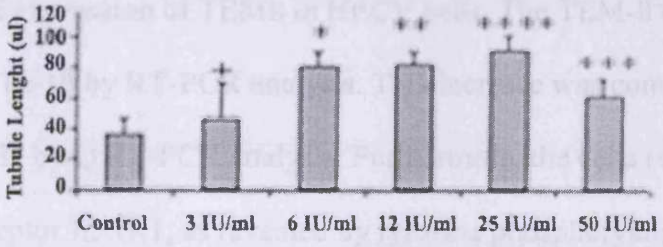
Figure7. 5. Quantitative analysis of TEM8 transcript using quantitative RT-PCR. Shown are the means of 4 independent experiments. IL-1ß increased the levels of TEM8 in endothelial cells. The most dramatic increase was observed after 1hr expoure to IL-1ß.

7.3.6 Enhancement of endothelial tubule formation by IL-1β

The vascular endothelial cells, HECV, formed capillary-like tubules over time (figure7.6A). The formation of tubules was substantially enhanced by the addition of interleukin-1ß, as shown in figure7.6B (tubule length was 36.87±10.71μm for the negative control and peaked to 91.64± 6.1μm at concentration 25 IU/ml p=0. 0185). The effects of IL-1ß on tubule forming was comparable to that of a known angiogenic factor, HGF (tubule length 57.02±21.51μm, p=0.02, vs negative control.

A





B

Figure 7. 6. Promotion of tubule formation by IL-1β. (**A**) Microscopic picture of tubules from endothelial cells and in response to IL-1ß. Insert: IL-1ß concentration at international unit per millilitre. (**B**) Quantitation of tubule length (mean±SD), $^{+}$p= 0.312 *vs* control, *p=0.0372 *vs* control, **p=0.0296 *vs* control, ***p=0.0422 *vs* control, ****p=0.0185 *vs* control.

7.5 DISCUSSION

The present study has, for the first time, demonstrated the effect of IL-1ß on the expression of the tumour specific endothelial marker, TEM8, and indicated its possible impact on angiogenesis.

Regulation of angiogenesis by cytokines is an attractive starting point as they are known to be involved in many intercellular autocrine and / or paracrine mechanisms (Hasday *et al*., 1990, Freiss *et al*., 1993, Duncan *et al*., 1994, Speirs *et al*., 1995, Adams *et al*., 1991, Ryan *et al*., 1993, Vanroozendaal *et al*., 1992). It has been documented recently for example that IL-8 mediates angiogenesis in vivo (Koch *et al*., 1992). We screened 13 different cytokines and found IL-1ß to be the cytokine that most constantly increased of expression of TEM8 in HECV cells. The TEM-8 transcript was increased in response to IL-1ß by RT-PCR analysis. This increase was convincing, as it was reproduced quantitatively by Q RT-PCR analysis. Furthermore, the cells reacted to IL-1ß via the specific receptor IL-1R1, as revealed by tyrosine phosphorylation of this receptor.

The biological importance of this change is worth mentioning. Firstly, the increased TEM-8 transcript has been clearly translated into a protein product, as the antibody specific to human TEM-8 clearly indicated the rise of TEM-8 protein, following IL-1ß treatment. Secondly the exact role of TEM-8 in angiogenesis is unclear. TEM-8 is a type I transmembrane protein, with a cytoplasmic tail sharing at least seven potential phosphorylation sites. This would suggest that TEM-8 is involved in transmitting signals into the cell. In the extracellular region TEM-8 has the von Willebrand factor type A domain containing a metal ion-dependent adhesion motif (I-domain) (Colombatti. 1991, Lee . 1995).

von Willebrand factor type A domains are often found with in extracellular domains of integrins where they constitute ligand-binding sites and have been shown to interact with vascular cell adhesion molecules via TEM-8 I-domain during leukocyte

trafficking(Wigelsworth *et al*., 2004). Thus, TEM-8 may regulate angiogenesis by interacting with the extracellular matrix and regulating vascular adhesions central to the angiogenesis process. The data shown here provides evidence that this may indeed hold true, as the increase in TEM-8 is associated with an increase in tubule formation, *in vitro*, from endothelial cells. In addition, in the study presented in this chapter it has been shown that IL-1β increases the expression of the markers specific to the tumour endothelium (TEM-8). This might be the beginning of understanding part of the regulation of expression of this marker. Low levels of TEM-8 expression in HECV cells were significantly increased by IL-1β. However, HECV cells without IL-1β treatment did express weak TEM-8 levels; a marker that is highly specific to tumour endothelium. Although, HECV cells are known to have blood vascular properties, which may explain its presence.

IL-1β exerts its maximal effects on the expression of these markers within 1–4 hours of incubation. It is unclear however, why the expression of these markers decreases at 24 hours. HECV cells are rapidly dividing transformed cells and this probably related to the instability of their gene transcription. The real time quantitative RT-PCR however, showed that the levels of expression of the TEM-8 in HEVC treated with IL-1β are significantly higher compared to their expression in the untreated cells. Although IL-8 and HGF increased the expression of TEM-8 in HECV cells, their effects were not significantly different from control cells.

IL-1β has been found to stimulate the proliferation of vascular smooth muscle cells and is involved in modifying a number of vascular functions by inducing autocrine production of chemotactic cytokines on endothelial cells, including IL-1 β itself (Libby *et al*., 1988, Mantovani *et al*., 1989, Mantovani *et al*., 1992). Moreover, IL-1β has been shown to enhance angiogenesis in melanoma cells (Koch *et al*., 1992, Gutman M 1995). IL-1β increased VEGF mRNA in rat aortic smooth muscle cells in a time and dose-

dependent manner (Li *et al*., 1995, Akagi *et al*., 1999). These findings suggest that IL-1β is an angiogenic factor. The current study shows that IL-1β enhances *in vitro* tubule formations, a process central to angiogenesis *in vivo*.

IL-1β is an inflammatory cytokine present in activated immune cells. Solid tumours are infiltrated by numerous immune effector cells, including macrophages and lymphocytes, through the expression of platelet-derived endothelial cell growth factor (Takahashi *et al*., 1996). This infiltration of immune effector cells occurs in both primary and metastatic cancer. Infiltrating immune cells may contribute to the angiogenesis of human colon cancer (Takahashi *et al*., 1996).

Taken together, IL-1ß may be one of the key factors involved in the development of the angiogenesis process in tumours, by way of induction of TEM-8 expression. Given the prime source of IL-1ß in tumour tissues (immune cells and stromal cells), this may be an important pathway by which infiltrating immune cells and stromal cells stimulate angiogenesis in malignancies. Therefore, it is concluded from this initial study that IL-1β increases the expression of TEM-8 in HECV cells. This suggests that TEM-8 has angiogenic properties in endothelial cells.

CHAPTER EIGHT

TEM-8 AND ITS ROLE IN ANGIOGENESIS

8.1 INTRODUCTION

As previous chapters have shown TEM-8 protein expression was significantly higher in tumour tissues compared to normal tissues. TEM-8 antibody identified more micro-vessels in colon tumour tissue by immunohistochemistry, than in normal colon tissue. More over, it was reported in chapter seven that, IL-1β significantly raised the level of TEM-8 at the protein level, as revealed by Western blotting. *In vitro* tubule forming assay, revealed that IL-1β significantly induced the formation of capillary- like tubules from HECV cells, accompanied by an increase in TEM-8 expression.This indicates that TEM-8 is potentially involved in angiogenesis and that IL-1β is a powerful regulator of the expression of TEM-8 in vascular endothelial cells, suggesting, an important pathway through which IL-1β regulates tumour-associated angiogenesis.

To date, it is not clear if TEM-8 directly induces angiogenesis, and if so, by what mechanisms it employs. In the current chapter, a TEM-8 knock-out cell was created and tested for its tubule forming capillary capability.

8.2 MATERIALS AND METHODS

8.2.1 Materials

Human endothelial cell line HECV cells was cultured as presented in previoues chapters. Pc DNA 3.1 GFP TOPO TA Expression Kit was obtained from Invitrogen. One shot competent *E-coli* Kit was obtained from Invitrogen. Gen Elute^{TM}plasmid, mini prep Kit for plasmid extraction were obtained from Sigma.

8.2.2 Methodology

8.2.2.1 Generation of TEM-8 null endothelial cells via construction of expression vectors and ribozyme transgenes

TEM-8 ribozymes were designed based on the secondary structure of the TEM-8 RNA (Gene bank accession No NM-0322087), generated from Zuker's m-Fold program (Mathews *et al*., 1999) and constructed as recently reported (Jiang *et al*., 2003c, Jiang *et al*., 2005) (Figure 8.1) .Three ribozymes were designed and engineered to target the AUC sites on positions 164,529 & 775 respectively which are with in the coding region of TEM-8 mRNA. Touch down PCR was used to generate the ribozymes, using Taq polymerase, this generated as A TA over-hang to PCR products. The products verified on 2% agarose.The following primer pairs were used as shown inTable8.1.

Table 8.1. Sequence of primers pairs used in the RT-PCR.

Primer	***Forward (5' – 3')***	***Reverse (3' – 5')***
TEM-8rib1	ctgcagagaccactggaagccctgatgag	actagttggagcggagaccctcggcatttcgtcctcacgca
TEM-8rib2	ctgcagtccatcagtcaaagcaatctgatg	actagtacaggacagccagcgtcatttcgtcctcacgga
TEM-8rib3	ctgcagtccatcagtcaaagcaatctgatg	actagttttcaggctctgcaaggcatttcgtcctcacgga

PCR products were T-A cloned into pcDNA3.1 GFP TOPO cloning vector according to the manufacturer's instructions (Invitrogen). Selection markers ampicillin and G418, for prokaryotic and mammalian cells respectively, and amplified using one-shot competent *E-coli* (Invitrogen) according to the manufacturer's instructions. Approximately 8-10 Colonies were screened at random to verify for the presence and correct orientation of the cloned products using the following primer pairs T7 F vs RB TopF and T7 F vs RBBm R. Application was carried out using the following conditions: denaturation at 94°C for 5 minutes; followed by 38 cycles at 94°C for 30 seconds; 54°C for 40 seconds; 72°C for 60 seconds and final extension period of 72°C for 10 minutes. PCR products were run on 2% gel and visualised by UV light after staining with stadium bromide. Positive colonies were cultured in LB broth over night at 37 °C and the E-coli cultures pelleted by centrifugation. Plasmids were extracted from *E-coli* pellets using gene Elute plasmid extraction kit according to the manufacturer's instructions. Purified plasmid was verified on a 0.8% agrose gel, and electrporated (Flowgen, Sussex, England, UK) into HECV cells as discussed in section 8.2.2.2. The following cell lines were generated and are cited through out the text as: $HECV^{\Delta TEM8a}$ for Rib 1, $HECV^{\Delta TEM8b}$ for Rib 2 and $HECV^{\Delta TEM8c}$ for Rib 3.

