VASCULAR ENDOTHELIAL GROWTH FACTOR IN BREAST CANCER AND NOVEL ANTI-ANGIOGENIC MECHANISMS OF TAMOXIFEN

A dissertation submitted for

the degree of Ph.D.

by

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work

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Abstract

Angiogenesis, the growth of new capillaries from preexisting blood vessels, is essential for tumour growth and the development of metastases. Vascular endothelial growth factor is the most potent angiogenic factor identified to date Tamoxifen has been shown to inhibit angiogenesis in a manner independent of the oestrogen receptor. The expression of VEGF in breast cancer, possible regulators, and evidence for a VEGF related inhibition of angiogenesis by tamoxifen was examined. BT474 and MDA-MB-231 cells are breast cancer cell lines of primary and metastatic origin, respectively. Both cell lines expressed VEGF protein and mRNA with the MDA-MB-231 cell line producing 5 fold more VEGF than the BT474 cell line VEGF protein was elevated in breast tumour tissue as compared to matched normal tissue. Serum VEGF levels were significantly elevated in patients with breast cancer compared to patients with benign fibroadenomas and age-matched healthy controls The expression of TGFβ-1 was found to be associated with the expression of VEGF in the serum and tumour tissue of breast cancer patients. TGFβ-1 increased expression of VEGF by both breast cancer cell lines. Tamoxifen significantly decreased serum levels of VEGF in breast cancer patients independently of oestrogen receptor status. Tamoxifen did not reduce VEGF production by either breast cancer cell lines or by macrophages, important contributors of breast tumour VEGF. Tamoxifen decreased monocyte transendothelial migration *in vitro* From these results we conclude that the expression pattern of VEGF suggest a role for this factor in breast cancer. TGFβ-1 is a regulator of VEGF expression in breast cancer. Tamoxifen reduces serum VEGF levels and in an *in vivo* situation this may be achieved by reducing endothelial migration and tumour recruitment of macrophages and, thereby reducing overall tumour production of VEGF.

Abbreviations

Ab	Antıbody
Abs	Absorbance
AFGF	acıdıc fibroblast growth factor
AP	Alkalıne phosphatase
ATCC	American Tissue Culture Collectıon
BCA	Bıcınchonınıc acid assay
BCIP	5-bromo-4-chloro-3-mdolyl phosphate
bFGF	basıc fibroblast growth factor
BSA	Bovıne serum albumın
cDNA	complementary deoxyrıbonucleıc acıd
CM	Condıtıoned medıum
CSF	Colony stımulatıng factor
DEPC	Diethyl pyrocarbonate
DMEM	Dulbelco's Modıfication Eagle's Medıum
DMSO	Dımethyl sulfoxıde
DNA	Deoxyrıbonucleıc acıd
DNase	Deoxyrıbonuclease I
dNTP	Deoxyrıbonucleosıde trıphosphates
DTT	Dithıothreıtol
EC	Endothelıal cell
ECACC	European Collectıon of Anımal Cell Cultures
ECM	Extracellular matıx
EDTA	Ethylenedıamıne tetracetic acıd
EGF	Epıdermal growth factor
EGM	Endothelıal growth medıum
ELISA	Enzyme lınked ımmunosorbent assay
ER	Oestrogen receptor
FCS	Foetal calf serum
HBSS	Hanks balanced salt solutıon
HCl	Hydrochloric acid
HMEC	Human mıcrovascular endothelıal cells
HRP	Horseradısh peroxıdase
HUVEC	Human umbilıcal veın endothelıal cell
IFN	Interferon
IGF-I	Insulin like growth factor I
IPA	Isopropyl alcohol

IL	Interleukın
PAGE	Polysacrylamıde gel electrophoresıs
PBS	Phosphate buffered salıne
PCR	Polymerase chaın reactıon
PD-ECGF	Platelet derıved endothelıal cell growth factor
PDGF	Platelet derıved growth factor
PMSF	Phenylmethylsulfonyl fluorıde
MOPS	3-[*N* - morpholmo]propanesulfonıc acıd
mRNA	Messenger rıbonucleıc acıd
MVD	Microvessel densıty
NBT	Nıtro blue tetrazolıum chlorıde
NP-40	Nonylphenoxy polyethoxy ethanol
PBS	Phosphate-buffered salıne
PAGE	Polyacrylamıde gel electrophorsıs
PCR	Polymerase chaın reactıon
PMSF	Phenylmethysulfonyl fluorıde
RNA	Rıbonucleıc acıd
RNase	Rıbonuclease
RT	Reverse transcrıptase
RT-PCR	Reverse transcrıptase - polymerase chaın reaction
RPMI	Roswell Park Memorıal Institute
SDS	Sodıum dodecyl sulphate
SSC	Standard sodıum cıtrate
SDS	Sodıum dodecyl sulfate
SDW	Sterıle dıstılled water
TAE	Trıs-acetate
TB	Terrıfic broth
TBS	Trıs-buffered salıne
TBST	Trıs-buffered salıne wıth Tween
TE	Tris EDTA
TEMED	*N,N,N',N'* – tetramethylethylenedıamıne
TGFα	Transformıng growth factor α
TGFβ	Transforming growth factor β
TMB	Tetramethylbenzıdıne
TNFα	Tumour necrosis factor α
UV	Ultravıolet

Units

bp	base pairs
°C	degress celsius
g	grams
hr	hours
µg	micrograms
µl	microlitres
µm	micrometers
µM	micromolar
M	molar
mA	milliamps
mg	milligrams
min	minutes
ml	mihhters
mM	millimolar
ng	nanogram
nm	nanometres
pg	picogram
sec	seconds
V	volts
w/v	weight per volume
v/v	volume per volume

Publications and presentations

Paper publications

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CHAPTER 1

INTRODUCTION

1.1 Cancer epidemiology

According to the World Health Organisation approximately 6.6 million people world-wide died of cancer in 1995 alone (WHO, 1996). Cancer is the second biggest killer with only heart disease killing more people each year. In Ireland there were nearly 20,000 cancer cases and approximately 7,500 cancer deaths in 1995 (Irish Cancer Registry, 1996). In this country the risk of developing cancer before 75 years is 39% for men and 35% for women while the risk of dying of cancer before 75 years is 17% for men and 13% for women

World-wide, breast cancer is the commonest malignancy amongst women with approximately 570 000 new cases each year (McPherson et al., 1994). In Ireland it is the most common cancer in women accounting for 16% of all cancer cases. There were over 1,500 cases and over 600 deaths from breast cancer in Ireland in 1995 Currently, the risk of developing breast cancer before the age of 75 is 7.3% and the risk of death before 75 years is 2.8%. The mortality rate for patients diagnosed with breast cancer is 41%.

1.2 Pathophysiology of breast cancer

Each breast is composed of 15 - 20 lobes, each of which then has a number of lobules. The lobules are connected to lobes via ducts. The most common form of breast cancer is ductal cancer, comprising 65% - 80% of all cases (American Joint Committee on Cancer, 1992). Lobular cancer is the second most common and there are a variety of other less common pathologies. Nearly all breast cancer is of epithelial origin although there are some rarer forms derived from connective tissue. Metastasis is a major feature of breast cancer. The most common site of metastases is to the axillary lymph nodes and other sites including the brain, liver, skeleton, skin and lungs. Approximately 60% of breast cancer cases are oestrogen receptor (ER) positive Oestrogen is believed to be mitogenic to all early stage breast cancer but as the disease advances many tumours lose this dependency and no longer express the oestrogen receptor.

1.3 Aetiology and treatment

No single factor has been definitively shown to initiate the malignant transformation of normal breast tissue. Epidemiological studies have identified a number of risk factors including age, early onset of menarche, late onset of menopause, elderly primi para (late

age of first birth), positive family history, exposure to ionising radiation, obesity and a high fat intake and previous benign disease. Up to 10% of cases are thought to be due to genetic predisposition. Most of these cases are due to inherited mutations in the BRCA1, BRCA2 and p53 genes (McPherson et al., 1994).

Breast cancer is a very heterogeneous disease and the type of therapy chosen will depend on a number of factors including the stage and histology of the lesion, age, menopausal status and overall health. At present the main treatment options are surgery, radiation therapy, chemotherapy and hormonal therapy. Surgical removal of the tumour is the most common treatment and is usually accompanied by adjuvant hormonal or chemo-therapy.

1.4 Cancer growth

The number of cells within a multi-cellular organism is tightly controlled by a complex mechanism of signals that control the proliferation, differentiation and survival of individual cells. Deregulation of these cellular processes may result in changes in the number of cells. In cancer a single cell clone undergoes a series of genetic changes that gradually result in increasingly deregulated growth. Genetic changes in a subset of genes, the oncogenes and tumour suppressor genes cause this deregulated proliferation of cells. Initially, a single cell is altered and proliferates too rapidly. Over time a hyperplastic lesion may be further altered until the proliferative rate, state of differentiation and relationship with the microenvironment result in a fully developed neoplasm capable of metastasising to distant organs. This uncoordinated growth of a heterogeneous population of tumour cells will ultimately result in organ destruction and loss of function leading to death. However, in addition to tumour cells, solid tumours contain non-transformed host cells including fibroblasts, smooth muscle cells, pericytes, immune cells and endothelial cells. Survival of the tumour cells requires the support of these host cells. One of the most striking examples of tumour dependence on host cells is seen in the reliance of tumours on host vasculature.

Similar to normal tissue, tumours require the delivery of oxygen and nutrients and the removal of toxic waste products. As the tumour expands its oxygen and nutrient requirements can no longer be met by simple diffusion from nearby blood vessels. Solid tumours induce the growth of new blood vessels into the tumour to provide oxygen and nutrients to the tumour tissue. Endothelial cells also support tumour growth

by releasing various paracrine growth factors, such as insulin-like growth factor (IGF) and granulocyte-colony stimulating factor (GMCSF) (Folkman, 1995) There is clear evidence that most solid tumours are dependent on the growth of new blood vessels, a process called angiogenesis. The evidence that tumour growth is angiogenesis dependent is comprehensively reviewed by (Folkman, 1990) and some examples are outlined here. A mouse sarcoma implanted sub-cutaneously, in transparent chambers, did not grow until the development of new blood vessels after which growth was nearly exponential (Algire et al , 1945). Similarly, a rat sarcoma implanted on the chick embryo chorioallanotoic membrane did not grow for the first 72 hr when there was no blood vessel supply but tumour growth increased rapidly within the first 24 hr of the in-growth of blood vessels (Ingber et al., 1986). When a rabbit epithelioma was suspended in the anterior chamber of the eye, where it is physiologically impossible to attract new blood vessels, it remained viable but over 34 days did not grow beyond 1 mm^3. When it was subsequently re-implanted on the iris, where it could induce new blood vessels, it reached 16,000 times its original volume within 2 weeks (Gimbrone et al., 1972) More recent experiments have show that avascular tumours remain small reaching a steady state size of approximately 2 mm^2, upon which the lesion forms a dense population of cells in which the rate of proliferation of tumour cells is balanced by the rate of apoptosis and necrosis of cells (Holmgren et al , 1995). These lesions are asymptomatic and are usually clinically undetectable until incidental discovery in post-mortem examinations (Folkman, 1995).

1.5 Angiogenesis

The vascular system is a very complex integrated organ providing essential transport functions such as the oxygenation and nutrition of all tissues, removal of the by-products of metabolism, the circulation of cells that mediate host defence, the transport of proteins and cells involved in wound healing, inflammatory response, homeostasis and thrombolysis. The vasculature is composed of around 10^{12} endothelial cells lining the inside of blood vessels covering an area of approximately 1000 m^2 in a 70 kg adult (Jaffe, 1987). At any time the cells in normal tissue are in close proximity to the endothelial cell of a blood vessel, which supplies all the necessary nutrients and removal of waste products. In the absence of neovascularisation, a tumour cell that is proliferating rapidly will soon be at some distance from the nearest endothelial cell due

to the increasing bulk of the tumour mass. Without nutrition and oxygenation by the vasculature its fate, and that of all its progeny, is starvation and death by necrosis or apoptosis.

Under normal physiological conditions, there is little need for new blood vessel formation, except in a few tightly controlled situations, such as wound healing, and the vasculature remains quiescent. However, the vasculature retains the ability to form new blood vessels, for example following injury (Edgington, 1995). The vasculature is formed in one of two ways, vasculogenesis and angiogenesis (Noden, 1989). Vasculogenesis is the process whereby simple angioblast cells, in the mesoderm, differentiate into endothelial cells and form primitive blood vessels (Risau and Flamme, 1995). Angiogenesis, however, is the sprouting of new capillaries from a pre-existing blood vessel (Wilting and Christ, 1996). Within the growing embryo the vasculature is formed by a combination of vasculogenesis and angiogenesis (Noden, 1989). In adults, however, angiogenesis is the sole method for new blood vessel formation (Tischer et al., 1991).

1.6 Physiological and pathological angiogenesis

Angiogenesis occurs in a highly regulated fashion during normal physiological events. In the female reproductive system angiogenesis is required for the development of the corpus luteum in the ovary, the endometrium during the menstrual cycle and in the placenta throughout gestation (Reynolds et al., 1992). Revascularisation of tissue in a wound bed following injury is essential for the delivery of nutrients and immune cells and removal of debris and is accomplished by angiogenesis (Arbiser, 1996). Angiogenesis is also required for development and healing of bones (Winet, 1996). Under these circumstances angiogenesis is briefly turned on and then completely shut down. Aberrant angiogenesis can occur in a number of pathological states other than tumour growth. In rheumatoid arthritis, new capillary blood vessels invade the joint and destroy cartilage (Koch, 1998). Ocular neovascularisation, including diabetic retinopathy, is the most common cause of blindness and dominates approximately 20 eye diseases (Folkman and Shing, 1992). Psoriasis is a chronic inflammatory disease that involves proliferation of the epidermis with nutritional support from increased angiogenesis in the dermis (Malhotra et al., 1989). Ischemic heart disease and

peripheral vascular diseases occur when there are insufficient blood vessels to feed the heart (Battegay, 1995).

1.7 Steps in angiogenesis

Angiogenesis is a complex multi-step process and although it is a continuous process it has been divided into six steps:

1) the release of angiogenic factors
2) activation of endothelial cells
3) release of proteolytic enzymes
4) endothelial cell migration and morphogenesis
5) endothelial cell proliferation
6) microvessel differentiation

A simplified version of the process is illustrated in figure 1.1. The angiogenic cascade begins in response to the release of angiogenic factors. Tumour cells and host cells can secrete a large number of factors that are capable of inducing angiogenesis. Table 1.1 provides a list of the most common endogenous protein angiogenic factors. In addition to these factors there are a variety of carbohydrates (lactic acid, hyaluronan fragments), lipids (prostaglandins) and other compounds (copper, nicotinamide) (comprehensively reviewed by Bouck et al., 1996). The precise role of each of these factors is still unclear as yet because of the wide range of mechanisms through which they can act. Some factors act nearly exclusively on endothelial cells, such as vascular endothelial growth factor (VEGF) (Jakeman et al., 1992), while other factors may act on a variety of different cells like basic fibroblast growth factor (bFGF), which may act on fibroblast, endothelial cells and smooth muscle cells (Gospodarowicz, 1990). Factors such as transforming growth factor β-1 (TGFβ-1) and tumour necrosis factor α (TNF-α) may inhibit the proliferation of endothelial cells but can still promote angiogenesis indirectly by involvement with other elements of the angiogenic cascade, such as the differentiation of endothelial cells (Pepper et al., 1993; Fràter-Schröder et al., 1987). Angiogenic factors, bFGF in particular, are also found bound to the extracellular matrix and are activated when released by proteases (Baird and Ling, 1987). Angiogenic factors also act with varying degrees of potency with factors such as VEGF and bFGF being the most potent. The effects of multiple angiogenic factors may be synergistic such as seen with bFGF and VEGF (Goto et al., 1993), and bFGF with TGFβ-1

(Gajdusek et al., 1993). This was demonstrated in *in vitro* experiments where the effect of both factors together was greater than the sum effect of each factor alone. Because of the large number of angiogenic factors and the interplay between them and other factors the exact response from release of angiogenic factors may be hard to predict.

The target vessels for angiogenic factors are the post capillary venules and small terminal venules (Guvakova and Surmacz, 1997). These vessels are comprised of endothelial cells that lie upon a basal lamina surrounded by a discontinuous layer of pericytes and smooth muscle cells embedded in the extracellular matrix. The morphology of the endothelial cells is altered in response to angiogenic factors. There is an increase in the amount of endoplasmic reticulum, Golgi apparatus and mitochondria and the endothelial cells form protrusions on the abluminal side (Fox et al., 1996).

In order to accommodate new capillaries and allow their formation the basement membrane and extracellular matrix must be degraded. This is achieved by the production of a variety of proteolytic enzymes including plasminogen activators (uPA and tPA) and metalloproteinases (MMPs), secreted by endothelial cells (Bacharach et al., 1992; Hiraoka et al., 1998). The urokinase plasminogen activator generates plasmin, which is a broad spectrum protease that is capable of degrading most matrix components (Fox et al., 1996) while the membrane type 1-MMP (mt-1 MMP) degrades fibrin and can also effectively degrade matrix in plasmin deficient mice (Hiraoka et al., 1998). Inhibition of urokinase activity inhibited angiogenesis and tumour growth of B16 cells in syngeneic mice (Brock et al., 1991). Protease inhibitors are also produced (Pepper and Montesano, 1990; Bacharach et al., 1992) so that a co-ordinated process of degradation and migration can be achieved. The angiogenic factor VEGF increases the number of uPA receptors (uPAR) on endothelial cells and this is believed to be important in co-ordinate regulation of matrix degradation and migration of endothelial cells (Mandriota et al., 1995). The matrix remodelling is a carefully controlled process balanced by the signals promoting and inhibiting proteolysis (Pepper and Montesano, 1990).

The breakdown of the basement membrane and extracellular matrix allows endothelial cells from post capillary venules to migrate and form sprouts. Projections form and as the gap between endothelial cells grows neighbouring cells follow from the parental vessel. The endothelium initially forms as a solid cord but eventually inter- and

intra-cellular spaces appear to form lumina (Folkman and Haudenschild, 1980). Very little is known about regulation of migration though VEGF is chemotactic for endothelial cells (Yoshida et al., 1996). Several cytokines have been reported that modulate migration but the signalling mechanism is only partly understood (Fox et al., 1996). Expression of various cell adhesion molecules including integrin $\alpha_v\beta_3$, E-selectin, sialyl Lewis-X and sialyl Lewis-A are essential for capillary morphogenesis (Brooks et al., 1994b; Nguyen et al., 1993).

As the endothelial sprout forms cells at the base proliferate to supply the elongating vessel. New capillaries therefore grow from the base and not from the tip (Fox et al., 1996). Endothelial cells are normally quiescent and divide very rarely, every 7-10 years or so, while tumour endothelium divides much more rapidly. Endothelial cells in a tumour environment have been found to proliferate approximately 40 times faster than in normal tissue (Vartanian and Weidner, 1994). Remodelling of surrounding tissue also leads to an increase in endothelial cell numbers within the tumour tissue (Fox et al., 1993).

Developing buds anastomose with other developing buds or pre-existing vessels to form capillary loops that facilitate transport of blood and tissue perfusion (Polverini, 1995). In normal tissue new vessels stabilise and persist for as long as they are required. In a tumour environment, however, the endothelium of the new capillaries have wide cell junctions, numerous trans-endothelial channels and a discontinuous lining (Fox et al., 1996). The vessels are often lined with tumour cells. An irregular basement membrane often forms which is composed of varying amounts of fibronectin, laminin, and collagen. Cells such as smooth muscle cells, fibroblasts, pericytes also contribute to the formation of the matrix (Paku and Paweletz, 1991). VEGF cause hyper-permeability of blood vessels (Senger et al., 1983). Because of both the structure of these new vessels and the action of VEGF on permeability these vessels are usually very leaky. Even after all these events have taken place there is a constant remodelling of formed vessels as well as the recruitment of new vessels so that the tumour vasculature is highly unstable (Fox et al., 1996). Endothelial cells are highly heterogeneous (McCarthy et al., 1991) and the morphology of the tumour vasculature is highly variable between tumours of different sites (Roberts et al., 1998).

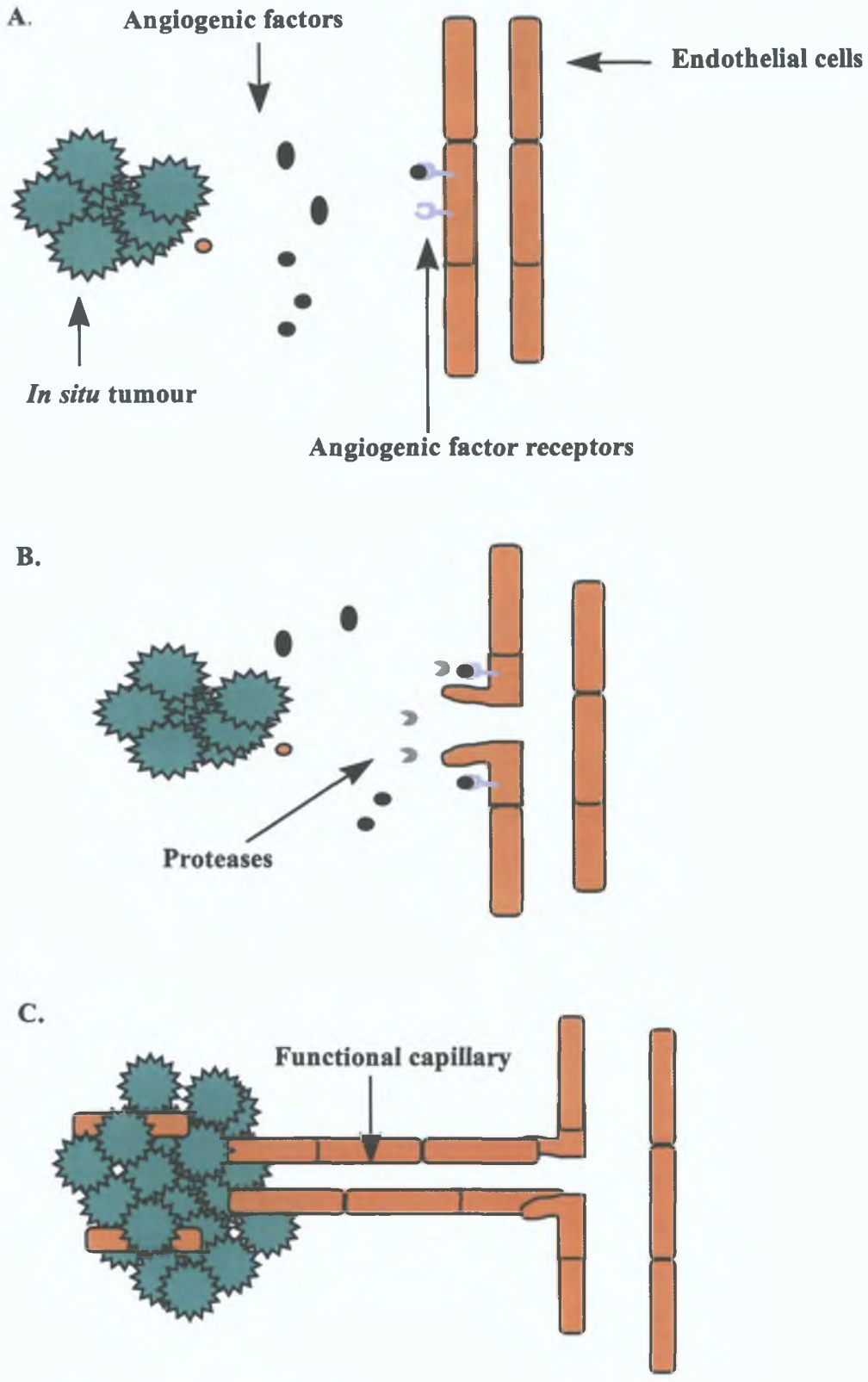


Fig 1.1: Steps in angiogenesis

Tumour cells release angiogenic factors which bind to receptors on endothelial cells (**A**). Endothelial cells release proteases to degrade the extracellular matrix and migrate along a chemotactic gradient (**B**). Capillaries mature and a functional lumen allows transport of oxygen and nutrients to the tumour (**C**).

Factor	Endothelial cell (EC) mitogen	Reference
Acidic fibroblast growth factor (aFGF)	Positive	(Thomas et al., 1985)
Basic fibroblast growth factor (bFGF)	Positive	(Gospodarowicz, 1990)
Angiogenin	no effect	(Kurachi et al., 1985)
Transforming growth factor-α (TGFα)	Positive	(Schreiber et al., 1986)
Transforming growth factor-β (TGFβ)	Inhibitor	(Roberts et al., 1986)
Tumour necrosis factor-α (TNF-α)	Inhibitor	(Fràter-Schröder et al., 1987)
Vascular endothelial growth factor (VEGF)	Positive	(Connolly et al., 1989)
Platelet-derived endothelial cell growth factor (PD-ECGF)	Positive	(Ishikawa et al., 1989)
Colony stimulating factor (CSF)	Positive	(Bussolino et al., 1991)
Insulin-like growth factor I (IGF-1)	n/a	(Grant et al., 1993)
Platelet-derived growth factor (PDGF)	Positive	(Risau et al., 1992)
Epidermal growth factor (EGF)	Positive	(Stewart et al., 1989)
Interleukin-8 (IL-8)	Positive	(Strieter et al., 1992)

Table 1.1: Endogenous protein angiogenic factors. n/a = not assessed

1.8 Switch to the angiogenic phenotype

Tumour progression is known to occur in a multi-step manner in which cells become increasingly deregulated and proceed through a number of stages from a hyperplastic lesion to a metastasising neoplasm. The acquisition of an angiogenic phenotype is believed to be a critical event in the progression of the tumour. Most malignancies of epithelial origin have a pre-neoplastic stage that may last for several years and these pre-neoplastic lesions are avascular compared with the highly angiogenic neoplasms. Studies of mice expressing oncogenes in the β-cells of pancreatic islets show that these cells readily become hyperplastic but only a few islets become angiogenic and this angiogenic activity is necessary for, and precedes, tumour formation (Folkman et al., 1989). In another mouse model, basal keratinocytes were transformed into a squamous cell carcinoma by the human papillomavirus type 16 oncogene and tumour progression was only seen after sudden onset of angiogenesis that had been quiescent until that point (Coussens et al., 1996). Examination of sections from cervical smears has demonstrated that angiogenesis precedes late stage disease but is absent in the earlier stages of this cancer (Guidi et al., 1995). This demonstrates that increased tumour cell proliferation is not sufficient for the development of angiogenesis but that another key step, the acquisition of an angiogenic phenotype is also required. This has led to the development of the concept that tumours must activate an angiogenic 'switch' before tumour growth can progress to the formation of a clinically relevant tumour (Hanahan and Folkman, 1996).

Under normal physiological conditions angiogenesis is regulated by the release of anti-angiogenic factors which counter the effect of angiogenic factors. For example, studies of physiological angiogenesis have demonstrated that it occurs in highly regulated fashion and can be effectively and completely shut down when not required. Table 1.2 lists some of the more common endogenous inhibitors of angiogenesis that have been identified. These anti-angiogenic molecules function to inhibit angiogenesis when it is actively taking place or to suppress angiogenesis under normal circumstances (Quinn et al., 1993).

Cells must have some mechanism whereby they can integrate the pro- and anti-angiogenic signals and determine whether they should proceed to an angiogenic phenotype. The switch to the angiogenic phenotype is controlled by the overall balance of angiogenic promotors and inhibitors (Hanahan and Folkman, 1996).

Thrombospondin, which inhibits angiogenesis, is produced constitutively by a normal hamster kidney fibroblast cell line but is downregulated during tumourigenesis (Rastinejad et al., 1989). By the time the tumour has become angiogenic these cells are only producing approximately 4% of the thrombospondin that their normal precursors produced (Rastinejad et al., 1989). This switch has been described in the progression of tumourigenesis by human fibroblasts cultured from Li Fraumeni patients (Volpert et al., 1997). Initially these cells produce abundant thrombospondin that overrides VEGF also produced and the cells have no net angiogenic activity. Loss of both p53 alleles and transformation by activated ras allowed the cells to become angiogenic in a stepwise fashion. Thrombospondin production was decreased and there was a large increase in VEGF production (Volpert et al., 1997).

Expression of many angiogenic and anti-angiogenic factors is under the control of oncogenes and tumour suppressor genes. Some oncogenes code for angiogenic factors such as bFGF which is coded by the *FGF-2* oncogene (Rodgelj et al., 1988) and PDGF-B coded by the *sis* oncogene (Waterfield et al., 1983). Other oncogenes can stimulate production of angiogenic factors. The oncogenes v-H-*ras*, v-*raf,* and v-*src* stimulate the production of VEGF by NIH3T3, human glioblastoma-astrocytoma cells and human fetal kidney cells, respectively (Grugel et al., 1995; Mukhopadhyay et al., 1995). Other angiogenic factors are also increased by oncogenes, for example, v-*erb*A increases PDGF-A expression by glial cells (Iglesias et al., 1995) and N-*ras* increases TNF-α production by human melanoma cells (Castelli et al., 1994). Various oncogenes can also aid angiogenesis indirectly, by increasing levels of the enzymes that assist angiogenesis, such as K-*ras* and N-*ras* that increase urokinase by human fibrosarcoma cells (Jankun et al., 1991). Activated oncogenes may decrease levels of angiogenesis inhibitors, such as v-*ras* that decreases levels of thrombospondin in bronchial epithelial cells (Zabrenetzky et al., 1994). Human mammary epithelial cells are not normally angiogenic but following transfection with the *int*-2 gene, which codes for FGF-3, these cells are capable of eliciting an angiogenic response (Costa et al., 1994). Tumour suppressor genes may also function to alter the levels of these agents. Loss of p53 decreases the levels of thrombospondin in fibroblasts (Dameron et al., 1994). VEGF is down-regulated by wild type (wt) p53 in a human foetal kidney cell line (Mukhopadhyay et al., 1995) while mutant (mt) p53 increases VEGF expression by NIH3T3 (Kieser et al., 1994). It would appear that the development of an angiogenic

phenotype is driven by the same processes of oncogene activation and tumour suppressor gene loss, that also results in the initiation of unregulated proliferation.

1.9 Tumour vascularity

The extent of angiogenesis may be measured by staining tumour sections for endothelial cell specific markers Endothelial cell specific markers that have been widely used to date include factor VIII-related antigen, platelet-endothelial cell adhesion molecule (PECAM or CD31) and the CD34 antigen (Weidner et al., 1991; Horak et al., 1992, Heimann et al., 1996). Microvessel density (MVD) can then be assessed by the extent and intensity of staining. Breast tumours have been shown to have significantly higher vascularisation than normal breast tissue and than benign tumours (Horak et al., 1992; Goede et al., 1998) Vessel count has been positively associated with various clinical outcomes. Horak *et al* (1992) found that the number of vessels in primary breast tumours was associated with nodal status, the size of primary tumour and the degree of differentiation of the tumour. Microvessel density has also been associated with metastatic disease (Weidner et al., 1991). Recent analysis of 531 breast cancer patients with a follow-up of greater than 6 years has found MVD to be a significant prognostic indicator for relapse-free survival and overall survival in a multivariate model (Gasparini et al., 1998). Clinical use of MVD as a prognostic factor may be delayed however because of variability of the methods used. Several papers have demonstrated that counts may be highly variable and of no prognostic significance (Gouldmg et al., 1995; Costello et al , 1995). Improvements m technology, such as automated image analysis systems, may overcome these difficulties as demonstrated by Simpson *et al* who found that such a system measuring overall endothelial area within a section to be more useful than manual vessel counts (Simpson et al., 1996)

Inhibitor	Antitumour Activity	Reference
Angiostatin	Yes	(Quinn et al , 1993)
Endostatin	Yes	(O'Reilly et al., 1997)
Angiostatic steroids	Yes	(Ingber et al., 1986)
Interferon-α	Yes	(Sidky and Borden, 1987)
Interferon-β	Yes	(Sidky and Borden, 1987)
Interferon-γ	Yes	(Norioka et al., 1994)
IFN-γ - inducible protein 10	n/a	(Strieter et al., 1995)
Interleukin-1	Yes	(Cozzolino et al., 1990)
Interleukin-12	Yes	(Voest et al., 1995)
Platelet Factor 4	Yes	(Maione et al., 1990)
Thrombospondin	Yes	(Rastinejad et al., 1989)

Table 1.2: Endogenous inhibitors of angiogenesis. n/a = not assessed.

1.10 Vascular endothelial growth factor

VEGF, also known as vascular permeability factor (VPF) and vasculotrophin, was originally isolated as a factor regulating vascular permeability in guinea pigs with implanted hepatocellular carcinomas (Senger et al., 1983). It was independently discovered in the conditioned medium of bovine pituitary folliculostellate cells as a factor that specifically increased the growth of adrenal endothelial cells (Ferrara and Henzel, 1989). Subsequent analysis showed that they were the same protein (Connolly et al., 1989). It is a basic, heparin-binding, homodimeric glycoprotein of 32-45kDa (Connolly et al., 1989). VEGF is inactivated by reducing agents but is heat and acid stable (Ferrara, 1995).

Analysis of VEGF clones shows that there are 5 different isoforms of 121, 145, 165, 189 and 206 amino acids generated by alternative splicing of 8 different exons (Figure 1.2)(Tischer et al., 1991; Poltorak et al., 1997). All isoforms contain a 26 amino acid signal sequence that is cleaved upon secretion. All isoforms contain the 115 amino acid amino-terminal exons 1-5 and the 6 amino acid carboxyl-terminal exon 8. There are 8 cysteine residues required for dimer formation and are all contained in exons 1-5. $VEGF_{206}$ contains an additional 17 amino acid stretch termed 6' which is derived from a contiguous downstream sequence of exon 6 and is due to the shift of the exon 6 splice donor further downstream into the intron and is not an independent exon.

These isoforms have different properties due the presence of the different exons. $VEGF_{121}$ has no heparin binding activity and is freely secreted by the cell (Houck et al., 1992). $VEGF_{189}$ and $VEGF_{206}$ contain exon 6 which is highly basic and readily binds heparin. These isoforms are secreted but are almost entirely bound to the cell surface or to the extracellular matrix. Addition of heparin, heparan sulphate and heparinase induces the release of $VEGF_{189}$ into the cell culture medium suggesting heparin-containing proteoglycans as likely binding sites for $VEGF_{189}$ (Houck et al., 1992). $VEGF_{165}$, which contains exon 7 that is basic and has weak heparin binding activity, displays an intermediate behaviour with some being freely secreted and some apparently bound also to the cell surface (Houck et al., 1992). Although $VEGF_{145}$ contains the heparin binding exon 6 it is also freely secreted but does remain bound to the extracellular matrix (Poltorak et al., 1997). $VEGF_{206}$ has, to date, only been detected in embryonal liver tissue (Houck et al., 1991). As yet, there is little information on which isoforms are expressed within tumours. Most studies have either measured total VEGF

mRNA or $VEGF_{165}$ protein. One recent study has demonstrated that mRNA for the 121, 165 and 189 isoforms were detectable in breast tumours but only the protein for $VEGF_{165}$ was detected (Scott et al., 1999).