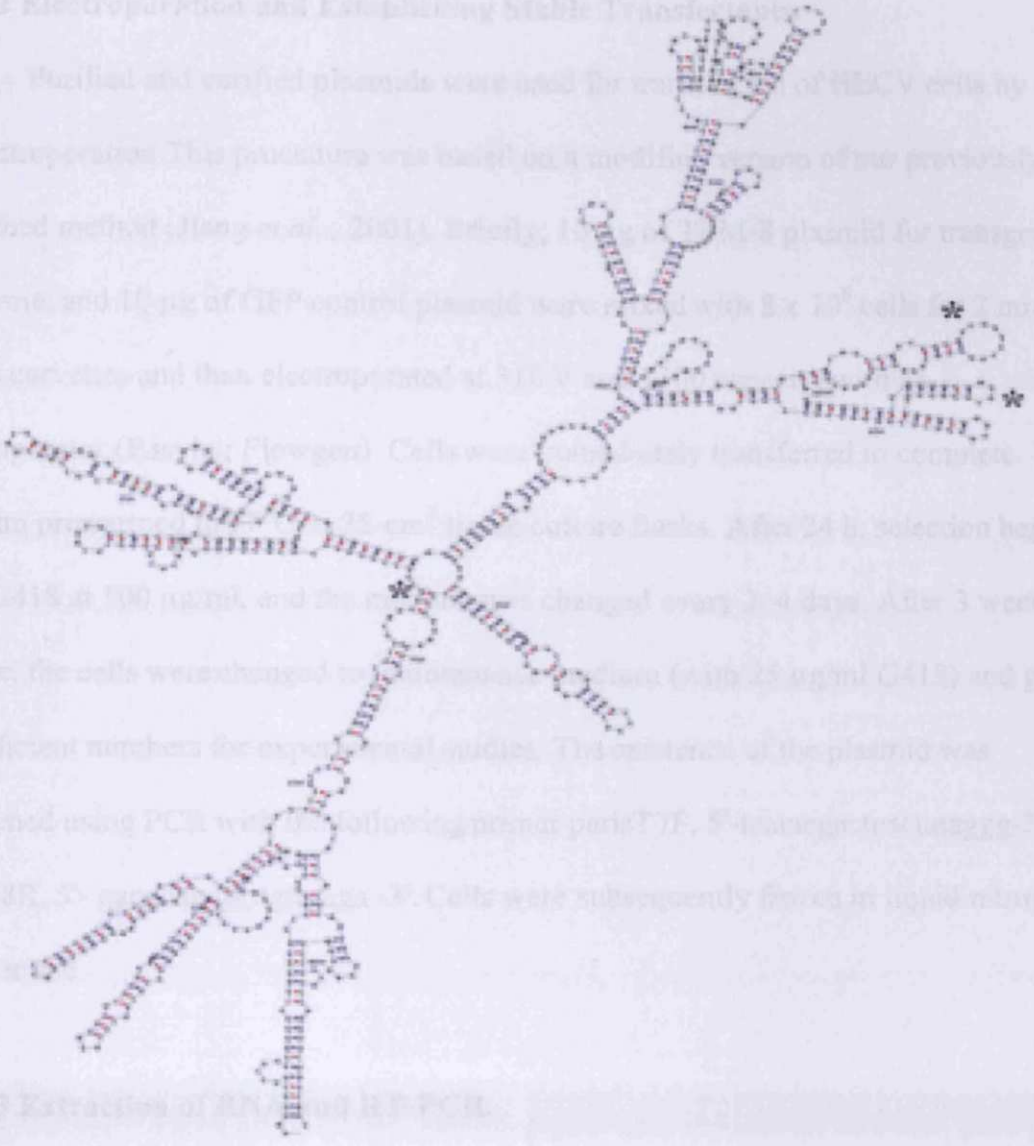


Figure 8.1. TEM-8. The secondary structure of human TEM-8. The coding sequence was folded using the Zucker's mFold programme. * Indicates the suitable sites for the hammerhead ribozyme to target (164,529 & 775).

8.2.2.2 Electroporation and Establishing Stable Transfectants.

Purified and verified plasmids were used for transfection of HECV cells by way of electroporation.This procedure was based on a modified version of our previously published method (Jiang *et al*., 2001). Briefly, 10 μg of TEM-8 plasmid for transgene ribozyme, and 10 μg of GFP control plasmid were mixed with 8 x 10^6 cells for 2 min in sterile curvettes and then electroporated at 310 V and 1500 capacity with an electroporator (Easyjet; Flowgen). Cells were immediately transferred to complete medium prewarmed to 37°C in 25-cm^2 tissue culture flasks. After 24 h, selection began with G418 at 100 μg/ml, and the medium was changed every 3–4 days. After 3 weeks of culture, the cells were changed to maintenance medium (with 25 μg/ml G418) and grown to sufficient numbers for experimental studies. The existence of the plasmid was confirmed using PCR with the following primer parisT7F, 5'-taatacgactcactataggg-3'; and TEM-8R, 5'- gacgcatattgttgttgaga -3'. Cells were subsequently frozen in liquid nitrogen for later use.

8.2.2.3 Extraction of RNA and RT-PCR.

Cellular RNA was extracted using an RNA extraction kit (AbGene, Ltd., London, United Kingdom) and quantified using a spectrophotometer (Wolf Laboratories). cDNA was synthesized using a first-strand synthesis with an oligo dt primer (AbGene). The PCR was performed using a Perkin-Elmer thermocycler and PCR mastermix reaction mixture (Abgene, Surrey, United Kingdom): denaturation for 5 min at 95°C followed by 38 cycles of 30s at 94°C,40s at 55°C ,and 60s at 72°C,with final extension of 72°C for 10 min. *ß-actin* was amplified simultaneously using primer pairs 5'gctgatttgatggagttgga-3' and 5'-tcagctacttgttcttgagtgaa-3'. PCR products were then separated on an 0.8% agarose gel, visualized under UV light, photographed using a Unisave camera (Wolf Laboratories, York, UK), and documented with Photoshop software.

8.2.2.4 Western Blotting Analysis of TEM-8.

TEM-8 knock-out cells ($HECV^{\Delta TEM8a}$, $HECV^{\Delta TEM8b}$ and $HECV^{\Delta TEM8c}$) were lysed in HCMF buffer as previousely mentioned. Equal amounts of protein from each cell sample (15 µg/lane) were added onto an 8% polyacrylamide gel. After electrophoresis, proteins were blotted onto nitrocellulose membrane and blocked in 10% skimmed milk for 40 min before probing with the antihuman TEM-8 or antihuman ß-actin antibody, and followed by labelling with peroxidase-conjugated secondary antibodies. A molecular weight marker mixture was used to determine the protein size. Protein bands were visualized with a chemiluminescence system. Exposed films were scanned with a scanner, and the density of protein bands was analyzed with the software Optimas.

8.2.2.5. Cell Proliferation assay using Crystal violet

Cell growth was measured using this assay to ascertain whether the differences in the cell lines were the result of change in cell proliferation. A fixed number of Wild type $HECV^{WT}$, GFP control $HECV^{pControl}$ and knockout TEM-8 $HECV^{\Delta TEM-8}$ cells were cultured in a 96-well culture plate in DMEM culture medium and incubated for 3 Days, 5 Days and 7 Days respectively. After discarding the medium, the cells were then fixed with 100µl per well of 4% formaldehyde and stained with 100µl / well of 0.5% crystal violet solution. After washing the cells with dH2O the dye was extracted using 100µl per well of 10% acetic acid solution. The level of absorbance was measured at a wavelength of 540nm using a spectrophotometer. The data obtained analysed in an *E*xcel spread sheet.

8.2.2.6. In Vitro Migration Assay

This assay was performed as previously reported(Jiang *et al .*, 2001). Briefly, $HECV^{WT}$, $HECV^{pControl}$ and $HECV^{\Delta TEM8}$ cells were resuspended in HEPES medium to

maintain a buffered environment for cells at PH 7.3, and were seeded into a 24-well plate and grown to confluence. Mineral oil was used to overlay the medium (HEPES Buffered environment for the cells, and to prevent evaporation occurring from each well (Jiang *et al* ., 1999). The degree of migration across the wounded cell surface was then examined microscopically (Leitz DMIRB; 10x lens objective) on a heated stage maintained at 37°C for up to 1 hr using a time-lapse video recording facility (Panasonic AG-4060, Japan).Wound closure between the leading cell fronts was then assessed at 15 min intervals in real time, using Optimas image analysis software (Version 6, Optimas,UK).

8.2.2.7. Vascular endothelial tubule formation assay.

This was based on a Matrigel-sandwich tubule forming assay developed in our laboratory and as reported chapter seven. Briefly, 200 μg of cold Matrigal solution in 100μl of medium (reconstituted basement membrane, Becton-Dickinson, Bristol, England) was added to a 96 well plate and allowed to air-dry at 37°C. Following dehydration of Matrigel to allow formation of a thin bottom layer. Wild type HECV ($HECV^{WT}$) GFP control plasmid ($HECV^{pControl}$) and TEM-8 transgene transfected HECV ($HECV^{\Delta TEM8}$) cells were seeded onto the Matrigel at 10,000 cells per well and allowed to attach for up to 2 hours. The medium was carefully removed. The second solution of Matrigel, which was mixed with test agents ie cytokines or medium, was added to the cells, followed by incubation at 37°C for 3 hours to allow the second layer of Matrigel to solidifiy.

Medium with matched IL-1β, VEGF, IL-8 and HGF as a positive control (40ng/ml) was added over the second Matrigel and the cells were then incubated at 37°C for 24 hrs. Microtubules were visualised microscopically and photographed using a digital camera. The length of tubules in a fix-sized frame was quantified using Optimas-6 software, as we have previously described in section 7.2.2.6.

8.3 RESULTS

8.3.1. TEM-8 Ribozymes Positive colonies and Plasmid detections.

TEM-8 ribozymes were inserted into the pcDNA3.1 GFP TOPO plasmid vector, as this vector will regulate the expression and secretion of the TEM-8 ribozyme proteins. Positive colonies whose TEM-8 was lost as the result of the ribozyme were selected; following amplification using RT-PCR of TEM-8 and the plasmid sequence, using the following condtions for each set (Figure 8.2A).The GFP colonies were identified as containing inserted nucleotide sequences in correct orientation, downstream of appropriate promoters (Figure 8.2B). As seen from PCR reactions (Figure 8.2B) , the GFP/TEM-8 ribozymes constructs showed strong PCR product expression of the gene. The digested plasmid constructs with restriction endonucleases; SnaB I and EcorR V for GFP TOPO was performed and showed that, only GFP constructs in lane 1,3 and 5 showed good incorporation of the gene (Figure 8.2C) .

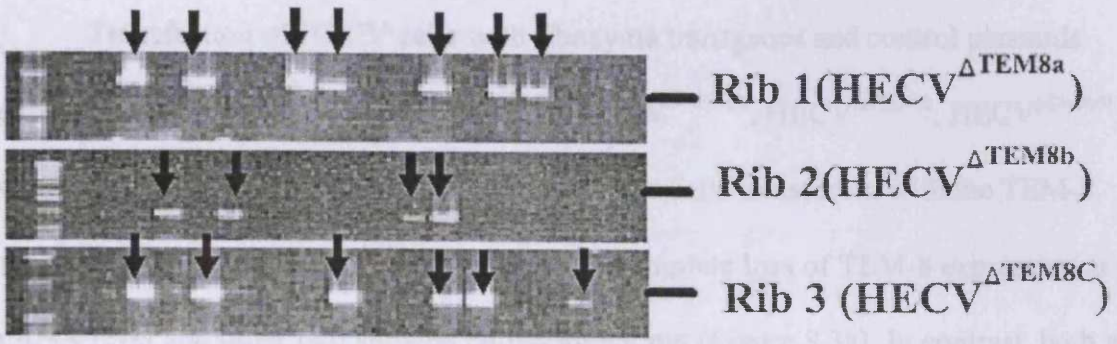


Figure 8.2A. Identification of positive colonies (Arrows) with correct orientation for the respective ribozymes of TEM-8, using PCR.