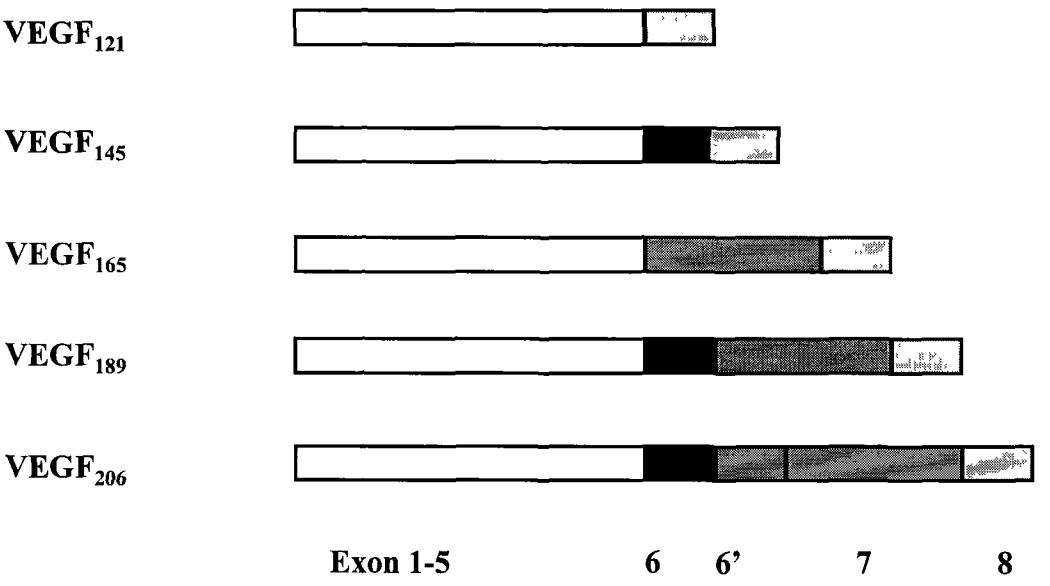


Figure 1.2 VEGF isoforms

1.11 VEGF family

VEGF is now understood to be just one member of a growing family of endothelial growth factors. The other members of the family are placenta growth factor (PlGF), platelet-derived growth factors (PDGF-A and PDGF-B), VEGF-B, VEGF-C, VEGF-D, VEGF-E, (Maglione et al., 1991; Olofsson et al., 1996b; Joukov et al., 1996; Yamada et al., 1997; Ogawa et al., 1998). All factors are secreted as dimers, have 8 conserved cysteine residues involved in inter- and intra-molecular disulphide bonds between the two subunits and share 20-45% homology. PlGF is alternatively spliced to give 2 isoforms and both isoforms are ligands for vascular endothelial growth factor receptor-1 (VEGFR-1 or flt-1) only (Park et al., 1998). PlGF is most highly expressed in the placenta and most likely regulates placental vasculature during gestation (Maglione et al., 1991). Although members of this family of growth factors tend to be homodimers, heterodimers of PlGF with VEGF have been identified and are capable of binding VEGFR-2 though there is diminution of response compared with binding of VEGF homodimers (Cao et al., 1996). PDGF is produced as two chains, PDGF-A and PDGF-B which dimerise to form a combination of PDGF-AA, -AB, -BB which have similar potencies (Hannink and Donoghue, 1989). VEGF-B is the most related member to VEGF, sharing approximately 45% homology to VEGF, and forms two isoforms but receptors for it have not yet been discovered (Olofsson et al., 1996a). It is expressed in a variety of human tumours including melanomas, sarcomas, head and neck tumours and breast tumours (Salven et al., 1998). VEGF-C binds to the VEGFR-2 and VEGFR-3 receptors (Joukov et al., 1997). VEGFR-3 is only expressed on lymphatic endothelial cells and overexpression of VEGF-C is associated with proliferation of lymphatic endothelial cells which suggests that VEGF-C is a regulator of lymph vessels (Jeltsch et al., 1997). VEGF-D is also a ligand for VEGFR-2 (flk-1) and VEGFR-3 (flt-4) and is most closely related to VEGF-C with both containing a cysteine rich C-terminus distinct from other members (Achen et al., 1998). VEGF-D is highly expressed in normal lung tissue, an organ containing a highly developed vascular system, and may be involved in the regulation of vasculature in this organ (Yamada et al., 1997). VEGF-E was originally identified as an open reading frame in the genome of the Orf virus NZ-7 strain (Ogawa et al., 1998). This virus causes contagious skin lesions that are highly vascular in nature (Ogawa et al., 1998). VEGF-E is a ligand for VEGFR-2 but has not yet been discovered in humans

1.12 Role for VEGF in angiogenesis

The essential role of VEGF in blood vessel formation can be seen even in the early stages of embryonic development. The formation of blood vessels was abnormal in mice embryos that had a heterozygous deficiency for the VEGF gene (VEGF +/-) (Carmeliet et al , 1996). In mice embryos that had a homozygous VEGF deficiency (VEGF -/-) there was even greater impairment of vascular development and embryos died mid-gestation (Carmeliet et al., 1996). Mice that had a homozygous deficiency for the VEGF receptors flt-1 and flk-1 (VEGFR-1 and VEGFR-2) also died *in utero* after approximately 9 days (Fong et al., 1995; Shalaby et al., 1995). There were no organised blood vessels within the embryo or yolk sac at any stage of development VEGF expression has been associated with, spatially and temporally, the physiological angiogenesis that occurs m the ovary (Ravindranath et al., 1992) and m healing wounds (Nissen et al., 1998) VEGF expression has also been associated with pathological angiogenesis associated with diabetic retinopathy and other ocular disorders (Miller et al., 1994) and the angiogenesis occurring during rheumatoid arthritis (Koch et al , 1994). Many tumours express abundant VEGF (Berse et al., 1992). Direct evidence for a role of VEGF in tumour angiogenesis has also been described. Mouse embryonic stem cells with homozygous deficiencies for VEGF have a dramatically reduced ability to form tumours in nude mice compared to normal stem cells and the tumours have a significantly reduced number of blood vessels (Ferrara et al , 1996). Administration of a monoclonal antibody against VEGF was found to inhibit the growth of a variety experimental tumours in mice including human rhabdomyosarcoma, glioblastoma and fibrosarcoma (Kim et al , 1993; Asano et al., 1995; Borgström et al , 1996) C6 glioma cells, transfected with a eukaryotic expression vector bearing an anti-sense VEGF cDNA, injected into nude mice were found to be significantly growth inhibited compared to control cells (Saleh et al., 1996). These tumours also had fewer blood vessels and a larger area of necrosis. The role of a VEGF-receptor system in tumour growth has been highlighted by experiments whereby tumour growth was inhibited by interfering with the signalling of the VEGF receptor VEGFR-2 using dominant-negative mutants (Millauer et al., 1994)

1.13 VEGF receptor system

The intracellular effects of VEGF are mediated by binding to one of two VEGF receptors. The receptors are *fms*-like tryrosine kinase (flt-1) and fetal liver kinase 1 (flk-1) otherwise known as kinase insert-domain containing receptor (KDR) (De Vries et al., 1992; Terman et al., 1992). Because of the confusion regarding the nomenclature of an increasing number of endothelial cell-specific tyrosine kinase receptors these receptors have recently been given the respective names VEGFR-1 (flt-1) and VEGFR-2 (flk-1) There is a third receptor, flt-4 (VEGFR-3), which is restricted to endothelial cells of lymphatic origin (Kaipainen et al., 1995) and is a ligand for VEGF-C (Joukov et al., 1996). Neither $VEGF_{121}$ nor $VEGF_{165}$ bind to VEGFR-3 (Wilting et al., 1996). VEGFR-1 and VEGFR-2 are predominately expressed on endothelial cells (Peters et al., 1993; Quinn et al., 1993) and are highly homologous to other tyrosine kinase receptors such as c-*fms* (receptor for colony stimulating factor 1 (CSF-1) and PDGF-Rα and PDGF-Rβ (receptors for PDGF) (Shibuya et al., 1990). Both VEGFR-1 and VEGFR-2 are type III receptor tyrosine kinases (Terman et al., 1992) Each receptor contains 7 immunoglobulin-like domains, a transmembrane domain and a long kinase insert in the kmase domain (Terman et al., 1992).

VEGFR-1 has a higher affinity for VEGF than VEGFR-2, K_d of 10-20 pM (≈ 30 – 60 pg/ml) and 100-125 pM (≈ 300 – 375 pg/ml), respectively (Terman et al., 1992) and the receptors are reported to have different signal transduction properties (Waltenberger et al., 1994; Seetharam et al., 1995). When both receptors were stably expressed in porcine aortic endothelial cells that lack endogenous VEGF receptors a different pattern of responses was observed between the two receptors. Cells expressing VEGFR-2 showed changes in morphology, actin reorganisation, membrane ruffling, chemotaxis and mitogenicity whereas the cells expressing VEGFR-1 showed none of these responses (Waltenberger et al , 1994). A similar experiment in the same endothelial cells demonstrated that binding of VEGF in VEGFR-2 expressing cells induced DNA synthesis and migration of cells but m VEGFR-1 expressing cells these responses did not exist (Landgren et al., 1998). In the VEGFR-1 cells, however, urokinase plasminogen activator (uPA) expression was induced which did not occur in the VEGFR-2 expressing cells (Landgren et al., 1998). NIH3T3 cells stably transfected with VEGFR-1 did not show an increase in proliferation upon binding VEGF (Seetharam et al., 1995). Monocytes express VEGFR-1 and binding of VEGF does

induce a chemotactic response in these cells (Clauss et al., 1996). Mutational analysis of both receptors show that mutations in VEGFR-2 disrupts VEGF stimulated proliferation of transfected Chinese hamster ovary (CHO) cells while mutations in VEGFR-1 had no effect (Keyt et al., 1996). The same study shows that both receptors bind different parts of $VEGF_{165}$ (Keyt et al., 1996).

Upon binding VEGF both receptors are autophosphorylated (Waltenberger et al., 1994). VEGF binding to bovine aortic endothelial (BAE) cells promotes the tyrosine phosphorylation of at least 11 proteins (Guo et al., 1995). VEGF promoted the tyrosine phosphorylation of several proteins that contain SH2 domains, including phospholipase C-γ (PLC-γ), ras GTPase activating protein (GAP) and phosphatidylinositol 3-kinase (PI-3 kinase) in bovine aortic endothelial cells (Guo et al., 1995). NIH3T3 cells transfected with VEGFR-1 phosphorylate PLCγ, GAP and mitogen activated protein kinase (MAPK) following ligand binding without increasing proliferation (Seetharam et al., 1995). The same cells transfected with VEGFR-2 also activate the same signal molecules but with a mitotic response (Takahashi and Shibuya, 1997). Activation of MAPK appears to be essential for the response of endothelial cells and the antiangiogenic mechanism of a 16 kDa fragment of prolactin appears to be by blocking MAPK activation (D'Angelo et al., 1995). The end molecules of VEGF induced transduction and the gene elements involved are as yet unknown. In general, it has been accepted that VEGFR-2 plays a greater role in tumour biology as it is responsible for the mitogenic and chemotactic responses of endothelial cells to VEGF and that the role of VEGFR-1 may be in development alone (Zachary, 1998).

The heparin binding capacity of VEGF appears to be important for binding. Cell surface-associated heparin-like molecules have been shown to be essential for the binding of bFGF to its high affinity receptors (Yayon et al., 1991). Although VEGF has a lower affinity for heparin than bFGF the interactions of VEGF with heparin are important for binding to its receptors. Cross-linking experiments have demonstrated that when the concentration of heparin-like molecules on the cell surface of BAE cells are reduced by heparinase, $VEGF_{165}$ no longer binds to its putative receptors (Gitay-Goren et al., 1992). Exogenous heparin restores binding. Low concentrations of exogenous heparin (0.1 - 1 μg/ml) have been shown to enhance the binding of $VEGF_{165}$ to both receptors in BAE cells and to endogenous receptors in NIH3T3 cells (Gitay-Goren et al., 1992; Tessler et al., 1994). Heparin however, at the same concentrations,

was found to inhibit binding and autophosphorylation of VEGF to a chimeric receptor containing the extracellular domain of VEGFR-2 fused to the transmembrane and intracellular domain of c-fms transfected into NIH3T3 cells (Tessler et al., 1994). Terman et al demonstrated that heparin had a stimulatory effect on VEGF binding to VEGFR-2 expressing BAE and CMT3 cell lines but had a inhibitory effect on VEGF binding on VEGFR-1 expressing SK-MEL-37 cells (Terman et al., 1994). This would indicate that the specific characteristics of the receptor as well as the heparin binding activity of VEGF itself is important in binding. One mechanism through which heparin seems to regulate the binding of VEGF to its receptors is by increasing or decreasing the number of receptors on the cell surface (Gitay-Goren et al., 1992; Terman et al., 1994). Heparin was also found to potentiate the binding of $VEGF_{165}$ to a soluble fusion protein containing the extracellular domain of VEGFR-2 fused to alkaline phosphatase, VEGFR-2/SEAP (Tessler et al., 1994). This experiment was conducted in a cell free environment and therefore excludes the effects of heparin sulphate proteoglycans already on the cell surface. Heparin sulphate had the same effect. Interestingly the degree of sulphation was found to be important as desulphated heparin had no effect and oversulphated heparin had a greater effect on VEGF binding than normally sulphated heparin (Tessler et al., 1994). This experiment demonstrated that heparin can also directly modulate the binding of $VEGF_{165}$ to VEGFR-2/SEAP, and that this modulation is probably due to the interaction of heparin with VEGF since heparin did not bind to the fusion protein (Tessler et al., 1994).

Hypoxia is known to increase VEGF expression but also increased VEGFR-2 expression in a cerebral slice culture system (Kremer et al., 1997). In addition, exogenously added VEGF increased VEGFR-2 expression in the same system (Kremer et al., 1997) and this effect was lost by the addition of anti-VEGF antibodies. The VEGF signal may also be regulated by the production of a soluble VEGFR-1 (Kendall and Thomas, 1993). This soluble receptor is coded from alternative splicing of the full VEGFR-1 and binds VEGF with high affinity (Kendall and Thomas, 1993). Transfection of soluble VEGFR-1 into human fibrosarcoma cells inhibited tumour growth, metastasis and mortality when implanted into nude mice (Goldman et al., 1998). These indicate possible mechanisms whereby VEGF receptor expression regulates angiogenesis.

1.14 Macrophages in breast tumours

Breast tumours are composed of a heterogeneous population of both host and tumour cells. In addition to endothelial cells there are other host cells present that include fibroblasts and a variety of immune cells. Macrophages form a major component of the immune infiltrate into many solid tumours and may account for up to 50% of breast tumour mass (O'Sullivan and Lewis, 1994). Macrophages are multifunctional cells capable of performing a number of functions including angiogenesis (Sunderkötter et al., 1994). Macrophages may mediate tumour cytotoxicity but many tumour cells can escape this immune surveillance by suppressing macrophage immune function by releasing suppressing factors such as TGFβ-1, interleukm-10 (IL-10) and prostaglandin E_2 (PGE_2) (Elgert et al., 1998). Macrophages can induce angiogenesis in wounds (Knighton et al., 1983) Conditioned medium from tumour associated macrophages isolated from experimental fibrosarcomas induced endothelial cell proliferation *in vitro* and angiogenesis *in vivo* (Polverini and Leibovich, 1984). Experimental murine fibrosarcomas were less vascularised when their hosts were depleted of monocytes (Sunderkötter et al., 1994). There is a significant positive correlation between breast tumour vascularity and macrophage content and increased macrophage content was associated with reduced relapse free survival and overall survival (Leek et al., 1996). Macrophages are capable of releasing a number of angiogenic factors including EGF, TNF-α, TGFβ, bFGF, lL-8 and VEGF (O'Sullivan et al., 1993, Leibovich et al., 1997; Lewis et al., 1995; Koch et al , 1998; Harmey et al., 1998). In addition to release of angiogenic factors, macrophages are also capable of releasing proteases to degrade the extracellular matrix such as plasminogen activators (Nathan, 1987) and can be involved in remodelling the matrix (Sunderkötter et al., 1994). Macrophages do, however, also produce metalloelastase which cleaves plasminogen to the angiogenesis inhibitor, angiostatin (Dong et al., 1997).

1.15 Anti-angiogenic therapy

Since angiogenesis is essential for tumour growth, the inhibition of angiogenesis offers a very attractive target for treating cancer. Since angiogenesis is a multi-step process, interruption of any of these processes may decrease tumour growth. Angiogenesis could be inhibited by decreasing or abrogating the effects of angiogenic factors, by increasing levels of angiogenesis inhibitors, inhibiting the activity of degradative

enzymes or by inhibiting endothelial cell proliferation, migration or tube formation. The huge advantage in altering angiogenesis alone is that side effects should be minimal since angiogenesis is normally quiescent throughout the body. A wide variety of experimental mechanisms have been employed to inhibit angiogenesis. Amongst these are the administration of anti-VEGF antibodies (Asano et al., 1995; Kondo et al., 1993) and antisense VEGF (Saleh et al., 1996). A fusion protein of VEGF with the diphtheria toxin injected into animals retarded the growth of Kaposi's sarcoma (Arora et al., 1999). Antibodies to $\alpha_v\beta_3$ integrin inhibited angiogenesis in the CAM assay by inducing apoptosis of proliferating endothelial cells (Brooks et al., 1994a). While these experiments are informative they are not practical as therapeutic options. Greater than 200 compounds have been reported as having anti-angiogenic activity (Holmgren and Bicknell, 1997). These compounds act in a wide variety of ways to inhibit various steps necessary for angiogenesis. Table 1.3 lists some of the more relevant compounds that are currently undergoing clinical trials for the inhibition of angiogenesis. Amongst the most promising of these drugs are angiostatin and endostatin. Both are endogenous inhibitors of angiogenesis (O'Reilly et al., 1994; O'Reilly et al., 1997; O'Reilly et al., 1997) that increase apoptosis of endothelial cells (Claesson-Welsh et al., 1998; Strieter et al , 1995). Results in mice have shown that these compounds greatly reduce tumour growth. However, all these compounds are still undergoing clinical trials to determine their safety and efficacy and will not be available for clinical use for several years. In the meantime the extended use of well characterised drugs as anti-angiogenic agents would be of huge benefit. One such drug is the anti-oestrogen tamoxifen.

Tamoxifen is used as the primary hormonal therapy for women with breast cancer It is primarily used as adjuvant therapy following surgical removal of a tumour. Tamoxifen reduces mortality, recurrence of disease and the incidence of contralateral breast cancer (Early Breast Cancer Trialist's Collaborative Group, 1992). Recently, prophylactic tamoxifen therapy has been shown to decrease the incidence of invasive breast cancer by 49% in women with an increased risk (Fisher et al., 1998). One mechanism through which tamoxifen may exert its effects is by reducing angiogenesis. Tamoxifen has been shown to inhibit angiogenesis in an *in vivo* model (Gagliardi and Collms, 1993). The major advantage of such a drug is that it is well characterised and has been used by millions of women over the last 20 years. Though there is an increased risk of endometrial cancer, thrombosis and visual disturbances the benefits are

believed to outweigh the risks (Assikis and Jordan, 1997) Tamoxifen is a well tolerated drug and in clinical trials less than 5% of patients discontinued treatment due to adverse side effects (Nolvadex, prescribing information, Zeneca Pharmaceuticals, Wilmington, USA) Being such a well characterised and well tolerated drug, adaptation of its uses to include prescription as an anti-angiogenic agent could be carried out with relative ease Exploration of its use as an anti-angiogenic agent may generate huge benefits in patients suffering from various angiogenic pathologies during the time that other more potent anti-angiogenic agents are undergoing developmental work and clinical trials

Drug	Mode of action	Trial	Reference
Marimastat	Inhibits MMPs	Phase II	(Pluda and Parkinson, 1996)
Thalidomide	Unknown	Phase II	(D'Amato et al , 1994)
Carboxyaminoimidazole (CAI)	Blocks Ca^{++} involvement m EC organisation	Phase I	(Alessandro et al , 1994)
Interleukin 12 (Il-12)	Induction of interferon inducible protein (IP-10)	Phase I	(Voest et al , 1995)
Interferon-α 2a	Downregulation of bFGF	Phase III *	(Folkman, 1989)
TNP 470 (formerly AGM-1470)	Inhibits EC proliferation and migration	Phase III	(Ingber et al , 1990)
Angiostatin	Induces EC apoptosis	Phase I	(O'Reilly et al , 1994)
Endostatin	Induces EC apoptosis	Phase I	(O'Reilly et al , 1997)

Table 1.3: Compounds undergoing clinical trials for use as anti-angiogenic therapy *, for infantile haemangiomas

1.16 Aim

Angiogenesis is essential for tumour growth and for the development of metastases VEGF is the most potent angiogenic factor identified to date but the degree of expression of VEGF and the regulation of expression are not fully described for breast cancer Altering the expression of VEGF, or disrupting any aspect of the angiogenic cascade, offers potential therapeutic benefits The aims of this thesis were, therefore, as follows

- Determination of the expression levels of VEGF in breast cancer by examining expression of VEGF by breast cancer cell lines, examination of levels of VEGF within breast tumours and determination of the levels of VEGF within the serum of breast cancer patients
- Identification of regulators of VEGF that are relevant m a breast tumour environment
- Determination of any anti-angiogenic effects of tamoxifen that may or may not be mediated through VEGF

CHAPTER 2

MATERIALS AND METHODS

2.1 Preparation and handling of reagents

All chemicals and reagents were stored according to the manufacturers instructions. General purchase chemicals were of analytical grade (Sigma Chemical Compay, St. Louis, MO, USA and BDH Chemicals Ltd., Poole, UK, unless otherwise stated). Solid chemicals were weighed using a Sartorius AC1215 electronic balance (Göttingen, Germany) and were prepared in distilled water with a conductivity of greater than 18mΩ. The pH of solutions was measured using a Radiometer PHM82 pH Meter (Copenhagen , Denmark). Solutions were autoclaved within a few hours of preparation. Liquid transfer for volumes of less than and including 1ml were carried out using Gilson pipettes and for volumes greater than 1 ml an electronic pipette aid (Drummond, Broomall, PA, USA) and disposable plastic pipettes were used.

2.2 Tissue culture

All tissue culture work was carried out in an aseptic manner within a laminar air flow cabinet (Holten LaminAir HB2436, Allerød, Denmark). The cabinet was switched on at least 20 min prior to use. The cabinet was thoroughly sanitised using 70% (v/v) isopropyl alcohol (IPA). All equipment and reagents were also sanitised before being brought into the cabinet. Disposable gloves and a clean lab coat with elasticated cuffs were worn at all times. The cabinet was cleaned again after completion of work and air was allowed to circulate for at least 20 min.

2.2.1 Breast Cancer Cell lines

Two human breast cancer cell lines were used for the duration of this project. BT474 (ATCC HTB20) cells, derived from a primary breast tumour, were received from the ATCC and MDA-MB-231 (ECACC92020424) cells, isolated from a pleural effusion, were received from the ECACC. BT474 cells were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% (v/v) foetal calf serum (FCS) and 10 μg/ml bovine insulin in 25 cm^2 or 75 cm^2 filter lid flasks at 37°C in a humidified atmosphere of 5 % CO_2 (Forma Scientific 3193 Water Jacket Incubator, Ohio, USA). MDA-MB-231 cells were maintained in Leibovitch L15 supplemented with 10% (v/v) FCS at 37°C. L15 is used independent of CO_2 and so sealed flasks were used with this medium. FCS was heat inactivated for 10 min at 56°C and stored in 50 ml aliquots at –20° C. Antibiotics were not used for routine culture of cells. For experiments, both cell lines were cultured in Dulbelco's Modification Eagle's Medium

(DMEM) with no loss of viability or obvious morphological changes. RPMI, L15, DMEM and FCS were from Gibco-BRL (Paisley, Scotland). Tissue culture flasks were from Sarsedt (Wicklow, Ireland).

2.2.2 Cell subculture

Cells were checked daily using an inverted phase contrast microscope (Nikon TMS, Tokyo, Japan) and were sub-cultured when they reached 80 - 90% confluency. Both cell lines grew as anchorage dependent monolayers and were detached by trypsin treatment for sub-culturing. For trypsinisation, the growth medium was decanted and cells were rinsed twice with 5 mls of 0.01 M phosphate buffered saline (13.8 mM NaCl, 2.7 mM KCl, pH 7.4) (PBS) to remove residual FCS, 1 - 1.5 ml of trypsin-ethylenediamine tetracetic acid (EDTA) (0.05% (w/v) trypsin, 0.02% (w/v) EDTA) (Gibco-BRL, Paisley, Scotland) was added to the flasks and placed at 37°C for several minutes until the cells had clearly detached from the flask surface. 5 ml of complete medium (medium containing 10% FCS) was added and cells were transferred to a sterile centrifuge tube and pelleted by centrifugation (MSE Instruments Mistral 2000, London, UK) at 300 × g for 5 min. The supernatant was discarded and the cell pellet was resuspended in 5 ml of full medium and used to seed fresh flasks at a ratio of between 1:3 to 1:6 for BT474 cells and between 1:6 to 1: 12 for MDA-MB-231 cells. Approximately 5 ml and 12 ml of full medium were added to cells in 25 cm^2 and 75 cm^2 flasks, respectively. Cells were maintained as described (2.2.1).

2.2.3 Preparation of frozen cell culture stocks

Cell stocks were stored under liquid nitrogen in a cyrofreezer (L'air Liquide RCB40A, Champigny, France). Frozen stocks were prepared from cells in log phase growth at less than 80 % confluency. Cells were washed twice with PBS and trypsinised as above (2.2.2). Trypsinised cells were recovered and pelleted through a 2 - 3 ml cushion of FCS by centrifugation at 300 × g for 5 min. FCS containing cell debris was discarded and the cell pellet was resuspended, with FCS, at a concentration of 1 x 10^6 cells / ml. An equal volume of 10% (v/v) dimethyl sulfoxide (DMSO) in FCS was added dropwise to the cell suspension with gentle mixing between additions. 1 ml aliquots of the cell suspension were added to cryovials and placed in a styrofoam rack at - 80°C for 24 hr before being transferred to the liquid nitrogen tank for long term storage.

2.2.4 Revival of stored cells

Cryovials were removed from the liquid nitrogen and rapidly thawed at 37°C Cells were transferred to a centrifuge tube, 10 ml of full medium was added and the cells were pelleted by centrifugation at 300 × g for 5 mm The supernatant was discarded and the cell pellet was resuspended in 5 ml of full medium and added to a 25 cm^2 flask Cells were incubated at 37°C for 16 - 24 hr Following incubation the medium was replaced with fresh medium Cells were then cultured and passaged as before (2 2 2)

2.2.5 Human microvascular endothelial cells

Primary cultures of human dermal microvascular endothelial cells (HMECs) were obtained from BioWhittaker (Niederlassung, Germany) The manufacturer supplied Endothelial Cell Growth Medium (EGM) containing the following supplements hydrocortisone, hFGF-B, R3-IGF, ascorbic acid, heparin, VEGF, hEGF, GA-100 and 5% (v/v) FBS Medium was changed every 2 days HMEC cell cultures are irreversibly contact inhibited and therefore were subcultured before they reached 85% confluency For trypsinisation cells were rinsed twice with Hanks Balanced Salt Solution (HBSS) and trypsinised as before Once cells were detached trypsin was inactivated by the addition of an equal volume of a trypsin neutralising solution HBSS, trypsin/EDTA and trypsin neutralising solution were also supplied with the cells These cells have a very limited life span and usually reached senescence within 5 passages

2.2.6 Cell Counting

Cell suspensions were counted using an Improved Neubauer haemocytometer slide Trypan blue dye exclusion was used to determine cell viability 20 µl of cell suspension was added to 180 µl of trypan blue, mixed, and allowed to stand for 2 mm A sample of this mixture was added to the counting chamber and the cells visualised and counted under a light microscope Viable cells only were included The four corner squares of the grid, each containing a volume of 1 mm^2, were counted and the number of cells calculated using the following equation

$$\text{cells / ml} = \frac{\text{count x 1,000 x 10 (dilution factor)}}{4}$$

2.2.7 Mycoplasma testing of cell cultures

On arrival into the laboratory all cell cultures were tested for mycoplasma contamination Routine testing of cell cultures was also carried out every 6 months thereafter Mycoplasma testing was carried out using a commercial PCR ELISA (Boehringer Mannheim, Mannheim, Germany) Mycoplasma testing was carried out according to the manufacturers instructions except that the ELISA step was omitted since it was unnecessary for simply confirming the presence or absence of mycoplasma, which could be easily confirmed by gel analysis alone In brief, a 1ml sample of medium from cells nearing confluency was taken Cell debris was removed by centrifugation at 200 × g for 10 mm Supernatant was removed and mycoplasma were pelleted by centrifugation at 13,000 × g for 10 min The supernatant was decanted and the pellet resuspended in 10 µl of sterile distilled water (SDW) and 10 µl of lysis reagent and incubated for 1 h at 37°C A positive control, supplied with the kit, and a negative control with SDW and lysis buffer alone were also included Following this incubation 30 µl of neutralisation buffer was added 10 µl of sample was mixed with 25 µl of a PCR ready-to-go solution and 10 µl of SDW in a thermo-tube The tubes were placed in a PCR thermal cycler machine (PTC 2000 Peltier Thermal Cycler, MJ Research, MA, USA) and product was amplified using the following program 5 mm at 95°C, 40 cycles of 95°C for 30 sec, 62°C for 30 sec, 72°C for 1 mm and a final extension of 10 mm at 72°C The resulting PCR product was visualised by ethidium bromide staining following electrophoresis (2 4 3)

2.2.8 Isolation of monocytes

Approximately 30 ml of blood from human volunteers was collect by venopuncture into heparinised tubes (Sarsedt, Wicklow, Ireland) A discontinuous gradient of 10 ml of Histopaque 1119, overlaid with Histopaque 1077 (Sigma, St Louis, MO, USA) was prepared and the 30 ml of blood layered onto this The gradient was centrifuged at 400 × g for 30 min at room temperature Following centrifugation the cells layered as seen in Figure 2 1 The plasma layer was recovered and filtered through a 0 2 µm syringe filter (Gelman, Ann Arbor, MI, USA) The lymphocyte/monocyte layer was collected and cells were washed twice in DMEM and pelleted by centrifugation at 220 × g for 10 mm. After washing the pellet was resuspended and cells were counted and viability assessed (2 2 6) Monocyte content of recovered cells was established using flow cytometry staining for CD14/CD45 (2 2 10) The cells were resuspended at a

concentration of 1 x 10^6 monocytes / ml in DMEM containing 20% (v/v) autologous plasma. The cells were seeded into tissue culture plates and monocytes allowed to adhere by incubating at 37°C for 2 h. Following this incubation the non-adherent lymphocytes were removed by washing twice with PBS. Fresh DMEM containing 20% (v/v) autologous plasma was added to the monocytes.

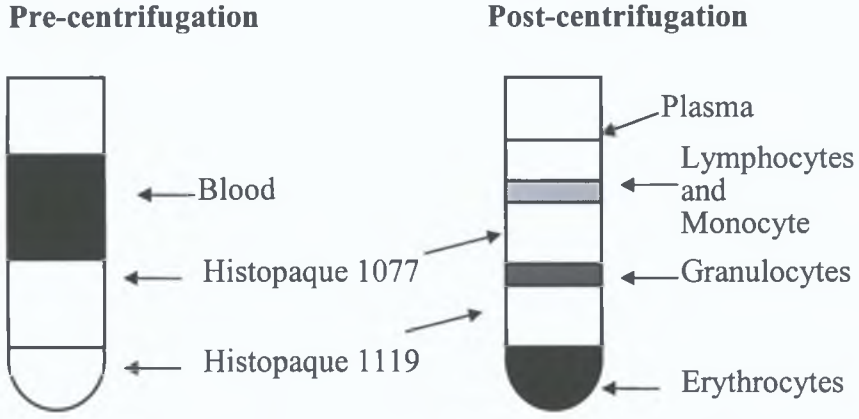


Fig. 2.1: Layers formed before and after centrifugation of whole blood through a Histopaque density gradient.

2.2.9 Preparation of monocyte derived macrophages

Monocytes isolated according to 2.2.8 were matured for 5 days in 96-well plates in DMEM containing 20% autologus plasma (AP). Over this period cells visibly increased in size. Following maturation monocytes were washed twice with PBS and activated for 24 hr by the addition of 100 ng recombinant human interferon γ (rh-IFNγ (10^3 units) (Gibco-BRL, Paisley, Scotland) in DMEM with 10% (v/v) FCS. Following activation the monocytes assume a macrophage like morphology.

2.2.10 CD14/45 Flow cytometry

Percentage monocytes present was assessed by determining the percentage of cells that expressed CD14 and CD45. CD45 is present on nearly all leukocytes while CD14 is specific for monocytes. A 100 µl aliquot of cells was incubated with 10 µl of CD14 and CD45 labeled monoclonal antibodies (Becton Dickinson, San Jose, CA, USA). The sample was mixed and incubated at 4°C for 30 min protected from the light. Cells were washed with 2 ml of cold PBS and centrifuged at 300 × g for 5 min. Cells were

resuspended with FACS fluid and analyzed (FACScan, Becton Dickinson, San Jose, CA, USA). Percentage of CD14 positive leukocytes was calculated. A sample dot plot and histogram for CD14/CD45 flow cytometric analysis can be seen in appendix 2.

2.2.11 Proliferation assay

For proliferation assays 200 µl of cells at a concentration of 1×10^5 cells/ml were seeded into 96-well plates in DMEM with 10% (v/v) FCS and incubated for 16 - 24 hr. Tamoxifen or 4-hydroxytamoxifen (Sigma, St.Louis, MO, USA) was added at various doses in 200 µl of DMEM with 10% (v/v) FCS and incubated for 72 hr. These drugs are very insoluble in aqueous solutions and must be initially solubilised in ethanol. An ethanol control containing 0.5% (v/v) ethanol in DMEM (equivalent to the volume of ethanol in the highest doses of tamoxifen) was also included. Cell proliferation was assessed using a CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) and was used in accordance with the manufacturers instructions. In brief, cells are incubated for 4 hr with 15 µl of a tetrazolium dye and living cells metabolise the tetrazolium to a formazan product. 100 µl of solubilisation solution was added and incubated at 37°C for 16 hr to solubilise the formazan product. Absorbance was measured at 570 nm in a plate reader. The absorbance reading is directly proportional to the number of cells present in each well. The proliferation of control cells which received no treatments was expressed as 100% and proliferation in other experimental cells was expressed as % of controls.