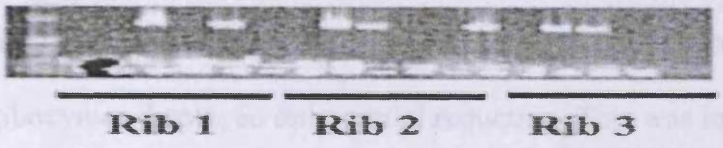


Figure 8.2B. TEM-8 ribozymes PCR of plasmid/vectors extracted using TEM-8F2 versus.Two from each GFP plasmid show strong expression of TEM-8 ribozymes (HECV$^{\Delta TEM8a}$, HECV$^{\Delta TEM8b}$ and HECV$^{\Delta TEM8c}$ respectively).

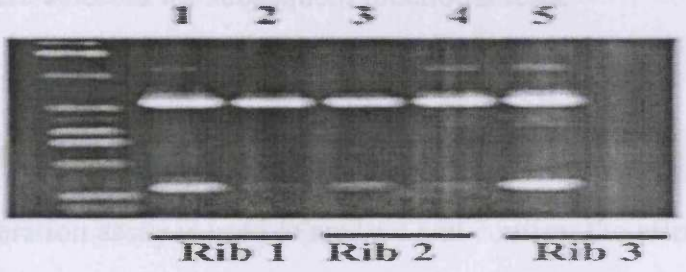


Figure 8.2C. Plasmid digestion confirms the presence of the full-length of TEM-8 ribozymes in GFP TOPO plasmid in lane 1 for HECV$^{\Delta TEM8a}$, lane 3 for HECV$^{\Delta TEM8b}$ and lane 5 for HECV$^{\Delta TEM8c}$.

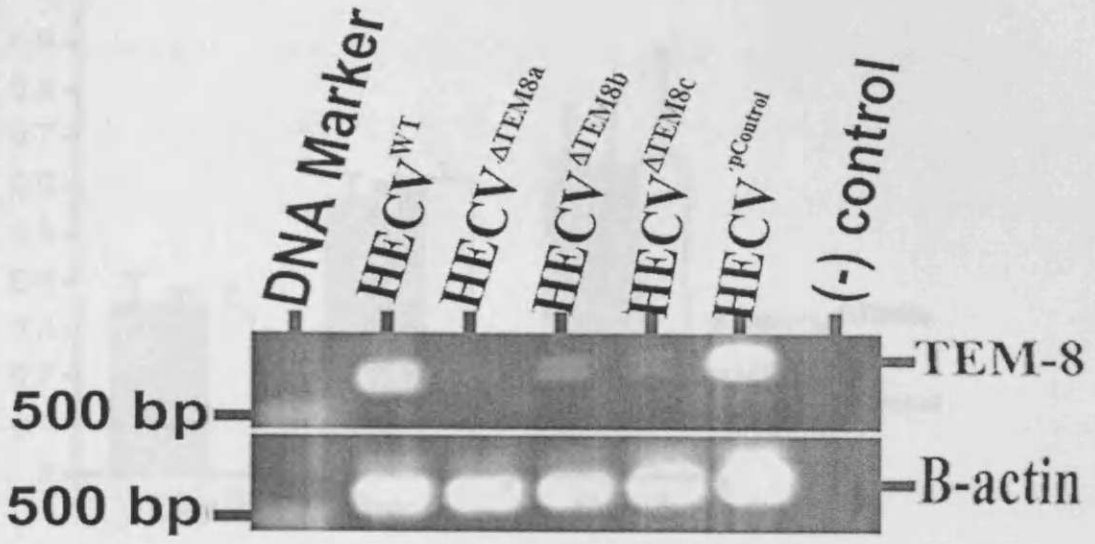
8.3.2 TEM-8 ribozyme transgene eliminated expression of TEM-8 in HECV cell

Transfection of HECV cells with ribozyme transgenes and control plasmids generated the following cells: $HECV^{\Delta TEM8a}$, $HECV^{\Delta TEM8b}$, $HECV^{\Delta TEM8c}$, $HECV^{pControl}$, together with the wild type $HECV^{WT}$. One of three cells transfected with the TEM-8 ribozyme transgene ($HECV^{\Delta TEM8a}$) exhibited a complete loss of TEM-8 expression at the mRNA level and other two showed partial knock out (Figure 8.3a). In contrast, both wild-type ($HECV^{WT}$) and control plasmid ($HECV^{pControl}$) exhibited strong transcript signals for TEM-8 expression. Only one cell type ($HECV^{\Delta TEM8a}$) of the three transfacted cells exhibited a dramatic reduction in the level of TEM-8 protein (Figure 8.3b), whereas the other two TEM-8 ribozymes displayed only partial reduction. This was in contrast to wild-type ($HECV^{pWT}$) and GFP control plasmid ($HECV^{pControl}$) which were both found to express high levels of TEM-8 protein as demonstrated by Western blotting. Therefore, $HECV^{\Delta TEM8a}$ cells were selected for subsequent functional tests.

8.3.3. The effect of knock-out TEM-8 on HECV cell growth

The cell proliferation assay is used to analyse and confirm the effects of elimination of TEM-8 on HECV cell geowth. The growth rates of transfected cell $HECV^{\Delta TEM-8}$, wild-type $HECV^{WT}$ and control $HECV^{pControl}$ cell lines were assessed over seven days using Crystal violet and the data plotted. The cell proliferation graph (Figure 8.4) showed $HECV^{\Delta TEM-8}$ cells to have no significant difference ($p>0.05$) in their growth rate when compared with wild-type $HECV^{WT}$ and cells expressing the control plasmid $HECV^{pControl}$.

A.



B

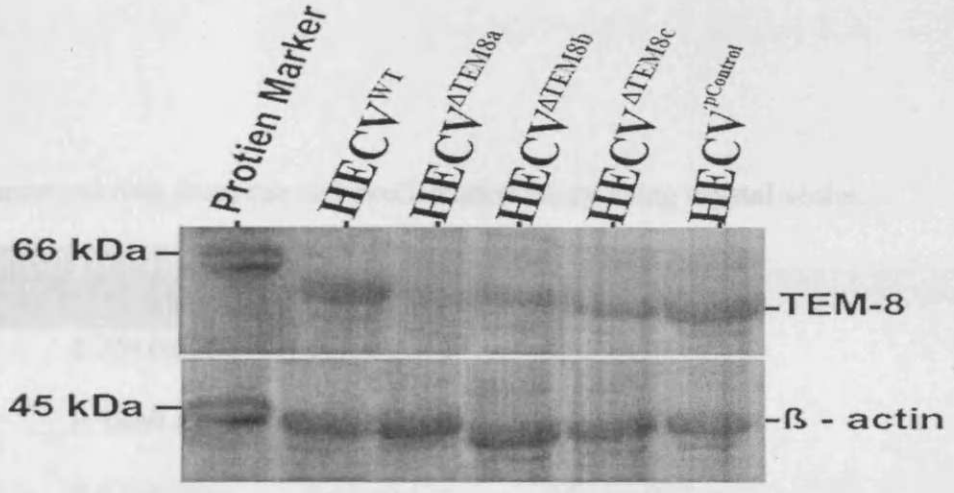


Figure 8.3. (A) RT-PCR analysis revealed TEM-8 mRNA was completely knocked out from the HECV$^{\Delta TEM8a}$ cell, but was only partially lost in HECV$^{\Delta TEM8b}$ and HECV$^{\Delta TEM8c}$ cells. β-actin was used as the housekeeping markers. (B) TEM-8 at protein level was dramatically reduced from one of three HECV cells used (HECV$^{\Delta TEM8a}$) compared to the partial loss of TEM-8 protien in HECV$^{\Delta TEM8b}$ and HECV$^{\Delta TEM8c}$ cells as shown by Western Blotting. Anti-actin was used as the housekeeping marker.

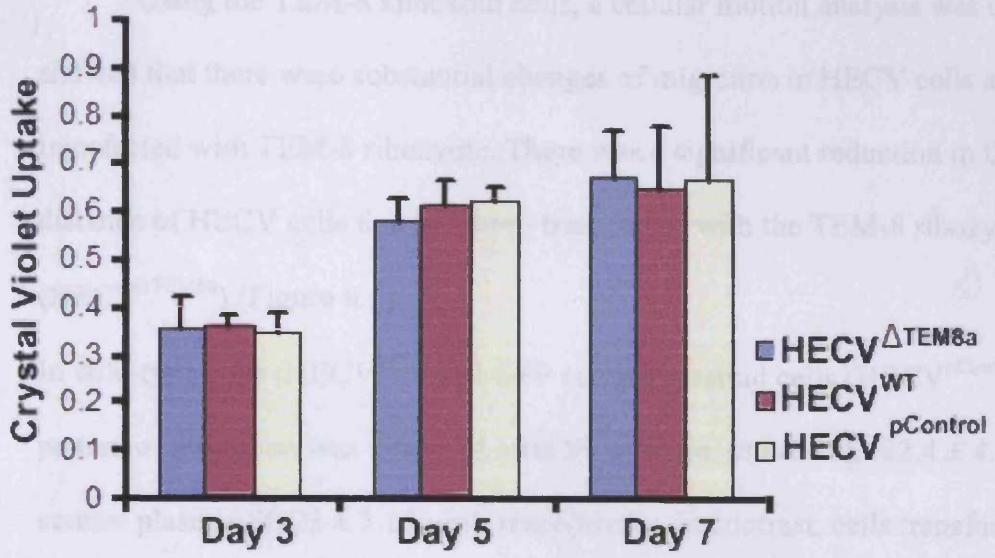


Figure 8.4. Growth rates of the transfected TEM-8 $HECV^{\Delta TEM8a}$ transgene, GFP control plasmid $HECV^{pControl}$ and wild-type $HECV^{WT}$ cells.

Table 8.2. Numerical data from the cell proliferation assay using crystal violet.

	$HECV^{\Delta TEM-8}$	$HECV^{WT}$	$HECV^{pControl}$
Day 3	0.35±0.065	0.35±0.023	0.35±0.039
Day 5	0.58±0.43	0.61±0.051	0.62±0.032
Day 7	0.67±0.096	0.65±0.125	0.66±0.027

8.3.4 Reduction of *in Vitro* Migration in TEM-8 knock-out Cell ($HECV^{\Delta TEM8a}$.).

Using the TEM-8 knockout cells, a cellular motion analysis was conducted; it showed that there were substantial changes of migration in HECV cells after being transfected with TEM-8 ribozyme. There was a significant reduction in the migration distance of HECV cells that had been transfected with the TEM-8 ribozyme transgene ($HECV^{\Delta TEM8a}$) (Figure 8.5).

In wild-type cells ($HECV^{WT}$) and GFP control plasmid cells ($HECV^{pControl}$), a similar pattern of migration was observed after 90 mintues (mean±SE 82.4 ± 4.45μm; $p>0.05$ vs. control plasmid 80.23 ± 3.19 μm), respectively. In contrast, cells transfacted with the TEM-8 ribozyme transgene ($HECV^{\Delta TEM8a}$) had a significantly reduced migration distance compared to wild-type and control plasmid cells (38.52± 2.17; $p<0.05$ vs. wild-type 82.4 ± 4.45 and vs. GFP control plasmid 80.23 ±3.19μm), respectively (Figure 8.6).