2.2.12 Human microvascular endothelial cell migration (HMEC)

Endothelial cell migration was assessed using Transwells® (Costar, Cambridge, MA, USA). These are sterile, disposable, 24-well plate inserts that form two different compartments within a well separated by membrane containing 8 µm pores (Figure 2.2). Cells on the upper surface of the membrane can migrate through the 8 µm pores onto the lower surface of the membrane. Migration of cells can be assessed by counting the number of cells that have migrated in response to a chemoattractant added to the bottom compartment. Endothelial cells were collected from tissue culture flasks and 10^5 cells in 250 µl of EGM added onto the top compartment of a Transwell® with 500 µl of EGM alone in the bottom compartment. Cells were allowed to recover overnight. Chemoattractant (20 ng/ml VEGF in EGM) was added to the bottom compartment and tamoxifen at various dose (0, 0.625, 1.25, 2.5, 5 µg/ml and a 0.5% (v/v) ethanol control)

was added to both compartments Cells were incubated for 48 hr Following incubation the inserts were removed and cells on the top surface of the porous membrane were removed by wiping the membrane with a wet cotton swab Cells on the lower surface of the membrane were fixed by incubating with 0 4% (v/v) formaldehyde for 30 min and stained with haemotoxylin for 1 mm Membranes were then removed from the insert housing using a scalpel and mounted on slides in DPX mounting medium Migrated cells on each membrane were counted using a light microscope under 400 × magnification

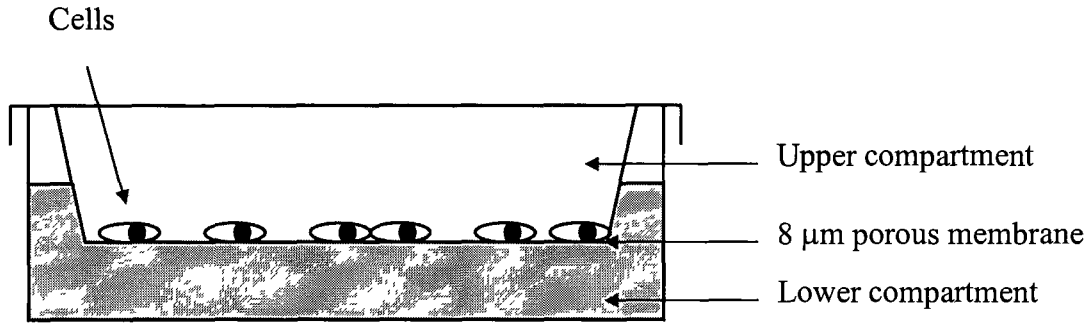


Fig. 2.2: Illustration of a Transwells®

2.2.13 Transendothelial migration of monocytes

Transendothehal migration of monocytes was assessed using collagen coated Transwells® (Costar, Cambridge, MA, USA) with a 3 μm pore membrane in 12-well plates HMEC were removed from culture with trypsin (2 2 5) and 5 × 10^5 cells in 0 5 ml of EGM was added to the top chamber and 1 ml of EGM to the bottom chamber Cells were allowed to recover overnight and were examined the following day to ensure that a confluent monolayer of endothelial cells had formed that left no parts of the porous membrane exposed Monocytes were isolated as described (2 2 8) and 5 × 10^5 monocytes added to the top compartment in 0 5 ml of EGM Tamoxifen (0, 2 5 μg/ml and a solvent control containing 0 5% (v/v) ethanol) was added to both the upper and lower reservoirs VEGF at a concentration of 20 ng/ml was added to the lower compartment to act as a chemoattractant for monocytes Cells were incubated for 5 hr and cells from the top compartment removed Cells in the lower compartment were collected by removing the medium and by gently washing the well and the underside of

the Transwell membrane in 0.0025% (w/v) trypsin, 0.01% (w/v) EDTA in PBS. Monocytes were counted using an a haemocytometer (2.2.6).

2.2.14 Preparation of cells

Cells required for an experiment were harvested by trypsinisation and counted. When looking at VEGF or TGFβ-1 protein, 200 µl of cells were seeded onto 96-well plates at a density 0f 100,000 cells/ml. For reverse transcription polymerase chain reaction (RT-PCR) 1 ml of cells at 100,000 cells/ml were seeded onto 12-well plates. Cells were allowed to recover for approximately 16 hr in DMEM with 10% (v/v) FCS. Experiments were usually carried out using DMEM containing 0.5% (w/v) tissue culture grade BSA (Sigma, St.Louis, MO, USA). Treatment with cytokines and tamoxifen was carried out by preparing these agents in DMEM and adding to the wells for the stated incubation period. Following incubation the conditioned medium was removed and stored at -20°C. Plates were also stored at -20°C for protein assay as described in section 2.7.3.

Hypoxic conditions were simulated by placing plates into a sealed chamber and sparging with 5% CO_2/95% N_2 for several minutes to expel air. This resulted in an aqueous O_2 concentration of approximately 2% as measured using a probe for dissolved O_2.

2.3 RNA Extraction

2.3.1 General Precautions

Since RNA is readily degraded by ubiquitous RNase enzymes a number of precautions were always taken when working with RNA. Gloves and a clean lab coat with elasticated sleeves were worn at all times.

All buffers were autoclaved or sterile filtered. Diethylpyro-carbonate (DEPC) inactivates RNases and DEPC-treated water was prepared by adding DEPC at 0.01% (v/v) for 16 hr. DEPC was then inactivated by autoclaving. Disposable plastics were used when possible. When using glassware it was soaked in 0.5 M NaOH for 2 hr, rinsed in DEPC-treated water, autoclaved and dried for 3 - 4 hr at 120°C. Non-disposable plasticware such as gel boxes and accessories were soaked in 0.5 M NaOH and rinsed in DEPC water. RNase free filter tips were used for pipetting. Solid chemicals were never weighed using a spatula but rather tipped out. All solutions were stored in aliquots.

2.3.2 Isolation of total RNA from cultured cell monolayers

RNA was isolated from cell cultures growing in 12 well plates or 25 cm^2 flasks Cells were washed twice with PBS at 4°C 600 µl of TRIZOL® LS (Gibco-BRL, Paisley, Scotland) was added to a single well of a 12 well plate and 2 ml of Trizol to a 25 cm^2 flask TRIZOL® LS was incubated for 5 mm at room temperature and removed to a clean microfuge tube 300 µl of chloroform was added per 600 µl of TRIZOL® LS, vigorously shaken and allowed to stand at room temperature for 2 - 15 mm Phases were separated by centrifugation at 12,000 × g for 15 min (Eppendorf Centrifuge 5417, Hamburg, Germany) Approximately 300 µl of the aqueous phase was removed and the RNA precipitated with 1 ml of isopropyl alcohol (IPA) for 10 min at room temperature RNA was pelleted by centrifugation at 12,000 × g for 10 min The supernatant was aspirated and the pellet washed m 1 ml of 70% (v/v) ethanol RNA was pelleted by centrifugation at 7,500 × g for 5 min, ethanol was carefully aspirated and the pellet was air dried over 2 - 4 min Pellets were resuspended in a suitable volume of DEPC water and immediately placed on ice The concentration and purity of RNA was assessed spectrophotometrically as outlined in 2 3 3 Integrity of RNA was also analysed by gel electrophoresis (2 3 4)

2.3.3 Quantification of RNA

RNA was assessed spectrophotometrically using a Genequant (Pharmacia, Cambridge, UK) by measuring the absorbance at 260 nm, the wavelength at which nucleic acids absorb maximally (λ_{max}) A 40 µg/ml sample of RNA has an absorbance value of 1 at 260nm The purity of RNA was determined by reading the absorbance at 260 nm, the λ_{max} of nucleic acids, and at 280 nm, the λ_{max} for proteins and obtaining the ratio of these absorbances Pure RNA has an A_{260}/A_{280} ratio of 1 6 - 1 8 Lower ratios often indicate presence of protein and higher ratios the presence of organic reagents Absorbance was measured in a quartz cuvette with a suitable dilution of RNA sample

2.3.4 Qualitative gel electrophoresis of RNA

The integrity of RNA was evaluated by electrophoresis The presence of two discrete strong bands, representing the 28 S and 18 S ribosomal RNA subunits, signified intact RNA RNA was electrophoresed in a similar manner as that described m section 2 4 3 except that 5 µl of RNA sample was mixed with 15 µl of sample buffer (50 % (v/v)

formamide, 8 3 % (v/v) formaldehyde, 0 027 M MOPS, 6 7 mM sodium acetate, 0 67 mM EDTA) and 3 µl of loading buffer (50 % glycerol (v/v), 1 mM EDTA, 0 4 % (w/v) bromophenol blue, 1 µg/ml ethidium bromide) RNA was denatured by heating to 70°C for 10 min (Techne Dri-Block DB-1M, Cambridge, UK) and electrophoresed as for DNA electrophoresis

2.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to detect and compare mRNA levels of VEGF in cells β-actm, a constitutively expressed gene, was also amplified and acts as an internal control for quantity of starting material and gel loading differences The ratio of VEGF to β-actin allows semi-quantitative comparison between samples The conditions used for amplification of VEGF and β-actin have been previously demonstrated to allow semi-quantitative analysis (Harmey et al 1998) Qualitative RT-PCR was also carried out to detect the isoforms of VEGF produced but was not quantitative

2.4.1 Reverse Transcription

1 µg of total RNA was used for the RT reaction DNase I treatment was carried out to remove contaminating DNA 1 µl of DNase I (Gibco-BRL, Paisley, Scotland) (1 U) was added to 1 µg of total RNA in 10 µl of 1 × DNase buffer in DEPC water and incubated at room temperature for 15 mm DNase I was inactivated by adding 1 µl of 25 mM EDTA and heating at 70°C for 10 mm 1 µl random primer (Promega, Madison, WI, USA)(500 µg/ml) was added to DNase treated RNA and incubated at 70°C for 10 mm and then placed on ice The following RT reaction components were prepared as a mastermix and added to each reaction sample 0 5 µl DEPC water; 0 5 µl RNasin (Promega, Madison, WI, USA) (19 U), 1 µl 5 mM dNTPs (1 1 1 1 dATP, dCTP, dGTP, cTTP)(Promega, Madison, WI, USA), 4 µl 5X RT buffer (supplied by the manufacturers with Superscript II™ reverse transcriptase), 2 µl 0 1 M DTT, 1 µl Superscript II™ Reverse Transcriptase (200 U)(Gibco-BRL, Paisley, Scotland) cDNA synthesis was carried out for 3 hr at 37°C and the cDNA either used immediately for PCR or stored at -20°C for a maximum of 48 hr

2.4.2 Polymerase Chain Reaction

The PCR reaction components were prepared as a mastermix and added to 1 5 µl of cDNA Each 50 µl reaction contained the following 5 µl 10 x buffer (supplied by the manufacturers with Taq polymerase enzyme), 2 µl 25 mM $MgCl_2$, 2 µl 5 mM dNTPs, 1 µl of forward and reverse primers (50 pmol)(Genosys, Cambridge, UK), 0 5 µl Taq DNA polymerase (2 5 U) (Promega, Madison, WI, USA) and DEPC water to a final volume of 48 5 µl Samples were placed in the PCR machine (PTC 2000 Peltier Thermal Cycler, MJ Research, MA, USA) and amplification carried out using the appropriate cycle The cycle used for amplification of VEGF was 30 cycles of 94°C for 1 mmute, 55°C for 2 minutes, and 72°C for 1 5 minutes using the following primers, which amplify all VEGF isoforms, as described previously (Berse et al 1992)

forward 5'-CGC AAG CTT AGG AGT ACC CTG ATG AG-3'

reverse 5'-CCG TCT AGA ACA TTT GTT GTG CTG T-3'

These primers amplify a 204 bp fragment The cycle used for amplification of β-actin was 32 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 1 5 minutes using a commercial actin primer mix (Stratagene, La Jolla, CA, USA) which amplifies a 661 bp fragment Specific isoforms were amplified by using primers that hybridise to opposite ends of the VEGF gene so that each PCR product will be of a different size depending on the number of exons present in each isoform Isoforms of VEGF were amplified using 30 cycles of 94°C for 1 min, 58°C for 1 5 min and 72°C for 2 mm The reaction conditions were same as for total VEGF reaction except the mastermix was comprised of 3 µl of 25 mM $MgCl_2$ for each 50 µl reaction and Taq was added just prior to the beginning of the cycle when the samples were at 94°C The following primers were used (Figure 2 3)

forward 5'-GGA GGG CAG AAT CAT CAC-3'

reverse 5'-CCG CCT CGG CTT GTC ACA-3'

Isoform specific primers were designed using Oligo 4 (Molecular Biology Insights, Cascade, CO, USA) and specificity was checked by comparing the primer sequence against the Genbank database to ensure that primers were specific to the VEGF gene All primers used span an intron so that if genomic DNA was amplified it would be of a far greater size than a product from cDNA PCR was visualised by electrophoresis on a 1 5 % agarose gel as described in 2 4 3 or for isoform specific PCR after southern blotting (2 6)

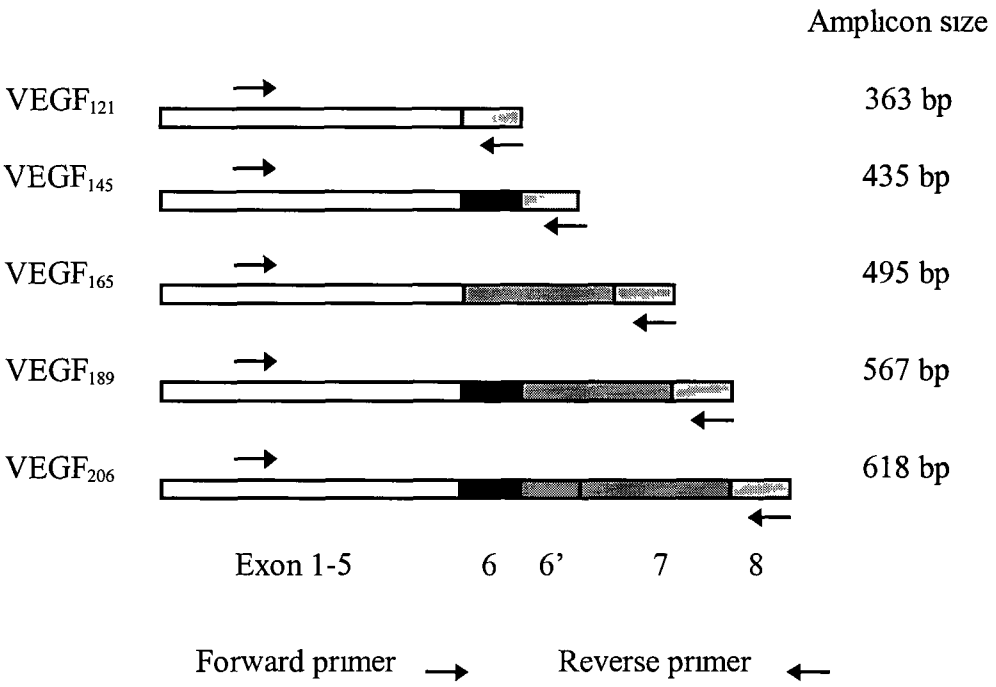


Fig. 2.3: Primers used for isoform specific PCR amplification Primers anneal to opposite ends of the VEGF transcript so that the size of the PCR product will be dependent on the number of exons in the transcript and therefore on the isoform present

2.4.3 Agarose gel electrophoresis of DNA

An agarose gel was prepared by boiling an appropriate amount of agarose in 50 ml of 1 x TAE buffer (40 mM Tris, 20mM acetic acid, 1 mM EDTA, pH 8 0) A 1 5 % (w/v) gel was the most commonly used The solution was allowed to cool to approximately 60°C (warm to the hand) and poured into a level gel mold of an Easi-E-Cast Gel System (Hybaid, Middlesex, UK) Bubbles were removed, the combs were put in place and the gel allowed to set Once the gel had set it was placed in the electrophoresis tank filled with 1 x TAE A suitable volume of 6 x loading buffer (30% (v/v) glycerol, 0 01% (w/v) bromophenol blue) was added to samples to give a final concentration of 1 × The gel was run at a constant voltage of 80 V (E-C Apparatus Corporation EC105, FL, USA) until the bromophenol blue in the loading buffer approached the end of the gel Bands were visualised on a UV transilluminator (Syngene, Cambridge, UK)

2.5 Densitometry

Blots and gel images were recorded using a Syngene UV transilluminator and white box in a Syngene darkroom with camera. The software used was GeneSnap version 2.6 by Syngene. Densitometric analysis was performed using GeneTools version 2.1.

2.6 Southern Blotting

2.6.1 Preparation of competent cells

Cells used for transformation were *E. coli* DH10B. Cells were streaked onto LB agar plates (0.005% (w/v) yeast extract, 0.01% (w/v) tryptone (both Bacto Laboratories, Liverpool, UK), 0.25 M NaCl, 0.015% (w/v) agar (Bacto Laboratories); pH 7.0) and incubated overnight at 37°C. Single colonies were selected the following day and cultured for 2 hr at 37°C with vigorous shaking at 200 rpm in an orbital shaker (Gallenkamp, Loughborough, UK). Contents were transferred to 75 ml LB broth (0.005% (w/v) yeast extract, 0.01% (w/v) tryptone, 0.25 M NaCl; pH 7.0) in a 250 ml flask and returned to the incubator. The absorbance at A_{550} was monitored until it reached 0.5 and the cells were placed on ice. Cells were collected by centrifugation at 1 500 × g for 5 min at 4°C. The cell pellet was resuspended in 30 ml of transformation buffer I (30 mM K-acetate, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15% (w/v) glycerol, final pH 5.8) and left on ice for 10 min. Cells were pelleted by centrifugation and resuspended in 3 ml transformation buffer II (10 mM Na-MOPS, 75 mM $CaCl_2$, 10 mM KCl, 15% (w/v) glycerol, final pH 7.0). Competent cells were aliquoted and flash frozen in liquid nitrogen and stored at –80°C.

2.6.2 Transformation of competent cells

pGEM3Z-hVEGF was a gift from Dr Larry Brown (Berse et al. 1992) and contains a 204 bp fragment of the VEGF gene cloned into a *Hin*dIII and *Eco*RI site of the multiple cloning site (Figure 2.4). An aliquot of competent cells was thawed on ice and 100 µl of cells incubated for 20 min on ice with 10 ng of plasmid in 1 µl. Cells were heat shocked for 90 sec at 42°C. 900 µl of LB broth was added and cells incubated for 1 hr at 37°C. 100 µl of transformed cells was spread on LB agar plates containing 50 µg/ml ampicillin and incubated overnight at 37°C. Transformed colonies were picked the following day and cultured in 10 ml of LB broth, containing 50 µg/ml ampicillin, for 16 hr at 37°C with vigorous mixing. Cells were then either frozen at –80°C or collected for

a miniprep of the VEGF plasmid. Transformation efficiency was approximately 1 x 10^7 colony forming units (cfu) /μg DNA.

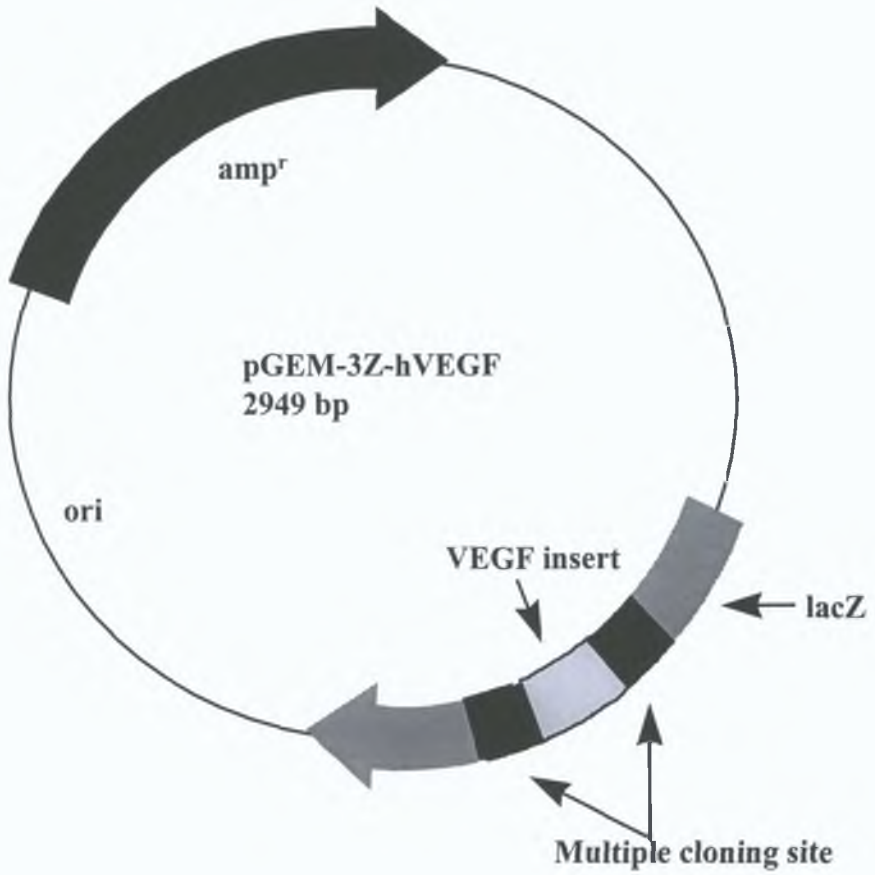


Fig 2.4: Plasmid map of pGEM3Z-hVEGF. A 204 bp fragment of the VEGF gene was cloned into the multiple cloning site between *Hin*dIII and *Eco*RI restriction sites. Interruption of the lacZ allows detection of positive clones since the ability to degrade X-gal is lost.

2.6.3 Preparation of probe

A single colony of transformed cells were cultured overnight in 10 ml of LB broth containing 50 μg/ml of ampicillin. Plasmids were isolated the following day using Wizard DNA Minipreps (Promega, Madison, WI, USA) according to the manufacturers instructions. In brief, 2 ml of cells were pelleted by centrifugation at 21,000 × g for 10 min. The pellet was resuspended in 200 μl of Cell Resuspension Solution. Cells were lysed by the addition of 200 μl of Lysis Solution. 200 μl of Neutralisation Solution was added and cellular debris was pelleted by centrifugation at 21,000 × g for 5 min. Cleared supernatant was aspirated and placed in a fresh tube. 1 ml of Wizard™

Miniprep DNA Purification Resin was added to the supernatant and the contents mixed. The Resin/DNA mix was added to a Wizard™ Minicolumn using a 5 ml syringe. The column was washed with 2 ml of Washing Solution and the Wash solution was completely removed by centrifugation in a microfuge tube at 21,000 × g for 2 min. The column was transferred to a fresh microfuge tube and 50 µl of SDW added. DNA was eluted by centrifugation at 21,000 × g for 20 sec. DNA was checked by electrophoresis and quantified by spectrophotometry. The VEGF insert was released by digesting 7.5 µg of DNA with 5 µl of *Eco*RI (50 U) and 10 µl of *Hin*dIII (100 U) (both enzymes from Gibco-BRL, Paisley, Scotland) with 25 µl of 10 × React 2 buffer in a total volume of 250 µl for 1.5 hr at 37°C. DNA was precipitated by adding 25µl of 3 M Na-acetate, pH 5.5 and 550 µl of 95% (v/v) ethanol and leaving at –20°C overnight. DNA was pelleted by centrifugation at 12,000 × g for 20 min and resuspended in 20 µl of TE (10 mM Tris, 1 mM EDTA, pH 7.4). Digested DNA was electrophoresed through a low melting point agarose gel (Gibco-BRL, Paisley, Scotland). The insert band was excised using a scalpel. Insert was then purified using Wizard PCR Preps (Promega, Madison, WI, USA) according to the manufacturers instructions. Briefly, the excised band was melted by heating to 70°C for 5 min. 1 ml of Wizard™ PCR Prep Resin was added and the contents gently mixed. The Resin/DNA mix was added to a Wizard™ Minicolumn using a 5 ml syringe. The column was washed with 2 ml of 80% (v/v) isopropanol and isopropanol was completely removed by centrifugation in a microfuge tube at 12,000 × g for 20 sec. The column was allowed to air dry for 2 min, transferred to a fresh microfuge tube and 50 µl of SDW added. DNA was eluted by centrifugation at 12,000 × g for 20 sec. Purified plasmid was examined by electrophoresis.

2.6.4 Southern blotting

DNA samples were electrophoresed on an appropriate agarose gel. The gel was photographed with a ruler placed along the side of the gel. DNA was denatured by washing the gel twice for 30 min each in 1.5 M NaCl, 0.5 M NaOH. The gel was rinsed briefly with de-ionised water and washed twice for 30 min each with neutralisation buffer (1 M Tris-HCl, ph 7.4, 1.5 M NaCl). To transfer the DNA, 0.45 µm nylon transfer membrane (Hybond, Amersham, Uppsala, Sweden) was cut to an appropriate size, pre-wetted in SDW and placed onto the gel. These were then placed in the sandwich depicted below (Figure 2.5). All items of the sandwich were carefully layered so as to ensure that there were no bubbles between any of the layers. The membrane

was marked with the orientation of the gel and the gel was surrounded with clingfilm to prevent the separate filter paper layers from contacting as the gel is reduced in size. The buffer reservoir was filled with 10 × SSC and DNA allowed to transfer by capillary diffusion for 16 hr. After transfer the membrane was removed and washed in 5 × SSC. DNA was fixed by baking at 80°C for 2 hr.

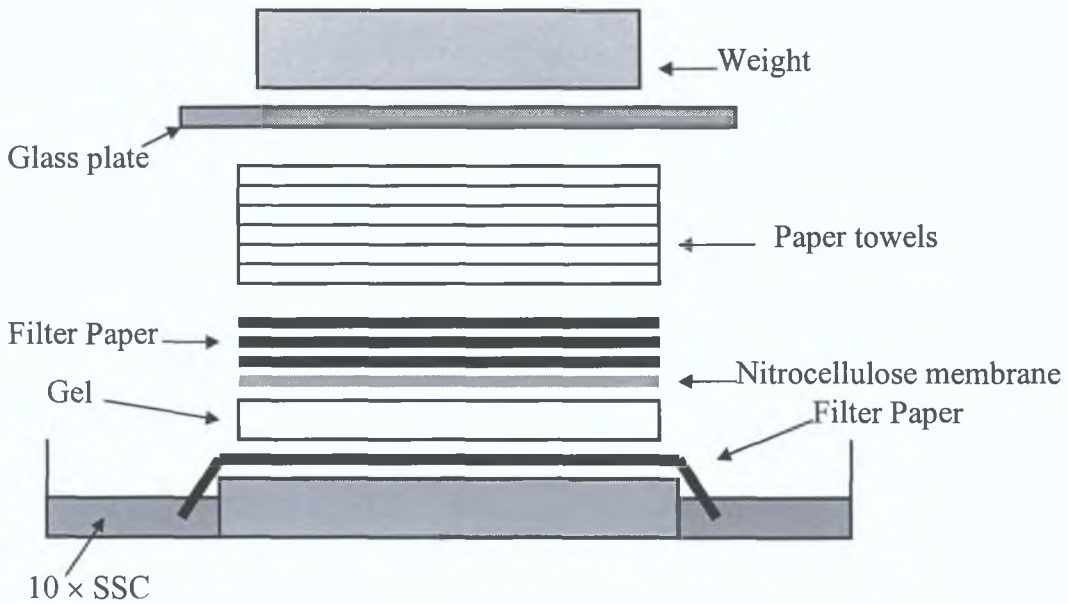


Fig. 2.5: Preparation of southern blot sandwich for transfer of DNA onto nitrocellulose

2.6.5 Probe labeling

Probe labeling was carried out using Prime-A-Gene system (Promega, Madison, WI, USA) according to the manufacturers instructions. 25 ng of gel purified VEGF insert in 10 µl of SDW was denatured for 5 min at 95°C and placed on ice. In a second screw cap centrifuge tube the following components were added in the order shown: 10 µl 5 × labeling buffer, 2 µl dNTPs, 10 µl of denatured DNA, 2 µl of BSA, 5 µl ^{32}P-dATP (50 µCi) (Amersham, Uppsala, Sweden), 20 µl SDW, 1 µl (5 U) Klenow fragment DNA polymerase I (Gibco-BRL, Paisley, Scotland). Probe labeling was carried out for 1 hr at room temperature. 2 µl of 0.5 M EDTA was added and the reaction heated to 95°C for 2 min to terminate the reaction. The labeling reaction was then placed on ice and the

probe purified by Nick® Columns Sephadex G-50 (Pharmacia, Cambridge, UK) according to the manufacturers instructions. In brief, excess liquid was poured off and the column rinsed once with TE (10 mM Tris-HCl, ph7.8; 1 mM EDTA). 3 ml TE buffer was added to the top of the column and allowed to run through. Labeled probe was then added to the column and 400 µl of TE added and allowed to run through. Probe was eluted with a further 400 µl TE buffer and collected. 100 µl of 10 mg/ml salmon sperm DNA, 80 µl SDW and 150 µl 1M NaOH was added to 400 µl of probe. Probe was then denatured at 95°C for 5 min and placed on ice. 150 µl 1M HCl and 800 µl 1M potassium phosphate, pH 6.5 were added, mixed and the probe added to hybridisation fluid.

2.6.6 Hybridisation

The membrane was prehybridised for 3 hr at 65°C in 20 ml of Rapid-Hyb buffer (Amersham, Uppsala, Sweden). Denatured probe was added to the hybridisation buffer and allowed to hybridise overnight. Hybridisation fluid was removed and the membrane was rinsed twice in 2 × SSC, 0.1% (w/v) SDS, washed twice for 15 min each in 2 × SSC, 0.1% (w/v) SDS at room temperature and then washed twice for 30 min each in 0.5 × SSC, 0.1% (w/v) SDS at 65°C. Membranes were sealed in plastic and exposed to X-ray film.

2.7 Protein electrophoresis

2.7.1 Protein isolation from cell cultures

Cell monolayers were washed twice with ice cold PBS. Cells were lysed in 1 X TNE (5 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 5mM EDTA) containing 0.5% (w/v) SDS, 0.5% (v/v) Triton X-100, 0.005% (w/v) deoxycholic acid and 1 mM phenylmethylsulfonyl fluoride (PMSF). Minimum volumes of lysis buffer were used to ensure maximum concentration of protein, typically 0.5 ml for a 75 cm^2 flask. The culture vessel containing the lysis buffer was placed on ice for 15 - 30 min. The lysate was passed through a 20 gauge needle several times to shear the genomic DNA and then boiled for 10 min. Debris was pelleted by centrifugation at 15,000 g for 10 min. The cleared lysate was stored at -20°C in aliquots. If the recovered protein extract was very dilute it was concentrated by precipitation overnight at -20°C in at least 4 volumes of methanol. Protein was recovered by centrifugation at 15,000 × g for 10 min and resuspended in 1 × TNE buffer.

2.7.2 Protein isolation from tissue samples

VEGF and TGFβ-1 were measured in normal and malignant breast tissue Tissue samples were removed from mastectomy samples by a pathologist Normal tissue was that which looked macroscopically normal and was sufficiently distant from the tumour margins as to be considered free of tumour Tissue samples were divided into separate cryotubes and flash frozen in liquid nitrogen within approximately half an hour of resection They were then stored at -80°C until they were required On the day samples were required they were ground to a fine powder using a mortar and pestle with liquid nitrogen The powder was resuspended m tris buffered saline (TBS) (25 mM Tris-HCl, pH 7 6, 150 m M NaCl) and homogenised using a Dounce homogeniser The homogenate was centrifuged at 15,000 g for 10 min and the supernatant removed to a fresh tube and assayed for VEGF and TGFβ-1 using a commercial ELISA (R&D Systems, Abingdon, UK and Genzyme, Cambridge, MA, USA, respectively) Protein levels were determined (2 7 3) and the levels of each factor expressed as pg VEGF/mg protein and ng TGFβ-1/mg protein

2.7.3 Protein Determination

The bichinoic acid (BCA) protein microassay (Pierce, Rockford, IL, USA) was used for the determination of total protein In this assay Cu^{++} reacts with the protein under alkaline conditions to give Cu^{+} which reacts with the BCA to give a coloured product The commercially available assay is provided as two reagents, A, an alkaline bicarbonate solution and B, a copper sulphate solution, which are mixed together at a 50 1 ratio prior to use 200 µl of working reagent was added to 10 µl of sample or standard in a 96-well plate and incubated at 37°C for at least 30 mm The absorbance of each well was read at 570 nm (Bio-Tek Microplate Autoreader EL311, VT,USA) The protein concentration was determined from a standard curve of known concentrations of BSA between 0 and 1000 µg/ml

2.7.4 Denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE)

PAGE was carried in the presence of SDS and 2-mercaptoethanol to denature proteins A 12% (w/v) gel was generally used for Western blot analysis A 7 5% (w/v) gel was used for Western blots analysis of the KDR protein Separating gel was prepared by mixing the following solutions

Separating	7 5 %	12 %
acrylamide bisacrylamide (37 5 1)	5 5 ml	9 2 ml
1 875 M Tris-HCl, pH 8 8	4 5 ml	4 5 ml
Distilled water	12 ml	8 3 ml
10 % (w/v) SDS	220 µl	220 µl
10 % (w/v) ammonium persulphate	150 µl	150 µl
N,N,N',N'-tetramethyl-ethylenediamine (TEMED)	10 µl	10 µl

The solutions were mixed and cast in vertical gel plates (Atto Corporation AE-6450 mini-slabs, Tokyo, Japan) A small volume of IPA was added to the gel to aid polymerisation by excluding oxygen, removing bubbles and keeping the top of the gel level Once the gel had set the IPA was poured off and the gel was rinsed in distilled water The stacking gel was prepared and added onto the resolving gel and combs inserted

Stacking Gel 1 7 ml_acrylamide bisacrylamide (37 5 1), 2 ml 0 6 Tris-HCl, pH 6 8, 6 ml distilled water, 100 µl 10 % (w/v) SDS, 150 µl 10 % (w/v) ammonium persulphate, 10 µl TEMED

An equal volume of 2 × Llaemmh buffer (2 ml 0 6 M Tris-HCl, pH 6 8, 5 ml 10 % (w/v) SDS, 1 ml 2-mercaptoethanol, 2 ml glycerol and 0 01% (w/v) of bromophenol blue) was added to sample or protein markers and denatured by boiling for 10 min The samples were added to the wells and electrophoresed at 20 mA /gel in electrode buffer (50 mM Tris base, 384 mM glycine and 0 1 % (w/v) SDS) until the dye front approached the bottom of the gel

2.7.5 Staining with Coomassie Brilliant Blue

Gels were stained for protein by immersion in Coomaisse brilliant blue (50% (v/v) methanol, 10% (v/v) acetic acid, 0 25% (w/v) Coomaisse brilliant blue G250) for 30 min Gels were destained with 5% (v/v) methanol with 7 5% (v/v) acetic acid until bands were visible

2.8 Western Blotting

Following electrophoresis the gel was overlaid with 0 2 µm nitrocellulose membrane and 3 layers of Whatman blotting paper stacked over the gel with no bubbles between any of the layers A wet transfer was carried out in transfer buffer (0 15 M glycine, 20 mM Tris, 0 01 % (w/v) SDS, 20 % (v/v) methanol) at 70 mA overnight or 400 mA for 3 hr in a Bio-Rad Trans Blotter (Hercules, CA, USA) During transfer temperature was maintained at 4°C using a cooling apparatus (Medingen KK5E, Bonn, Germany) Following transfer the membrane was blocked for at least 1 hr with 5 % (w/v) dried milk (Marvel) in TBST (25 mM Tris-HCl, pH 7 6, 150 m M NaCl, 0 05 % (v/v) Tween 20) and the primary antibody (VEGFR-2 from Santa Cruz, CA, USA) was added at a suitable dilution (1 100) in blocking buffer for 2 hr Following incubation with primary antibody the membrane was washed 3 times for 10 min each in TBST The appropriate secondary antibody was diluted in TBST (1 750 for anti-rabbit IgG alkaline phosphatase (AP) conjugate (Promega, Madison, WI, USA) and 1 2,000 for anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Dako, Cambridge, UK)) and added to the membrane for a 2 hr incubation with gentle agitation The membrane was washed for 10 min each in TBST For alkaline phosphatase (AP) conjugated antibodies the bound antibody was detected using a colorimetric substrate (0 335 mg/ml BCIP, 0 67 mg/ml NBT (Promega, Madison, WI, USA) in substrate buffer of 100 mM Tris-HCl, pH 9 5, 100 mM NaCl, 5 mM $MgCl_2$) The reaction was stopped, when bands were visible, by pouring off substrate solution and adding water For horseradish peroxidase (HRP) conjugated antibodies a chemiluminescent substrate system was used (Pierce, Rockford, IL, USA) The reagents are provided as two solutions which are mixed equally just before development and added to the blot for 5 min Following this incubation the membrane was sealed in plastic and exposed to an X-ray film in a dark room and developed Exposure times varied between a few seconds and several minutes depending on the amount of starting protein

2.9 Serum processing

Preoperative blood was collected from patients and also from healthy controls. Blood was collected by venopuncture into serum blood tubes (Sarstedt, Wexford, Ireland). Blood was centrifuged at 1,100 × g for 30 minutes. Serum was collected and filtered through a 0.2 μm Acrodisc (Gelman Sciences, Ann Arbor, MI, USA) and stored in aliquots at -80°C. Ascites fluid, freshly drained, was processed in the same way.