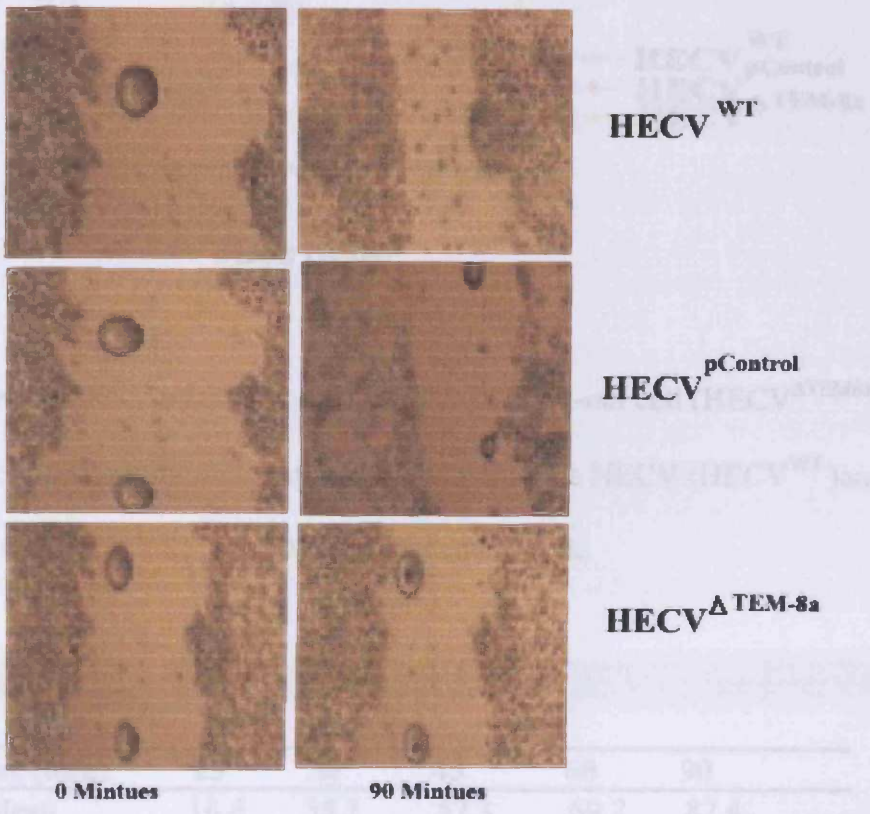


Figure 8.5. Migration assay pictures of different cell lines at the beginning and after 90 mintues showing the migratory capacity of $HECV^{WT}$, $HECV^{pControl}$ and $HECV^{\Delta TEM8a}$ cells.

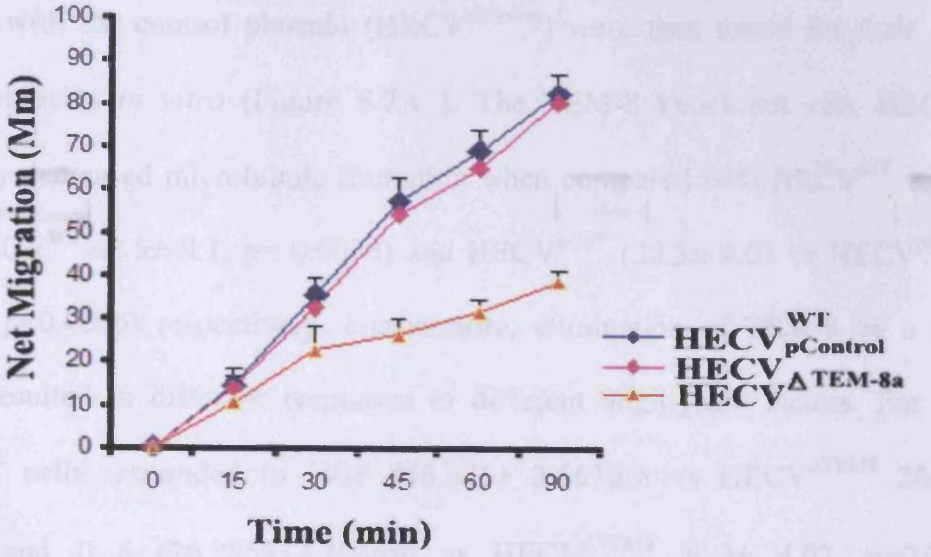


Figure 8.6. *In vitro* HECV migration assay. TEM-8 knock-out cell ($HECV^{\Delta TEM8a}$) significantly decreased migration compared to the wild type HECV ($HECV^{WT}$)and CFP control plasmid ($HECV^{pControl}$) . * $p<0.05$ by student's *t* test.

	Time (min)	15	30	45	60	90
$HECV^{WT}$	Mean	14.4	35.3	57.3	69.2	82.4
	SD	3.92	4.15	5.34	4.89	4.45
$HECV^{pContro}$	Mean	13.7	32.4	54.3	65.2	80.23
	SD	4.67	2.05	1.89	4.26	3.19
$HECV^{\Delta TEM8a}$	Mean	10.34	22.42	26.12	31.43	38.52
	SD	6.14	5.76	3.77	2.96	2.17

Table 8.3. Numerical data from the Migration assay.

8.3.5. Reduction of *in Vitro* tubule formation in TEM-8 knock-out Cells ($HECV^{\Delta TEM8a}$).

The TEM-8 knock-out cells ($HECV^{\Delta TEM8a}$), wild type cells ($HECV^{WT}$), and cells transfected with the control plasmid ($HECV^{pControl}$) were then tested for their ability to form microtubules *in vitro* (Figure 8.7A). The TEM-8 knock-out cell, $HECV^{\Delta TEM8a}$, significantly decreased microtubule formation when compared with $HECV^{WT}$ and (20.3± 4.03 vs $HECV^{WT}$ 42.5± 9.1, p= 0.0078) and $HECV^{pGFP}$ (20.3± 4.03 vs $HECV^{pGFP}$ 39.5± 10.1 μm , p=0.0086) respectively. Furthermore, elimination of TEM-8 by a ribozyme transgene resulted in different responses to different angiogenic factors. For example, $HECV^{\Delta TEM8}$ cells responded to HGF (56.321± 3.563μm vs $HECV^{\Delta TEM8}$ 20.3± 4.03, p=0.0095) and IL-8 (26.385±14.396μm vs $HECV^{\Delta TEM8}$ 20.3± 4.03, p=0.0581) by increasing tubule formation, but had lost their responsiveness to IL1 ß (21.434± 4.236μm vs $HECV^{\Delta TEM8}$ 20.3± 4.03, p=0.164) and VEGF (21.67± 3.55 μm vs $HECV^{\Delta TEM8}$ 20.3± 4.03,p=0.173) (Figure 8.7B). There was no difference in tubule formation between the $HECV^{WT}$ and $HECV^{pGFP}$ (42.5± 9.1 vs. control plasmid 39.5± 10.1 39.5± 10.1 μm μm p=0.306), respectively.

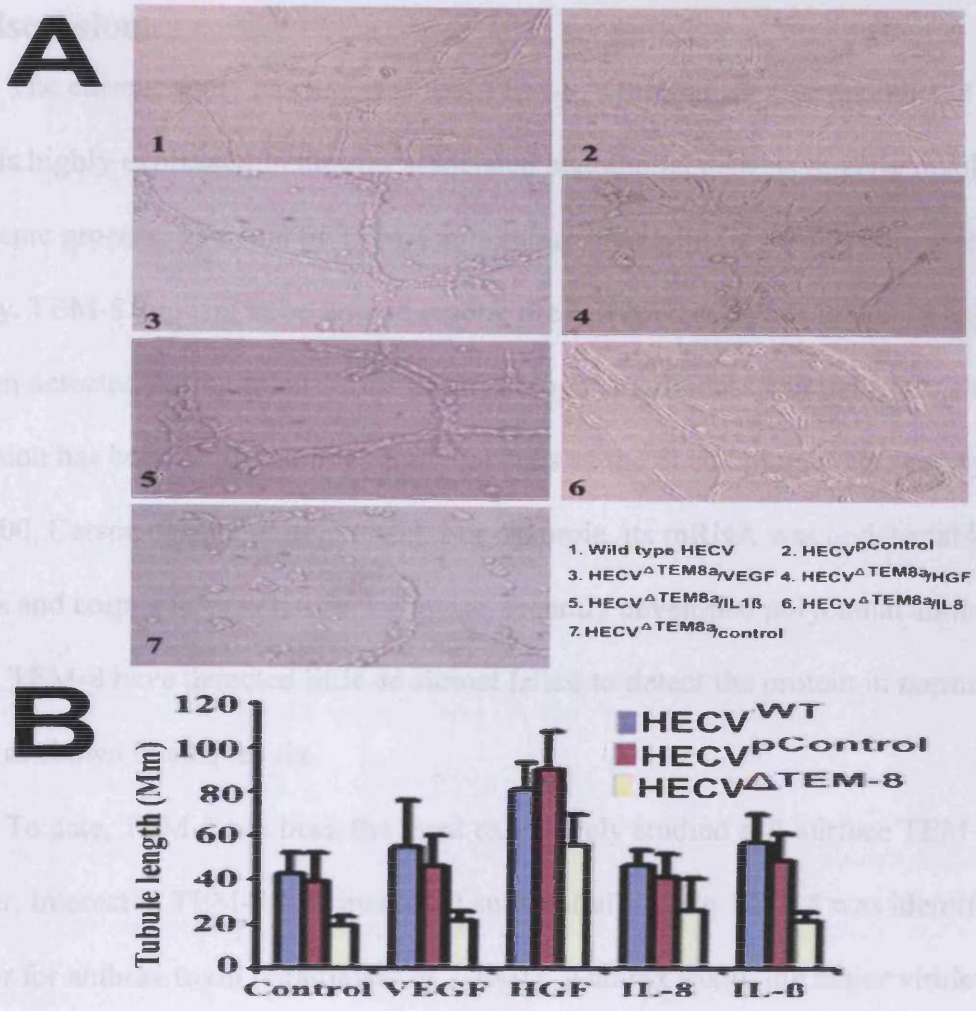


Figure 8.7. *In vitro tubule formation assay.* (A)TEM-8 knock-out cell ($HECV^{\Delta TEM8}$) significantly decreased the ability of HECV cells to form tubules (7) compared to wild type HECV cells ($HECV^{WT}$) (1) and GFP control plasmid ($HECV^{pControl}$) (2). $HECV^{\Delta TEM8}$ treated with HGF (4) and IL-8(6) showed increased in micro-tubule formation. In contrast $HECV^{\Delta TEM8a}$ cells treated with VEGF (3) and IL-1β (5) showed no significant change from TEM-8 knock-out cell ($HECV^{\Delta TEM8a}$) (7). (B) The bar graph represents the means of tubule length in each condition.

8.5 Discussion

The current study has reported that TEM-8, a tumour specific endothelial marker which is highly expressed in tumour associated endothelial cells, is directly involved in the angiogenic process. The role of TEM-8 in tumour angiogenesis involves the changes of cell motility. TEM-8 appears to be unique among the cell surface TEMs in that its expression has not been detected during other forms of physiologic angiogenesis in the adult, although expression has been observed in endothelial cells of the developing mouse embryo (Croix *et al*., 2000, Carson-Walter *et al*., 2001). For example, its mRNA was undetectable in healing wounds and corpus luteum tissue. Likewise, recently developed polyclonal antibodies against TEM-8 have detected little or almost failed to detect the protein in normal colorectal tissues as shown in chapter six.

To date, TEM-8 has been the most extensively studied cell-surface TEM family member. Interest in TEM-8 has increased substantially since TEM-8 was identified as a receptor for anthrax toxin (Bradley *et al*., 2001). Anthrax toxin, the major virulence factor produced by Bacillus anthracis, consists of three polypeptides called protective antigen, lethal factor, and oedema factor. Protective antigen mediates binding of the complex to TEM-8 whereas lethal factor and oedema factor are responsible for eliciting toxicity. Recently, capillary morphogenesis protein 2 (CMG2), the closest homologue to TEM8 , was identified as a second receptor for anthrax toxin (Scobie *et al*., 2003).