2.10 VEGF ELISA

VEGF protein levels were determined using an ELISA. For cell culture supernatants a sandwich ELISA using two antibodies was developed. Plates were prepared by adding 100 μl of the coating antibody (R&D Systems Anti-human VEGF neutralising antibody) at a concentration of 0.7 μg/ml in PBS and incubating overnight at room temperature in sealed plates. The following day the plates were washed, 4 times, with wash buffer (0.05% (v/v) Tween-20 in PBS, pH 7.4) and blocked by incubating with blocking buffer (1% (w/v) BSA and 5% (w/v) sucrose in PBS) for 1 hr at room temperature. Following blocking, plates were washed again and at this stage could be used directly or could be stored by drying completely and storing sealed, with desiccant, at 4 - 8°C for a few months. Samples or standards were prepared in diluent (0.1% (w/v) BSA, 0.05% (v/v) Tween-20 in TBS, pH 7.3) and 100μl added to the wells for 2 hr at room temperature. The plates were washed 4 times with wash buffer and 100μl of biotinylated detection antibody (R&D Systems Biotinylated Anti-human VEGF antibody) at a concentration of 500 ng/ml in dilution buffer was added for a 2 hr incubation at room temperature. The plate was washed again 4 times with wash buffer and 100μl of streptavidin HRP (Zymed Laboratories, San Francisco, CA, USA) added for 30 min at room temperature. The plate was washed 4 times with wash buffer and 100 μl of substrate was added (0.1 mg tetramethylbenzidine (TMB), 10% (v/v) DMSO, 0.045 M phosphate-citrate buffer, 0.006 % (v/v) hydrogen peroxide, pH 5.0) for 40 min at room temperature in the dark. The reaction was stopped by the addition of 50 μl of 0.5 M H_2SO_4. Absorbance was read at 450 nm with a correction wavelength of 570 nm. VEGF levels in samples were determined from a standard curve constructed from known standards (Appendix 1A)

The VEGF ELISA used for cell culture supernatants was found to be unsuitable for measurements of VEGF in serum or tumour homogenates and a commercial ELISA was used instead (R&D Systems, Abington, UK). This was used in accordance with the manufacturers instructions. In brief, 100 μl of assay diluent and 100 μl of sample or

standard was added to each well and incubated for 2 hr at room temperature. The contents were aspirated and the wells washed 4 times by filling each well with 400 µl of wash buffer. 200 µl of an antibody conjugate was added for a 2 hr incubation at room temperature. The wells were washed 4 times with 400 µl of wash buffer and 200 µl of substrate added for 25 min. The reaction was stopped by the addition of 50 µl of stop solution and the absorbance for each well read at 450 nm with a 570 nm correction. VEGF levels in samples were determined from a standard curve constructed from known standards (Appendix 1B).

2.11 TGFβ-1 ELISA

TGFβ-1 levels were measured in culture supernatants, serum and tumour homogenates using a commercial ELISA (Genzyme, Cambridge, MA, USA). The assay was performed in accordance with the manufacturers instructions. In brief, TGFβ-1 was activated by acidification. For serum samples 450 µl of assay diluent was added to 10 µl of serum sample and 20 µl of 1M HCl for 1 hr at 4°C. For cell culture samples 200 µl of sample was mixed with 200 µl of sample diluent and 20 µl of 1M HCl for 1 hr at 4°C. The acid was neutralised by the addition of 20 µl of 1M NaOH for the serum samples and 15 µl of 1M NaOH for cell culture samples. 100µl of activated sample was added to a 96-well plate for 1 hr at 37°C. Sample was removed and the wells were washed 5 times by filling the wells with 400µl of 1 × wash buffer. 100 µl of an anti-TGFβ-1 HRP-conjugate was added to each well for 1hr at 37°C. Conjugate was aspirated and the wells washed again, 5 times, by the addition of 400 µl of 1 × wash buffer. 100 µl of a substrate reagent was added for 20 min at room temperature. Substrate reaction was stopped by the addition of 100 µl of stop solution and the absorbance at 450 nm was measured in each well. TGFβ-1 levels were calculated following the construction of a standard curve for TGFβ-1 of known concentration (Appendix 1C).

2.12 Statistical Analysis

Data is graphically presented as mean ± standard error of mean (SEM). Normal probability plots were prepared to determine whether the data was normally distributed. For normally distributed data statistical significance was determined using students t-test and ANOVA for samples with more than 2 groups. A Scheffe post-hoc test was carried with the ANOVA to determine differences between groups. Data that was not normally distributed

was analyzed using Wilcoxon signed rank in place of paired t-test and a Mann-Whitney U for a students t-test Correlation coefficients were determined using Pearson Product Moment (normally distributed data) or Spearman Rank (abnormally distributed data) Data was taken to be significant where $p < 0\ 05$ Data was analysed using DataDesk 4 1 for a Macintosh computer (Data Description Inc , Ithaca, NY, USA)

CHAPTER 3

VEGF EXPRESSION IN BREAST CANCER

3.1 Introduction

Solid tumours, such as breast tumours, cannot grow beyond $2mm^3$ without new vessel formation Angiogenesis occurs when the net balance of angiogenic promotors is greater than that of the angiogenesis inhibitors Constitutive expression of VEGF by breast cancer cells and a consequent increase of VEGF within the tumour tissue would be indicative of a shift in the balance of angiogenic promotors and suggest a role for VEGF in breast tumour mediated angiogenesis VEGF was first identified in the ascites fluid of guinea pig bearing syngeneic hepatocellular tumours (Senger et al , 1983) VEGF has since been shown to be overexpressed in many different cancers including brain tumours (Hatva et al , 1995), colon cancer (Takahashi et al , 1995), kidney and bladder cancer (Brown et al , 1993), hepatocellular cancers (Suzuki et al , 1996), ovarian cancers (Mesiano et al , 1998) hemangioblastomas (Wizigmann-Voos et al , 1995) and breast tumours (Toi et al , 1995, Anan et al , 1996, Yoshiji et al , 1996) Many different cancer cell lines have also been found to express VEGF (Senger et al , 1993) These cell lines are grown under normal cell culture conditions and as such have abundant nutrients and oxygen available It is likely therefore that the overexpression of VEGF by these cell lines may be due to mutations, such as mutations to p53, resulting in deregulated production of VEGF Northern blot analysis showed that VEGF mRNA levels were increased in breast tumour tissue relative to adjacent normal breast tissue (Yoshiji et al , 1996) *In situ* hybridisation demonstrated that malignant epithelium stained strongly for VEGF mRNA while only weak staining was found in normal epithelium of the breast (Brown et al , 1995)

The VEGF gene is alternatively spliced as described earlier to give 5 different isoforms (Figure 1 2) The isoforms are named according to the number of ammo acids they contain ($VEGF_{121}$, $VEGF_{145}$ $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$) (Tischer et al , 1991, Plouet et al , 1997) All isoforms contain a signal sequence which direct it for secretion from the cell $VEGF_{121}$ is the only isoform that is freely available $VEGF_{165}$ contains exon 7 that is slightly basic and has some heparin binding activity and this isoform is loosely bound to the cell membrane Exon 6 which is present in $VEGF_{145}$, $VEGF_{189}$ and $VEGF_{206}$ is highly basic and maintains these isoforms tightly bound to the cell membrane (Houck et al , 1992) The isoforms that a tumour cell produces determine its

bioavailability (Houck et al , 1992) Clearly synthesis of soluble isoforms would result in an immediate angiogenic response but synthesis of the larger isoforms may result in delayed angiogenesis

Receptors for VEGF were originally identified on endothelial cells (Jakeman et al , 1992) and were initially thought to be exclusive to these cells VEGF receptors, however, have been found on other cell types The VEGFR-1 receptor has been found on monocytes and is involved in the chemotactic response of monocytes to VEGF (Shen et al , 1993) VEGF also binds to cultured osteoblasts and induces differentiation of these cells (Macaulay et al , 1995) Both the VEGFR-1 and VEGFR-2 receptors are expressed on uterine smooth muscle cells (Brown et al , 1997) Binding experiements with ^{125}I-VEGF have demonstrated that human melanoma cells but not normal melanoctyes bind VEGF (Gitay-Goren et al , 1993) though it is not yet known whether VEGF acts as an autocrine growth factor in these cells VEGF is an autocrine mitogenic growth factor for human retinal pigment epithelial cells (Guerrin et al , 1995)

The aim of this chapter was to establish VEGF expression levels in breast cancer cell lines and determine which isoforms BT474 and MDA-MB-231 cells produced In order to do this a VEGF ELISA and a method for determining the isoforms present was required Since the VEGFR-2 receptor is considered to play a greater role in tumour development (Waltenberger et al , 1994), expression of this receptor by breast cancer cell lines was examined VEGF protein levels in breast tumour tissue and matched normal breast tissue were measured Circulating levels of VEGF in breast cancer patients were also measured to determine whether there is an elevation of VEGF in the serum of patients with breast cancer and the relationship between the stage of the disease and the level of serum VEGF

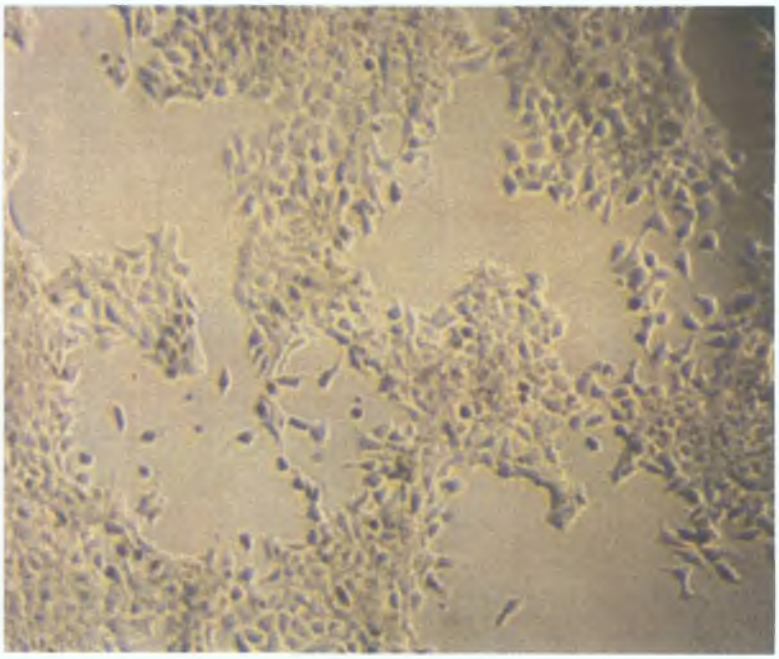
3.2 Results

3.2.1 BT474 and MDA-MB-231 cell lines

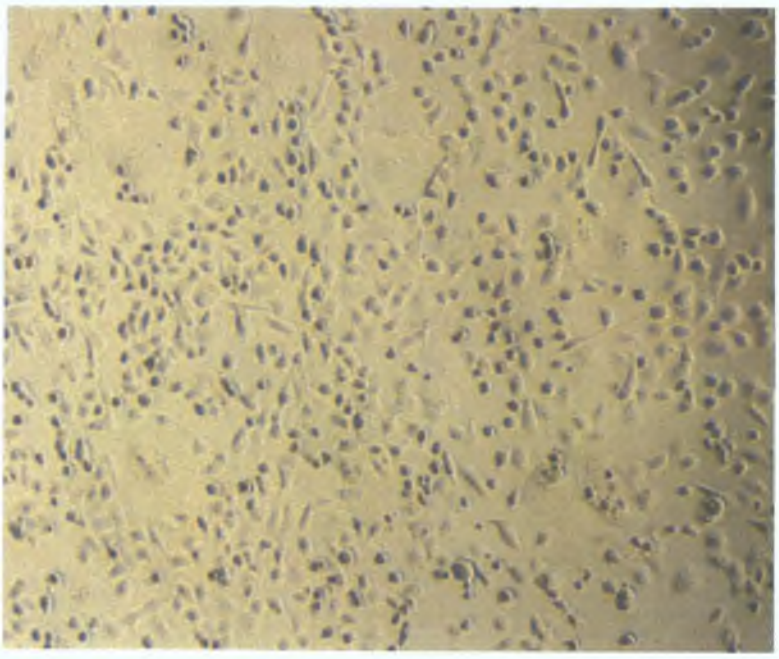
Figure 1A and 1B are representative photographs of cultured BT474 and MDA-MB-231 cell lines, respectively The BT474 cell line is a cell line is derived from a ductal carcinoma BT474 cells grow slowly outward from islands of cells, a typical morphological feature of breast cancer cell lines (Leibovitz, 1994) MDA-MB-231 cell were derived from a malignant pleural effusion in a woman with primary breast cancer and are considered to be representative of metastatic breast cancer cells These cells grew rapidly in an epithelial like manner Both cell lines are ER negative

3.2.2 Mycoplasma testing of BT474 and MDA-MB-231 cell cultures

Mycoplasma is a common contaminant of cultured cell lines All cell lines were routinely tested for mycoplasma contamination as described (2 2 7) Cell lines coming into the laboratory were tested upon arrival and cell lines positive for mycoplasma were discarded Culture supernatants were centrifuged to pellet any mycoplasma present Resuspended pellet was treated with lysis buffer and mycoplasma DNA was amplified by PCR Figure 3 2 is representative of an agarose gel of PCR products following amplification for mycoplasma-specific DNA Lane 1 shows molecular weight markers, ϕX174 digested with *Hae*III A negative control (no added template) and a positive control (mycoplasma DNA) were included with the samples for PCR There is no amplified product in the negative control (lane 2) demonstrating that no contamination of the samples occurred A 660 bp PCR product was amplified from the positive control (lane 3) Smearing of this sample indicates some degradation of template probably as a result of repeated freezing and thawing Mycoplasma specific PCR reactions from BT474, MDA-MB-231 and HMEC cells are shown in lanes 4, 5 and 6 Absence of PCR product indicates that these cell lines are free from mycoplasma contamination PCR products from a mycoplasma positive cell line are shown in lane 7



A.



B.

Fig. 3.1: Monolayer cultures of BT474 **(A)** and MDA-MB-231 cells **(B)**.

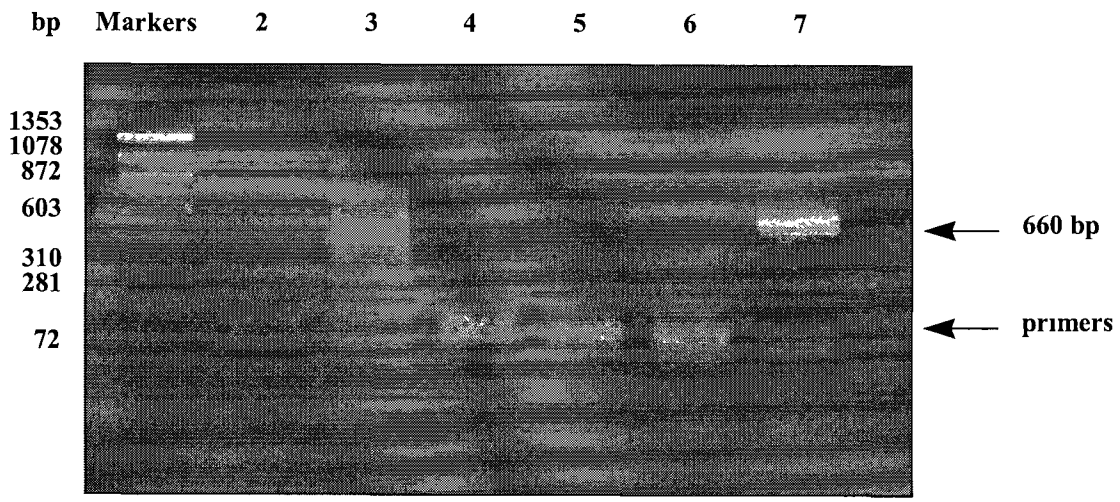


Fig. 3.2: Mycoplasma specific PCR reaction The PCR products were resolved on a 1 5% (w/v) agarose gel and photographed under UV light Markers are ϕX174 DNA digested with *Hae*III Lanes 2 and 3 contain a negative and positive control, respectively Lanes 4, 5 and 6 contain samples from BT474, MDA-MB-231 and HMEC cells, respectively Lane 7 contains a sample for a cell line found to be contaminated with mycoplasma Positions of primers and mycoplasma specific band (660 bp) are indicated