In the current study, we investigated the impact of genetically eliminating the expression of TEM-8 from endothelial cells on the formation of microtubules *in vitro*. Of the three ribozyme transgenes we constructed, one was highly effective in that it completely eliminated the expression of the transcript as revealed by RT PCR. Furthermore, it also substantially reduced the level of the protein, suggesting that the transgene was highly active and that the genetically modified cell generated served as a good cell model in functional based tests, such as the *in vitro* tubule forming assay and cell migration assay.

Indeed, using the TEM-8 knockout cells, ($HECV^{\Delta TEM8}$) cells resulted in a significant reduction in both tubule formation, and in cell motility compared with wild-type $HECV^{WT}$ and GFP control plasmid ($HECV^{pControl}$) cells respectively.

Furthermore, transfected TEM-8 HECV expressing cells ($HECV^{\Delta TEM8}$) did not display any change in cell proliferation compared with the $HECV^{WT}$, suggesting that the reduction in both tubule formation and in cell motility in $HECV^{\Delta TEM8}$ cells is unlikely to be that of reduced cell proliferation.This has indicated the following, firstly, TEM-8 is directly contributing to the formation of microtubules from endothelial cells, and secondly, TEM-8 is linked to the motile nature of endothelial cells, a cell function critical to the angiogenic process. An additional point extracted from these functional tests was that TEM-8 may selectively regulate angiogenic factor regulated tubule formation. For example, IL-1β upregulates TEM-8 and tubule formation, whereas, knock-out TEM-8 cells showed reduced or almost no responsiveness to IL-1β. However, the latter failed to impact on HGF and IL-8 induced micro-tubule formation, in both $HECV^{WT}$ and $HECV^{\Delta TEM8}$ which responded almost equally. Thus, TEM-8 is an important tumour endothelial marker and contributes to the angiogenic process. This has raised important questions as to its candidacy in anti-angiogenic therapies in cancer. The highly specific expression of the molecule in cancer has indeed made it an ideal target as recently suggested (Croix *et al* ., 2000, Carson-Walter *et al* ., 2001). Indeed, the current study has already shown that ribozyme transgene can be effective in this aspect. Other factors, such as neutralising antibodies, siRNA (bearing similarities to the ribozyme approach in the current study), and indeed a modified version of anthrax toxin may prove to be highly valid options.

We conclude that, TEM-8, a tumour specific endothelial marker, is a regulator of angiogenesis *in vitro*. Targeting the TEM-8 by way of hammerhead ribozyme encoding antisense to TEM-8, is an effective method to reduce the micro-tuboule formation and migration potential of endothelial cells, and may have important therapeutic implications.

CHAPTER NINE

THE POTENTIAL ROLE OF THE TEM-8 vW/TM DOMAINS IN TUBULE FORMATION.

9.1 INTRODUCTION

The survival of tumours and thus their metastases are dependent on the balance of endogenous angiogenic and antiangiogenic factors such that the outcome favours increased angiogenesis. Targeting the endothelial cells that line tumour vessels is a promising anticancer strategy that has generated widespread excitement among biologists and clinicians (Kerbel and Folkman. 2002). However, most angiogenic factors, angiogenic markers and intended targets are not entirely specific to tumours, i.e. they exist in both normal and tumour tissues, although may be more prevalent in the later. Targeting these molecules would undoubtedly bring side effects that are naturally associated with influencing these molecules in normal circumstance (tissue and organs).

TEM-8 is of particular interest also because of its cell–surface localization, conservation in mice, and unique pattern of expression(Carson-Walter *et al .*, 2001). TEM-8 is a type I transmembrane protein 564 amino acids in length, and has been postulated to be involved in the interaction of cells with the surrounding extracellular matrix(Van der Vieren M*et al .*, 1999, JO*et al .*, 1995), and has been indicated to be potentially angiogenic.

Recently, capillary morphogenesis protein 2 (CMG2), the closest homologue to TEM-8, was identified as a second receptor for anthrax toxin(Scobie *et al .*, 2003) . The extracellular region of both receptors contains a von Willebrand factor type A domain. A metal ion-dependent adhesion site resides within the von Willebrand factor type A domain and appears to be necessary for interaction with protective antigen(Bradley *et al .*, 2003).

As shown in the previous chapter, knock out of TEM-8 from human endothelial cells (HECV) significantly reduced tubule formation, and tumour endothelial marker-8 (TEM-8) has been found to be selectively up regulated in tumour-associated endothelial cells.
Thus TEM-8 is implicated in tumour specific angiogenesis. However, its molecular mechanism in angiogenesis is not defined. In the current study, we have shown that, the key

role of the vW domain together with transmembrane domain of the TEM-8 in the angiogenesis process.

9.2 MATERIALS AND METHODS

9.2.1 Materials

Chinese Hamster Ovarian Cells (CHO) was obtained from ATCC's Cell Biology Collection, Middlesex, UK. Cells cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma)(PH 7.3) containing; 2Mml-gluamine, 15Mmhepes and 4.5mM$NaHCO_3$, DMEM was supplemented with 10% heat inactivated foetal calf serum (PAA Laboratories, Yeovil, England, UK), 50units/ml of Benzopenicillin (Britannia Pharmaceuticals Ltd) and 50µg/ml of streptomycin (Gibco BRC, Paisley, Scotland). Matrigel (extracted from Engelbreth-Hom-Swarm (EHS) sarcoma) was purchased from Collaborative Biochemical (Bedford, MA). RNA extraction and reverse transcription kits and PCR mix were purchased from Abgene (Surrey, England, UK).

9.2.2 Methodology

9.2.2.1 Construction of expression cassettes for various TEM-8 Domains

As shown in Figure 9.1, a series of primers were designed in order to generate various fragments (domains) and full length cDNA for TEM-8, the vW domain, ED (the extracellular domain), ED-TM (Extracellular domain plus transmembrane domain), and ICD (intracellular domain and FL (full length). Primer sequences are given in (table 9.1) and similarly synthesised by (Invitrogen life technology). cDNA template from HUVEC cells were used for generating the domain, by using PCR. Following verification of each product, each domain of TEM-8 was T-A cloned into a mammalian expression vector (pEF6/V5-His) and followed by transformation of E.coli. The colonies with the corrected

insert were selected using orientation specific PCR. Plasmid was extracted using the standard plasmid extraction kits. Following re-verification using PCR and restriction digestion, the Purified plasmid or the respective control plasmids were used to transfect CHO cells, using an electroporator (V.340 v). Cells were immediately transferred to complete medium pre-warmed to 37°C and plated into 25-cm^2 tissue culture flasks. After 24 h, selection began with Blasticidin S HCl at 10μg/ml, and medium was changed every 3–4 days. After 3 weeks of culture, the cells were changed to maintenance medium (with 2μg/ml Blastmyocin) and grown to sufficient number for experimental studies.

primers	Sense primer (5' – 3')	Antisenes primer (5' – 3')
TEM-8vW	attggccacggcggagggagagccctcgg	aattgagtggatgatgccttg
TEM-8TM	attggccacggcggagggagagccctcgg	agagtcccagatg
TEM-8 EDvW/TM	attggccacggcggagggagagccctcgg	ccagaaccaccagaggagag
TEM-8ED	attggccacggcggagggagagccctcgg	accgtcagaacagtgtgt
TEM-8IC	attggccacggcggagggagagccctcgg	tcccattcttttaatgcct
TEM-8CD	attggccacggcggagggagagccctcgg	gttgttcttgaccctggtg
TEM-8FL	attggccacggcggagggagagccctcgg	gacagaaggcctggaggag

Table 9.1. Primer sequences of the synthesized various TEM-8 domains.

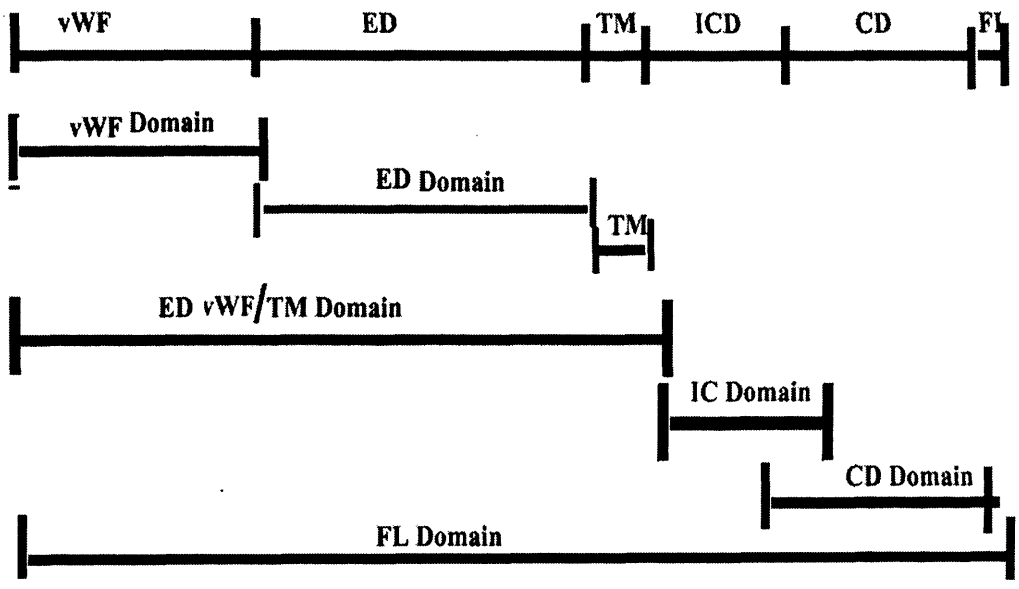


Figure 9.1. The graph shows the different designed TEM-8 Domains been synthesized.

9.2.2.2 Micro-tubule Formation Assay From CHO Transfected Cells.

This was based on a Matrigel-sandwich tubule forming assay developed in our laboratory (Jiang *et al*., 1999, Cai *et al*., 1999, Martin *et al*., 1999). Briefly, 200 µg of cold Matrigel solution in 100µl reconstituted basement membrane, (Becton-Dickinson, Bristol, England) was added to a 96 well plate and allowed to air-dry at 37°C. Following rehydration of Matrigel to allow formation of a thin bottom layer, wild CHO (CHO^{WT}), pEF6 control plasmid and various TEM-8 domain (CHO^{vW}, CHO^{TM}, $CHO^{vW/TM}$, CHO^{AE}, CHO^{AC}, CHO^{IC} and CHO^{FL} respectively) transfected CHO cells were seeded onto the Matrigel at 10,000 per well and allowed to attach for up to 2 hours. The medium was carefully removed. The second solution of Matrigel was added to the cells, followed by incubation at 37°C for 3 hours when the second layer of matrigel solidified. Medium was added over the second Matrigel and the cells were then incubated at 37°C for 24 hrs. Microtubules were visualised microscopically and photographed using a digital camera. The length of tubules in a fix-sized frame was quantified using the Optimas-6 software, as previously described (Jiang *et al*., 1999, Martin *et al*., 1999).

9.3 RESULTS

9.3.1 Generation of Expression Domains Using RT-PCR.

The designed TEM-8 domains after been synthesised, their expression been checked by RT-PCR using HECV cells. Strong signal of variant domains of TEM-8 with right size of the expression shown in HECV cell (Figure 9.2).

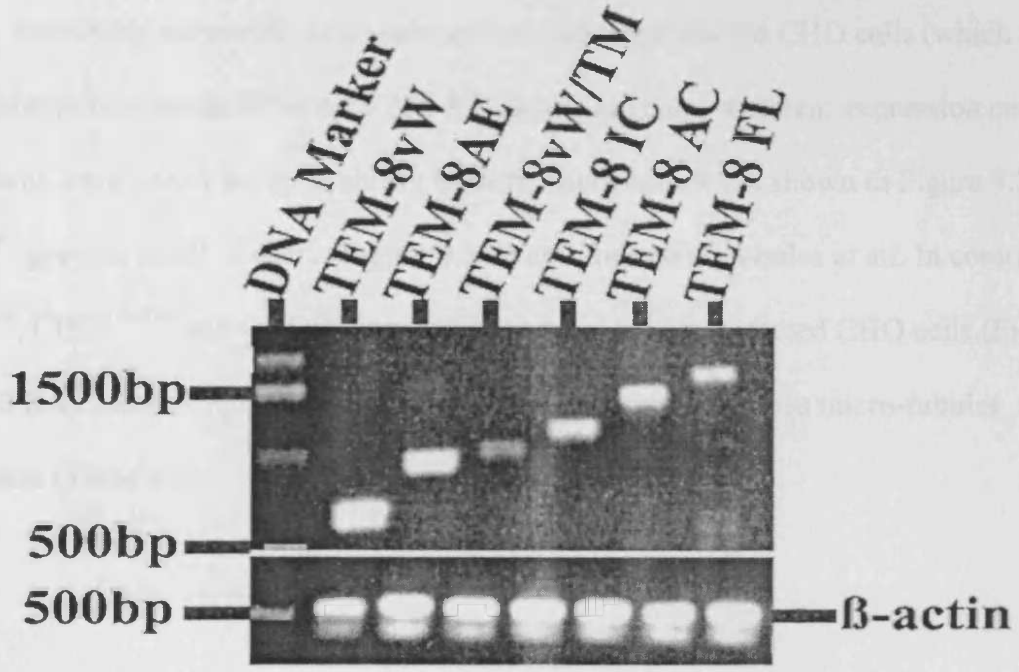


Figure 9.2. RT-PCR shows the strong expression with correct size of various TEM-8 domain primers synthesized.

9.3.2 vW domain with Transmembrane domain Portion of TEM-8 have a role in the Micro-vessels formation of CHO cells.

Following successful establishment of stably transfected CHO cells (which do not form tubules or express different TEM-8 Domains),to carry different expression cassettes, these cells were tested for their ability to form micro-tubules as shown in Figure 9.3. CHO^{WT} grew in small clusters (Figure 9.3-9) and formed no tubules at all. In contrast, CHO^{vW}, $CHO^{vW/TM}$ and CHO^{FL} formed micro-tubules in transfected CHO cells (Figure 9.3-1, 3 &6). Interestingly, CHO^{TM} ie TM domain alone show less micro-tubules formation (Table 9.2).

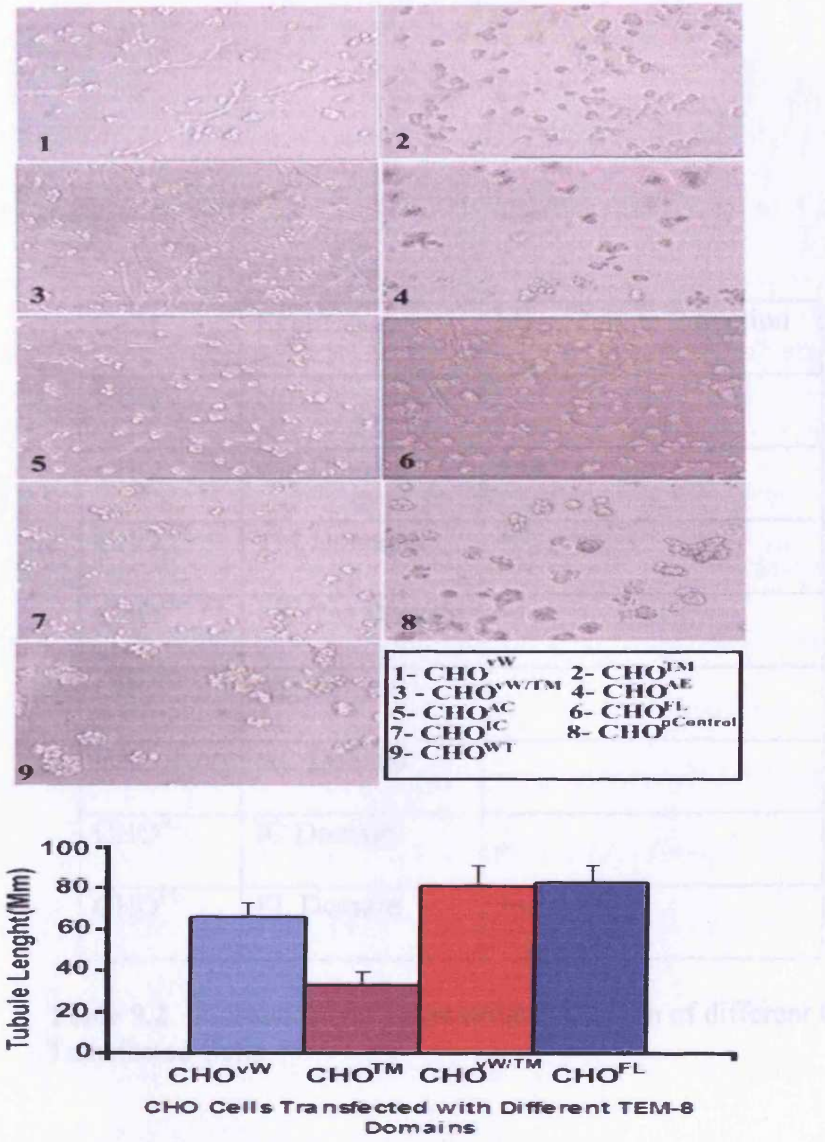


Figure 9.3. ***In vitro*** **tubule formation assay**. vW Domain of TEM-8 induces the ability of CHO to form micro-tubules (1). Expression constructs including the vW together with TM domains (2&3) and FL domain (6) increased micro-tubule formation in CHO cells. On other hand, the wild CHO (9) and PEF6 control plasmid (8) together with other transacted TEM-8 domains (AE (4), AC (5) and IC (7) show no micro-tubule formation.

Cells	Expressiom	Micro-tubule formation
CHO^{WT}	N/A	–
CHO^{vW}	vW Domain	+++
CHO^{TM}	TM Domain	+/-
$CHO^{vW/TM}$	vW/TM Domain	++++
CHO^{AE}	AE Domain	–
CHO^{AC}	AC Domain	–
CHO^{IC}	IC Domain	–
CHO^{FL}	FL Domain	+++++

Table 9.2. Expression and microtubles formation of different CHO Transfected Cells.

9.4 Discussion

Angiogenesis is a complex process involving multiple endothelial cell activities working in concert to permit blood vessel formation. Key processes that contribute to angiogenesis include endothelial cell migration, proliferation, apoptosis, and differentiation(Zachary 2003). The current study has reported that TEM-8, a tumour specific endothelial marker which is highly expressed in tumour associated endothelial cells, is directly involved in the angiogenic process through its vW domain.TEM-8 has some very interesting features in its protein structure. It has a vW domain, which has been indicated in the vasculature integrity (Nanda *et al*., 2004). It also shares homology with CMG-2, a protein known to regulate the morphology of capillaries(Scobie *et al*., 2003). These properties, together with the observations in the study have prompted us to further examine the potential role of the protein domains in tubule forming activities of TEM-8. Selection of the domains was based on the following consideration, to include the vW domain without TM domain as vW may act as a secreted protein, construct the vW by inserting the TEM-TM domain, in order to test the necessity of TM domain, the intracellular domain to test if intracellular part of the protein is valid and full length TEM-8.

For these expression cassettes, we chose CHO to test for the following reasons: first CHO is superb in protein expression; second, CHO does not express TEMs; and third, CHO does not form tubules in the experimental conditions used here. The current study has shown that the vW part of TEM-8 contributes the main pro-angiogenic function of TEM-8 and this domain needs to act on the cell surface, as vWTM has resulted in substantially more tubule forming from the CHO cells.

Von Willebrand factor (vWF) is defined as a multimeric glycoprotein, and is synthesized exclusively in endothelial cells and megakaryocytes and stored in Weibel-Palade bodies in ECs and in platelet-granules(Mannucci 1995).vW factor has been used as

a confirmatory marker for vascular differentiation, particularly in well-differentiated tumours (Millard PR and AR. 1985). Vessel density in tumour specimens, as determined by immuno-histochemical staining for vWF or other endothelial cell markers, is a prognostic factor for selected solid tumours such as colon cancer. vWF is heterogeneously distributed throughout the vasculature. Transcriptional control in response to the tissue microenvironment is thought to be responsible for local variations in endothelial cell levels of vWF(Millard and Heryet 1985).

The effect of TEM-8 on the endothelial phenotype is highly specific. As been showed in previous chapters TEM-8 expression primarily influences endothelial cells motility (chemotaxis and chemokinesis) on collagen, and has no effect on proliferation or survival. TEM-8 is sufficient and necessary to stimulate migration on collagen. One of the most important molecular regulators of migration is cell–matrix interactions. Indeed, antagonism of such interactions prevents neovascularization(Byzova *et al .*, 2000, Friedlander *et al .*, 1995). The presence of an extracellular vWA domain in TEM-8 indicated that altered cell–matrix interactions were a likely mechanism by which TEM-8 stimulated migration and tuble formation.

These observations may also help to explain the tumour regression observed when anthrax toxin is injected into tumour-bearing mice at nontoxic doses (Liu *et al .*, 2003, Duesbery *et al .*, 2001, Koo *et al .*, 2002). Before the identification of TEM-8 as the anthrax toxin receptor, inhibition of angiogenesis was postulated to mediate the antineoplastic effects of anthrax toxin, because the treated tumours appeared "white" and were found to be deficient in CD31-positive blood vessels(Duesbery *et al .*, 2001) . Given the TEM-8 protein expression patterns in tumour endothelium described here, it seems likely that TEM-8 is responsible for this effect.

We conclude that TEM-8, a tumour specific endothelial marker, is a regulator of angiogenesis in vitro, potentially via its vW/TM domains. Targeting the TEM-8 by way of neutralising antibodies to this domain, may be an effective method to reduce micro-vessel formation and may have important therapeutic implications. Together with observations in colon cancer that TEM-8 is excessively expressed in tumour endothelial cells, but not in endothelial cells from the normal tissues, the validity of TEM8 as a target in antitumour and anti-angiogenesis therapies should be explored.

CHAPTER TEN

GENERAL DISCUSSION.

Metastatic spread is a common feature of almost all malignant neoplasms (Hanahan and Weinberg 2000). It is well established that one of the initial metastatic routes for carcinomas is the vascular system (Pepper 2001, Weaver 2001). The incidence of cancer metastasis and angiogenesis are closely linked and tumour angiogenesis is associated with poor prognosis (Bricknell and Harris. 1991, Chodac *et al*., 1980).Vascular invasion and metastasis is considered to be a major determinant of prognosis in almost all solid cancers.

Angiogenesis, defined as the sprouting of new capillaries from pre-existing vessels, is characterized by expansion of the endothelium by proliferation, migration and remodelling. Angiogenesis is a key to cancer development and particularly metastasis (Kerbel and Folkman. 2002). Despite the rapid progression in understanding the biology and the clinical significance of angiogenesis, there has been very little information on markers that are specific to tumour endothelium until recently, when biological markers 'specific only' to tumour-associated endothelium were reported. This has made studying tumour specific angiogenesis feasible.

Expression of VEGF family members and their receptors in colon cancer.

The first part of the current study examined the expression and pathological prognosis of a number of angiogenic factors namely VEGF-A,-B,-C and VEGF-D and their receptors VEGF-R1,-R2 and VEGF-R3, as well as the recently identified tumour endothelial markers (TEM1-9) (Croix *et al*., 2000). The latter part of the study had two main purposes: firstly to investigate the scope of the abnormalities, if any, of angiogenic factors and their receptors and markers linked to tumour related angiogenesis and secondly, to identify the key molecule(s) that may have potential value in predicting prognosis and in molecular targeting.

Also for the first time, the study has employed a quantitative approach to studying the transcript of the newly identified family of tumour endothelial markers (TEMs) in colorectal cancer tissues and correlated that with tumour stage.

The results have shown that VEGF-B, VEGF-C and their receptors VEGF-R1 (Flt-1) and VEGF-R2 (KDR) mRNA were expressed at a much higher level in colorectal tumours than in normal mucosa. Although VEGF-A, VEGF-D and FEGF-R3 (Flt-4) expression were not significantly different between colorectal cancer and normal tissues, their levels were higher in early stage of colon cancer ie, Dukes A without lymph node involvement. VEGF-B and VEGF-R2 levels were found significantly higher in colorectal cancer with regional lymph node involvement (Dukes C). Levels of VEGF-C and VEGF-R1 were similar in Dukes B and Dukes C tumour.

These data strongly point to the fact that, the expression of angiogenic factors in the VEGF family are widely aberrant. This occurs together with the aberrant expression of their receptors. These changes have indicated that these factors and their expression pattern have potential prognostic value. For example, high levels of VEGF-B and its receptor VEGF-R2 and VEGF-C may indicate regional involvement and aggressive tumours. Limited by the size of the cohort of the current collection and limited information on followup, these data were not able to provide information of these factors and clinical outcome/survival. A large sample size with longer followup would certainly assist to clarify this issue.

Expression of TEMs in clonical colon cancer and the TEM-8 context.

Our study has shown that TEMs are elevated in colorectal cancer tissues compared with normal background tissues. Although in the current study TEM-2 and TEM-6 are expressed at a very high level in colorectal cancer tissues, they may not be clear indicators for assessing the degree of tumour angiogensis in colorectal cancer, since almost over half

the normal tissues screened in this study were also positive for TEM-2 and TEM-6. On the other hand, TEM-1, TEM-7, TEM-7R and TEM-8 appear to be superior tumour endothelial markers, since their expressions were significantly higher in tumour tissues, with a tiny proportion of normal tissues being positive. Although TEMs were initially thought to be specific only to endothelial cells in tumour tissues (Croix *et al .*, 2000, Eleanor *et al .*, 2001), this result failed to be reproduced in the current study. Instead, only TEM-8 was found to be almost absent in normal tissues, but significantly raised in colon cancer tissues. The potential reason(s) for the inconsistency are not clear.

However, a number of possibilities exist. Firstly, *in-vitro* and *in-vivo* differences; the initial discovery of TEMs and the difference between normal and tumour associated endothelial cells were based on cells isolated from tissues. These isolated (*ex-vivo*) cells have lost their contact with the neighbour cells and environment and may behave differently. Secondly, factors derived from tumours and normal tissues may vary *in-vitro* and *in-vivo*. Thirdly, potential differencs in message and protein expression *in-vitro* and *in vivo*. Despite the lack of information and further evidence from the literature, the same conclusion has been reached in a separate study to this in breast cancer (Davies *et al .*, 2004, Davies *et al .*, 2005), in that TEM-8 was highly expressed in breast tumours and virtually absent in normal mammary tissues.

Taken together, we conclude that TEM-8 is one factor that is uniquely expressed in tumour associated endothelial cells. Furthermore, we decided to concentrate further study on TEM-8, with the following approach; firstly to raise an antibody to TEM-8 in order to investigate TEM-8 at protein level; secondly, to investigate the potential regulators of TEM-8 expression and to investigate the molecular implications of TEM-8 in angiogenesis. We also used a TEM-8 antibody for identification of micro-vessels in human colon cancer

Limited clinical studies from previous reports on TEMs, including TEM-8, were assessed only at the mRNA level (Croix *et al .*, 2000, Davies *et al .*, 2004), due largely to the

lack of antibodies to these newly discovered markers. By using our newly developed antibody to TEM-8, we found that TEM-8 expression was significantly elevated in colorectal cancer tissues at protein level ascertained by western blotting. In addition, the use of TEM-8 antibody identified more positive vessels in tumour tissues than in normal tissues, using imunohistochemistary. This is particularly interesting, as an endothelial marker that is non-specific to tumour associated endothelial cells, Factor-VIII related antigen (vonWillebrand Factor), failed to reveal more micro-vessels in tumour tissues. This provides further evidence that TEM-8 does indeed recognise micro-vessels (endothelial cells) that are specific to tumour angiogenesis.

Previously, St. Croix *et al* (Croix et al ., 2000) evaluated the expression of tumour endothelial marker transcripts at mRNA level by using RT-PCR in purified cultures of endothelial cells derived from both normal and tumour colon tissues. They found that the level of tumour endothelial markers was predominantly expressed in tumour endothelium, however, in normal endothelial cells the level of tumour endothelial markers were either absent, or barely detectable. In a later study conducted by Carson-Walter *et al* (Carson-Walter *et al* ., 2001), this result of elevated TEMs levels in colon cancer was corroborated using *in situ* hybridisation. These finding suggests that TEM-8 may play a pivotal role, via mechanisms yet to be identified, in tumour associated angiogenesis, making it an ideal endothelial marker for assessing the level of tumour specific angiogenesis.

IL-1β is a regulator of the expression of TEM-8; implications in angiogenesis.

Although TEM-8 is selectively upregulated in tumour associated endothelial cells, there has been no information to indicate the regulatory pathways for its increase in tumour tissues. Here, 13 different cytokines were screened, and only IL-1ß was found to notably increase expression of TEM-8 in HECV cells. IL-1β has been found to stimulate the

proliferation of vascular smooth muscle cells and is involved in modifying a number of vascular functions by inducing autocrine production of chemotactic cytokines on endothelial cells, including IL-1β itself (Libb *et al .,* 1988, Mantovani and Dejana 1989, Mantovani *et al .,* 1992). Moreover, IL-1β has been shown to enhance angiogenesis in melanoma cells (Koch *et al .,* 1992, Gutman 1995). IL-1β increased VEGF mRNA in rat aortic smooth muscle cells in a time and dose-dependent manner (Li *et al .,* 1995, Akagi *et al .,* 1999). These findings suggest that IL-1 β is an angiogenic factor.

Anthrax toxin, the major virulence factor produced by *Bacillus anthracis,* consists of three polypeptides known as protective antigen, lethal factor, and oedema factor. Protective antigen mediates binding of the complex to TEM-8 whereas; lethal factor and edema factor are responsible for eliciting toxicity. Lethal factor (LF) of *Bacillus anthracis* is known to induce the production of IL-1 β and tumour necrosis factor (TNF-α) from macrophages (Hanna *et al .,* 1993).

Thus a possibility exists that IL-1β and LF can mutually regulate the expression of each other, via TEM-8 (Antrax receptor), in that LF stimulate the production of IL-1β which in turn upregulates the expression of TEM-8 (anthrax receptor), thus allowing amplification of the LF signal and enhancing angiogenesis. Given that the prime sources of IL-1ß in tumour tissues are immune cells and stromal cells, this may be an important pathway by which infiltrating immune cells and stromal cells stimulate angiogenesis in malignancies. Therefore, it was concluded from this initial study that TEM-8 might have an impact on the process of angiogenesis via IL-1β signals, and further investigations were carried out using this cytokine.

TEM-8 is a critical molecule in tubule forming from endothelial cells.

Although TEM-8 is critically raised in tumour associated endothelial cells, it is not clear if this molecule is critical to angiogenesis, or merely a marker that is upregulated in

tumour tissues. The current study created a cell model to test the hypothesis, that as well as being a marker, TEM-8 is also an important angiogenesis related molecule. We constructed a ribozyme transgene which allowed elimination of the expression of TEM-8. Using the cell model (TEM-8 null cells), we have shown that loss of TEM-8 from HECV cells resulted in significant reduction in both tubule formation and in cell motility. This has indicated that TEM-8 is directly involved to the formation of microtubules from endothelial cells, and is also linked to the motile nature of endothelial cells, a cell function critical to the angiogenic process.

Moreover, no significant change was seen in growth between TEM-8 Knock-out HECV, Wildly HEVC and GFP control plasmid cells, further suggesting that TEM-8 is an angiogenesis related factor, rather than a growth -related molecule in endothelial cells.

Furthermore, given the results that the TEM-8 knock-out only affected IL-1β induced tubule forming, but did not influence that of HGF and IL-8, it can be concluded that TEM-8 is key to IL-1β induced angiogenesis.

TEM-8 and its molecular structure in angiogenesis.

One of the critical questions related to TEM-8 is whether the structure of TEM-8 is related to the angiogenic effects of the molecule and, if so, which part is responsible. TEM-8 is a type I transmembrane protein 564 amino acids in length, and has been postulated to be involved in the interaction of cells with the surrounding extracellular matrix (Van der Vieren *et al* ., 1999, JO *et al* ., 1995), and has been indicated as potentially angiogeneic. The extracellular region of TEM-8 contains a von Willebrand factor type A domain. A metal ion-dependent adhesion site resides within the von Willebrand factor type A domain, and appears to be necessary for interaction with protective antigen (Bradley *et al* ., 2003).

We first constructed a series of expression vectors that allowed expression of different domains of TEM-8 including the full length of the molecule. We then chose CHO

cells as a recipient for these expression vectors. The selection of CHO cells was based on: (a) CHO is superb for protein expression; (b), CHO do not express TEMs; and (c), CHO do not form tubules *in-vitro*. Our results have shown that the vW part of the TEM-8 contributes to the angiogenic function of TEM-8. Furthermore, these domains need to act on the cell surface, as vW/TM resulted in substantially more tubule forming from transfected CHO cells.

The vWF/TM domain may provide a potential and selective target for anti-angiogenesis in tumours. The validity of TEM-8 as a target is supported by several recent studies using anthrax toxin as an antitumor agent. The expression pattern of TEM-8 may help to explain the tumour regression observed when anthrax toxin is injected into tumor-bearing mice at nontoxic doses (Liu *et al*., 2003, Duesbery *et al*., 2001, Koo *et al*., 2002). This idea is interesting and may provide further explanation to the phenomena observed. Before the identification of TEM-8, it was observed that anthrax toxin (at non toxic dose) had anticancer effects and that 'treated tumours' appeared "white" and were deficient in CD31-positive blood vessels (Duesbery *et al*., 2001). Given the TEM-8 protein expression patterns in tumour endothelium described here, it seems likely that TEM-8 is responsible for this effect.

However, the current study has a number of weakness that I was not able to address during the period of the research. First, the cohort of the colorectal tissues used in the study was small. The follow-up was not fully available. This was due to the fact that the collection was made a few years ago and that a number of samples were already used up in previous studies in the Department. A larger collection would undoubtedly help to clarify the statistical weakness as seen in part of this study. Second, the findings of the current study have not had support from in vivo models. This is due largely to the time restrain and HO regulations on this issue. An interesting approach would be to establish a tumour model using TEM-8 modified cells and to evaluate the angiogenic aspect of the tumours.

Potential questions and future studies.

This study has highlighted the effects of TEM-8 on angiogenesis; However, further queations required to be answered.

How does IL-1β up-regulate TEM-8 over-expression? If it occurs at gene transcription level for example, then it would be interesting to know whether using a transcription blockade could interrupt this pathway. TEM-8 is an important tumour endothelial marker and contributes to the angiogenic process. This has raised important questions as to its candidacy in anti-angiogenic therapies in cancer. The highly specific expression of the molecule in cancer has indeed made it an ideal target as has recently been suggested (Croix B ST *et al* ., 2000, Carson-Walter *et al* ., 2001). What would the effects be of using of TEM-8 inhibitors like neutralising antibodies or selective anthrax receptor blockades on angiogenesis?

Although this study has highlighted the effects of TEM-8 on angiogenesis, there is clearly a need for more work to be done in this area in order to fully understand the role of this gene as a regulator of this process. Prevention of metastasis of cancers, including colorectal cancer, remains a long-term goal.

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Appendix 1: Reagents, buffers and solutions

Preparation of reagents and solutions

Preparation of Amido Black

- *Working Solution*: Dissolve 0.1g in Napthalene black, 10ml Acetic acid and 25ml Ethanol made up to 100ml with distilled water. Store at room temperature.

Preparation of Amido Black black destain

- *Working Solution*: Mix 100ml acetic acid and 250ml ethanol and make up to 1 litre with distilled water.

Preparation of 10% Ammonium Persulfate (APS)

- *Working Solution*: Dissolve 1g of Ammonium persulfate in 10ml of distilled water. Store at 4°C

Preparation of Antibodies

- *Stock Solution*: Antibodies supplied from the manufacturer are dilution in 0.1% BSA in BSS buffer and stored in 100µl aliquots at -20 °C.

Preparation of 10% Blocking Solution:

- *Working Solution*: Dissolve 10g of semi-skimmed milk powder, 100 µl (0.1%) of Tween 20 in 100ml of TBS working buffer. Make fresh as required.

Preparation of 1% Bromophenol Blue

- *Working Solution*: Dissoolve 0.1 g Bromophenol Blue in 10ml of 70% Ethanol.

Preparation of BSS buffer

- *Working Solution*: Dissolve 794.6 g of NaCl, 22.35g KCl, 21.45 g KH_2PO_4, 113.6g Na_2HPO_4 in 10 Litres of distilled water. Store at Room temperature.

Preparation of Complete Cell culture medium

- 500 ml DMEM/F12 with 2mM L-glutamine (Sigma)
- 50ml of FCS
- 250 µl of Penicillin and 200 µl of streptomycin.
- Store up to a month at 4°C

Final concentrations of Penicillin and Streptomycin are 100 Units/ml and 100 µg/ml respectively.

Preparation of Cell Lysis buffer

- *Stock solution:* Cell Lysis Buffer is prepared as a 2x concentrate. Dissolve 0.48 g Tris-Cl, 0.87 g NaCl, 2ml of Triton x-100, 0.5g Sodium deoxycholate, 0.02 g Sodium azide, 0.27 g Sodium orthovanadate in 100 ml of distilled water. Store at 4 °C.
- *Working solution:* A working solution is prepared by diluting 5 ml of the stock solution in 3.33ml of Inhibitor buffer, 100 µl of PMSF, 40µl 10mM CaCl, 1.5ml of 10% Triton X-100 made up to 10ml with distilled water.

Preparation of 0.1% Comassie Blue

- *Working Solution*: Dissolve 1.0 g Comassie, 400ml Methanol and 100ml Acetic acid in a final volume of 1 litre of distilled water. Filter and store at room temperature.

Preparation of DiI

- *Stock solution*: Dissolve 50mg DiI in 5ml DMSO (dimethylsulphoxide) to give a 5mM stock solution. Store in 500µL aliquots at -20°C in the dark.
- *Working solution*: A working solution is prepared by adding 100 µL of stock solution to 10ml of DMEM/F12.

Preparation of 0.5 M EDTA

- *Working solution:* Dissolve 18.6g EDTA in 100ml of distilled water. Stir vigorously and adjust pH to 8.0 with NaOH.

Preparation of Ethidium Bromide (EtBr) Staining Solution

- *Working solution*: Dissolve 0.1g Ethidium Bromide in 10 ml distilled water. Mix well and wrap in aluminium foil.

Preparation of fluoroscein

- *Working solution:* Dissolve 3mg in 10ml of acetone. Prepare fresh.

Preparation of G418 Selection Media

- *Working solution*: Mix 250 of G418 (200 mg/ml) to a bottle of complete cell culture medium. Use 75 µl of G418 for maintenance medium Protect from light by wrapping in aluminium foil.

Preparation of Hydroquinone (10 mM)

- *Working solution:* Dissolve 0.011g in 10ml of distilled water. Prepare fresh.

Preparation of Inhibitor Buffer

- *Stock solution:* Inhibitor buffer is prepared as a 3x concentrate. Dissolve 2.76 g sodium nitrate, 5.58 g EDTA, 630 mg sodium Fluroide, 10g Na_2H_2PO4, 300,000 Units of Aprotonin madde up to 1 litre with distilled water. Store at 4 °C.

Preparation of Loading dye

- *Working solution*: Loading dye is prepared as a 10 x concentrate. Dissolve 0.125g Bromophenol Blue, 20g sucrose in 50ml of distilled water. Dispense to microcentrifuge tubes and freeze until needed.

Preparation of 4% Paraformaldehyde

- *Working Solution*: Dissolve 4g of Paraformaldehyde in 90 ml of distilled water. Heat in water bath at 75^{O} C for 2-3 hours, stirring occasionally. Once dissolved, allow the solution to cool before adding 10 ml of concentrated PBS (dissolve 1 PBS tablet in 10mls distilled water). Store at 4°C.

Preparation of Ponceau S

- *Working Solution:* Mix 20ml of Ponceau S concentrate (Sigma) in 180ml of distilled water. Store at Room temperature.

Preparation of Proteinase K

- *Working Solution:* Dissolve 10mg in 0.5 ml of distilled water. Dispense to microcentrifuge tubes and freeze until needed.

Preparation of Reaction Buffer

- *Working Solution:* Mix 5ml of 10% SDS, 1ml of 1 M Tris, 1 ml of).5 M EDTA and make up to 100ml with distilled water. Prepare fresh.

Preparation of Running Buffer

- *Stock solution:* Running Buffer is prepared as a 10x concentrate. Dissolve 303 g Tris (Cl), 1.44 kg Glycine and 100 g SDS in 10 Litres of distilled water. Store at Room temperature.
- *Working solution:* A working solution is prepared by diluting 100 ml of the stock solution in 900ml of distilled water.

Preparation of Sample Buffer

- *Working Solution*: Sample Buffer is prepared as a 2x concentrate Mix 50ml 0.5M Tris-Cl (pH=6.8), 5 ml 2-mercaptoethanol, 20 ml glycerol, 20 ml 10% SDS, 2 ml 1% bromophenol blue and 3ml of distilled water. Keep at 4°C

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Preparation of SDS-PAGE Gels:

	Resolving gel composition for 10mls	
	8%	10%
Distilled water	4.6	4.0
30% Acrylimide/0.8% Bisacrylamide	2.7	3.3
1.5M Tris (pH=8.8)	2.5	2.5
10% SDS	0.1	0.1
10% APS	0.1	0.1
TEMED	0.006	0.004

	5% Stacking gel for 2mls
Distilled water	1.4
30% Acrylimide/0.8% Bisacrylamide	0.33
1.0M Tris (pH=6.8)	0.25
10% SDS	0.02
10% APS	0.02
TEMED	0.002

Preparation of 3M Sodium acetate pH5.2

- *Working solution:* Dissolve 40.82g sodium acetate (Trihydrate) in 8ml of distilled water (DEPC treated for RNA) adjust pH to 5.2 with glacial acetic acid make up to 100ml in distilled water.

Preparation of sodium bisulfite (3 M)

- *Working solution:* Dissolve 4.14g of sodium bisulfite in 8ml of distilled water adjust pH to 5.0 and make up to 10ml with distilled water. Prepare fresh

Preparation of sodium phosphate buffer

- *Working solution:* Dissolve 0.89g $Na_2HPO_4.2H_2O$ and 0.78g NaH2PO4.$2H_2O$ in 100ml of distilled water. Stir vigorously and adjust pH to 8.0 with NaOH. Store at room temperature.

Preparation of TAE Electrophoresis Buffer

- *Stock solution:* Running Buffer is prepared as a 10x concentrate. Dissolve 48.4g Tris-base, 11.4ml glacial acetic acid and 20ml of 0.5 M EDTA (pH=8.0) in a final volume of 1 Litre of distilled water. Store at Room temperature.
- *Working solution:* A working solution is prepared by diluting 200 ml of the stock solution in 800ml of distilled water.

- **Preparation of TBE Electrophoresis Buffer**
- *Stock solution:* Running Buffer is prepared as a 5x concentrate. Dissolve 540g Tris-Cl, 275g Boric acid and 46.5g of di-sodium EDTA (or 20ml of 0.5 M EDTA; pH8.0) in a final volume of 10 Litres of distilled water. Store at Room temperature. Discard any buffers that develop a precipitate.
- *Working solution:* A working solution is prepared by diluting 200 ml of the stock solution in 800ml of distilled water.

Preparation of TBS Buffer
- *Stock solution:* TBS Buffer is prepared as a 10x concentrate. Dissolve 121 g Tris-Cl, 400.3g NaCl in 5 Litres of distilled water. Store at Room temperature.
- *Working solution:* A working solution is prepared by diluting 500 ml of the stock solution in 4500ml of distilled water.

Preparation of TE buffer
- *Stock solution:* TE Buffer is prepared as a 10x concentrate. Dissolve 12.11 g Tris-Cl, 3.72g EDTA in 1 Litre of distilled water. pH to 8.0 with conc HCl . Store at Room temperature.
- *Working solution:* A working solution is prepared by diluting 2 ml of the stock solution in 18ml of distilled water.

Preparation of Transfer Buffer
- *Working solution:* Transfer Buffer is prepared as a 1x concentrate. Dissolve 15.15 g Tris-Cl, 72g Glycine and 1 litre of methanol in 4 Litres of distilled water. Store at Room temperature.

Preparation of 1M Tris-Cl (pH=8.8)
- *Working Solution*: Dissolve 60.6 g Tris in 400ml of distilled water then adjust pH to 8.8 using NaOH. Add water to make final volume of 500 ml. Store at Room temperature.

Preparation of 1.5M Tris-Cl (pH=6.8)
- *Working Solution*: Dissolve 90.8 g Tris in 400ml of distilled water then adjust pH to 6.8 using concentrated HCl. Add water to make final volume of 500 ml. Store at Room temperature.

Preparation of 1% Tween 20
- *Working solution*: Dissolve 100µl of Tween20 in 10mls of Phosphate-buffered saline (PBS).

Preparation of 3% Wash Solution:
- *Workinf Solution:* Dissolve 3g of semi-skimmed milk powder, 100 µl (0.1%) of Tween 20 in 100ml of TBS working buffer. Make fresh as required.

Qiagen Buffers

QIAGEN buffer	Composition
Buffer P1 (Resuspension Buffer)	50 mM Tris·Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A
Buffer P2 (Lysis Buffer)	200 mM NaOH; 1% SDS (W/V)
Buffer P3 (Neutralization Buffer)	3.0 M potassium acetate, pH 5.5
Buffer QBT (Equilibration Buffer)	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v) 0.15% Triton® X-100 (v/v)
Buffer QC (Wash Buffer)	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)
Buffer QF (Elution Buffer)	125 mM NaCl; 50 mM Tris·Cl, pH 8.5; 15% isopropanol (v/v)