3.2.3 Development of an ELISA to measure VEGF

In order to measure VEGF produced by cell lines a sandwich ELISA using two anti-VEGF polyclonal antibodies was developed The final protocol developed that was used for VEGF measurements is described (2 10) The principle of the assay, in brief, is a sandwich ELISA m which a 96-well plate is coated with an anti-VEGF polyclonal antibody (R&D Systems AF-293-NA) The samples to be assayed are added to the well and are bound by the antibody Following this incubation a second biotinylated anti-VEGF antibody is added (R&D Systems BAF293) and also binds VEGF A streptavidm molecule conjugated to horseradish peroxidase (HRP) binds to biotin and HRP converts a substrate (tetramethylbenzidine – TMB) to a coloured product, the intensity of which is dependent on the quantity of VEGF present The optimum concentration of each element was determined empirically by repeatedly performing the assay with different concentrations of each component Table 3 1 represents some data from several assays, performed m duplicate, where the concentration of each component was varied A regression coefficient (r^2) of greater than 0 95 was required for the assay to be considered linear within the concentration range used (0 - 1000 pg/ml) The intra-assay precision was calculated by performing the assay 8 times with the same sample on one plate and the inter-assay precision was calculated by performing the assay with the same sample on four different plates in duplicate The intra-assay coefficient of variance (CV) was 5 7%, while the inter-assay CV was 9 7% A CV of less than 10% is usually sufficiently precise for an ELISA Samples were suitably diluted so that they were typically at a concentration roughly equivalent to the mid-point of the concentration standards A representative standard curve for the assay is illustrated in appendix 1A Unfortunately, this assay did not accurately measure VEGF levels in serum samples as determined by measuring serum containing known levels of VEGF Other factors were also found to be essential for the accurate performance of the assay and they included, 1) incubation of plates on a shaker during binding steps and 2) filter tips, such as those used for DNA work could not be used with TMB substrate as they caused an immediate reaction with this substrate VEGF levels were reported as pg/ μg total cellular protein m conditioned medium, pg/ μg protein in tissue and as pg/ml serum for serum samples

Primary (Coatıng) Ab (μg/ml)	**Biotinylated Secondary Ab (μg/ml)**	**Streptavidin - HRP (dilution)**	**Absorbance Range**	**Regression Coefficient (r^2)**
0 4	0 2	20 000	0 281	0 914
0 4	0 2	8 000	0 240	0 975
0 4	0 2	4 000	0 492	0 992
0 4	0 2	2 000	0 579	0 995
0 4	0 2	1 000	0 32	0 679
0 5	0 2	2000	0 643	0 988
0 6	0 2	2000	0 791	0 986
0 7	0 2	2000	0 870	0 993
1	0 2	2000	0 132	0 85
0 7	0 3	2000	921	0 985
0 7	0 4	2000	1 002	0 987
0.7	**0.5**	**2000**	**1.132**	**0.995**

Table 3.1 Optımısatıon of ELISA protocol The assay was performed ın duplıcate wıth dıfferent concentratıons of each component The absorbance range and correlatıon coefficıent were calculated for each assay and the optımum concentratıon of each component was empırically assessed The optımum concentratıons are shown ın bold

3.2.4 Breast cancer cell line expression of VEGF protein

VEGF protein production by BT474 and MDA-MB-231 cell lines was examined Both cell lines were found to produce VEGF protein (Fig 3 3) These experiments were performed in serum-free medium so as to avoid induction of VEGF by agents in serum, indicating that this represents constitutive expression by these cells VEGF protein produced was measured in the conditioned medium of the cells over 24 hr Without the presence of other carrier proteins in the medium VEGF may degrade over time, and 0 5% (w/v) BSA was therefore added to the medium to stabilise VEGF protein Levels of VEGF were expressed as pg VEGF/μg of total cellular protein The metastatic MDA-MB-231 cells produced almost 3 fold more ($p = 0\ 001$) VEGF protein (12 1 ± 2 4 pg/μg protein) than did the primary cell line BT474 (4 09 ± 0 69 pg/μg protein)

3.2.5 VEGF mRNA in BT474 and MDA-MB-231 cell lines

Semi-quantitative PCR was carried out to establish VEGF mRNA levels in cells lines (Fig 3 4) Conditions used have previously been shown to result in semi-quantitative amplification of VEGF (Harmey et al , 1998) The PCR primer pair used amplifies a 204 bp fragment from all VEGF isoforms As a control, constitutively expressed β-actin mRNA was also amplified by RT-PCR, resulting in a 661 bp product Primers used in both cases span intronic sequences, allowing differentiation from PCR products arising from amplification of genomic DNA PCR products were quantified by densitometry and the VEGF product normalised against β-actin and expressed as the ratio of VEGF/β-actin Cells were cultured in medium without FCS so that there would be no interference from agents in the FCS MDA-MB-231 (lane 1) cells appeared to produce more VEGF mRNA than the BT474 cell line (lane 2) (0 96 vs 0 29 densitometric units) This represents a 3 3 fold increase in VEGF mRNA in MDA-MB-231 cells compared to BT474 cells Figure 3 4B shows the VEGF/β-actin ratio for 3 independent experiments MDA-MB-231 cells produce significantly higher levels of VEGF mRNA than the BT474 cells

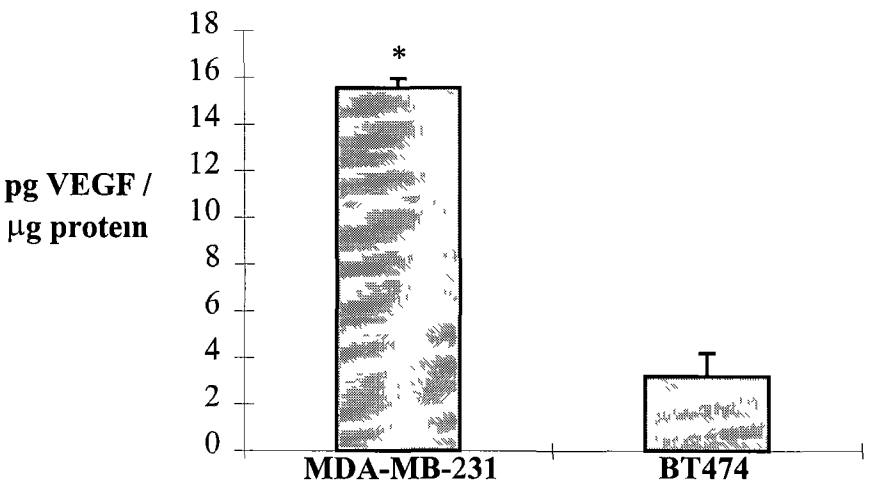
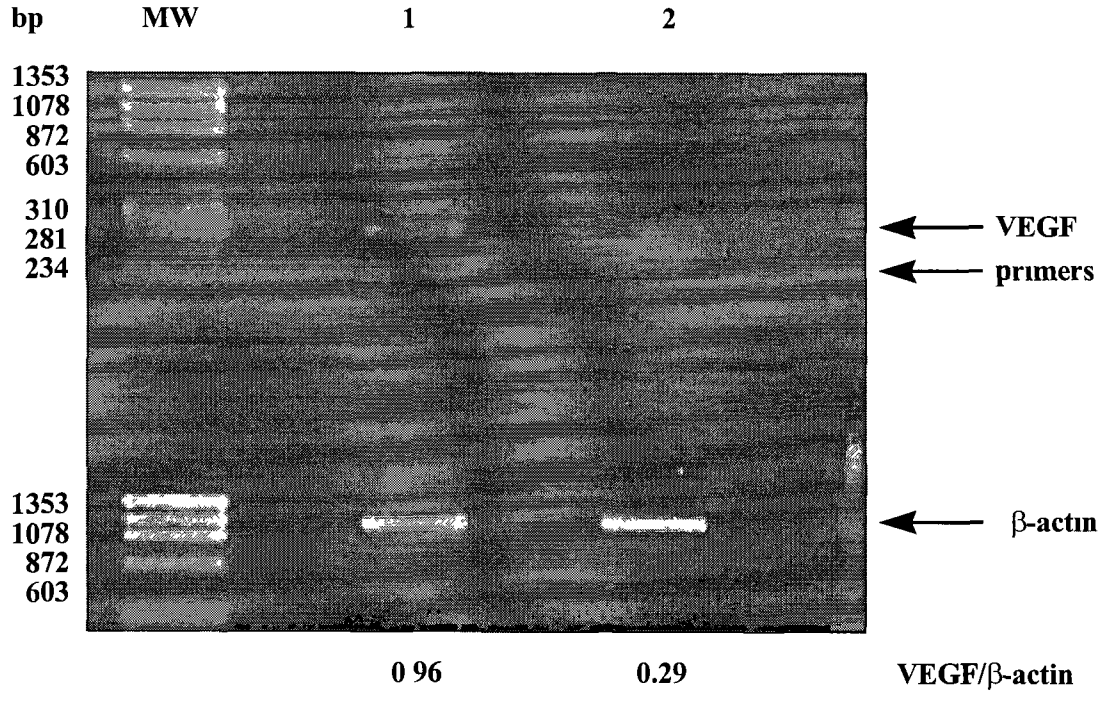
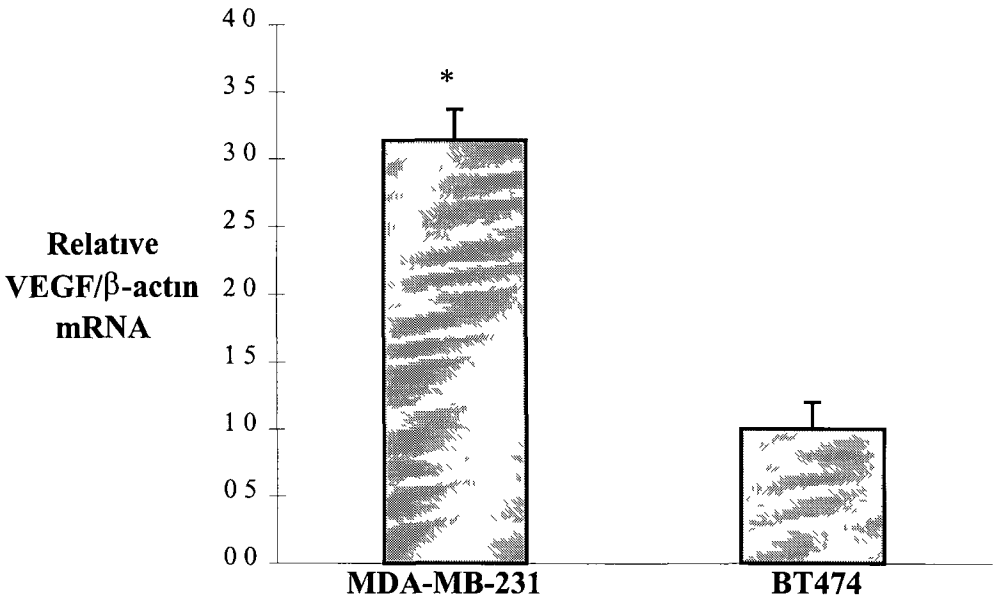


Fig 3.3: VEGF protein production by BT474 and MDA-MB-231 cells over 24 hr Data represents 3 independent experiments carried out in triplicate and are expressed as mean ± SEM Statistical analysis was by unpaired students t-test *, p = 0 001 MDA-MB-231 vs BT474



A.

Fig 3.4A: 1 5% (w/v) agarose gel electrophoresis of VEGF and β-actin PCR products generated by RT-PCR of RNA from MDA-MB-231 (lane 1) and BT474 (lane 2) cells Cells were cultured for 24 hr in fresh medium and RNA isolated and amplified by semi-quantitative RT-PCR Position of molecular weight markers, ϕX174 digested with *Hae*III, are shown (bp) Position of VEGF product and primers and β-actin product are indicated



B.

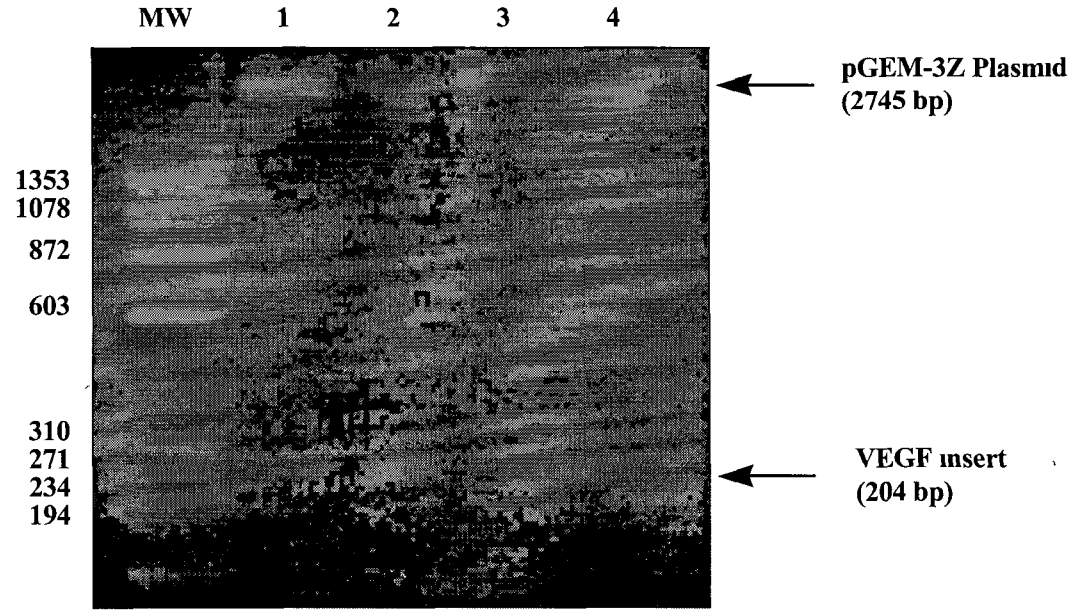
Fig 3.4B: Relative VEGF/β-actin mRNA levels in BT474 and MDA-MB-231 cells as assessed semi-quantitatively by densitometry Data is expressed as relative VEGF/β-actin with the BT474 cells arbitrarily taken as 1 0 and is from 3 independent experiments Statistical analysis was by students t-test *, $p < 0\ 005$ vs BT474 cells

3.2.6 VEGF isoform expression by BT474 and MDA-MB-231 cells

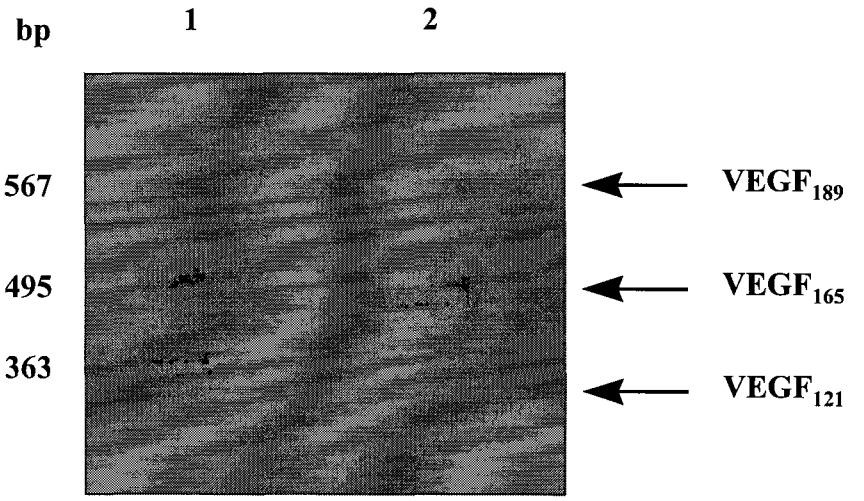
VEGF isoform expression in BT474 and MDA-MB-231 cells was examined by RT-PCR (2 4) followed by hybridisation with a VEGF probe (2 6) This combination of techniques was used because of problems with weak signal following the PCR step To prevent non-specific amplification a reduced number of PCR cycles (25) combined with a hot start (heating template to 94°C for 10 min before adding Taq polymerase) was carried out The increased specificity resulted in weak amplification of PCR products Thus, to visualise PCR products southern blotting using a hVEGF probe was carried out The probe used for southern blotting was a 204 bp fragment of the VEGF gene released by *Eco*RI and *Hin*dIII digestion of the pGEM3Z-hVEGF plasmid (Figure 3 5A) Lanes 1-4 show plasmid DNA following digest and a 204 bp fragment that was released This fragment was purified, labelled with P^{32} and used as a probe in southern blot analysis of isoform specific PCR products The VEGF isoforms produced by both cell lines is shown in figure 3 5B The hVEGF probe bound to two bands of the BT474 PCR products at 363 and 495 bp, which correspond to the $VEGF_{121}$ and $VEGF_{165}$ isoforms The hVEGF probe bound to two bands of the PCR products of MDA-MB-231 cells at 495 bp and 567 bp which correspond to the $VEGF_{165}$ and the $VEGF_{189}$ isoforms

3.2.7 Western blot analysis of VEGFR-2 expression by BT474 and MDA-MB-231 cells

The BT474 and MDA-MB-231 cell lines were examined for the presence of VEGFR-2 (KDR) to determine whether there was a possibility that VEGF may act as an autocrine growth factor for breast tumour cells Western blots were performed on both cell lines using an antibody directed against VEGFR-2 Figure 3 6 shows the results of a western blot analysis for VEGFR-2 protein Coomaisse staining of polyacrylamide gel was used to determine the integrity of the protein, which was satisfactory A positive control, the carboxyl terminus of VEGFR-2 (lane 1) was detected at 47 and 42 kDa The VEGFR-2 receptor is highly glycosylated and the two different bands in the positive control lane is most likely due to alternative glycosylation VEGFR-2 expression was not detected in cell lysates of either BT474 (lane 2) or MDA-MB-231 (lane 3) cells



A.



B.

Fig. 3.5: Southern blot analysis of isoform specific RT-PCR (**A**) shows a 1.5% agarose gel of plasmid pGEM3Z-hVEGF digested with 50U *Eco*RI and 100U of *Hin*dIII for 3 hr. Position of molecular weight markers (MW), ϕX174 digested with *Hae*III, are shown (bp). Lanes 1-4 show digested plasmid (2745 bp) and released VEGF insert (204 bp). (**B**) Southern blot analysis of VEGF isoform cDNA generated by RT-PCR. PCR product was resolved on a 1.5% agarose gel, transferred to nylon membrane and hybridised with P^{32} labelled hVEGF probe. Lanes 1 and 2 show a sample from BT474 and MDA-MB-231 cells, respectively. Molecular weight markers (MW) and VEGF isoforms present are indicated. The figure is of two autoradiographs aligned along side each other.

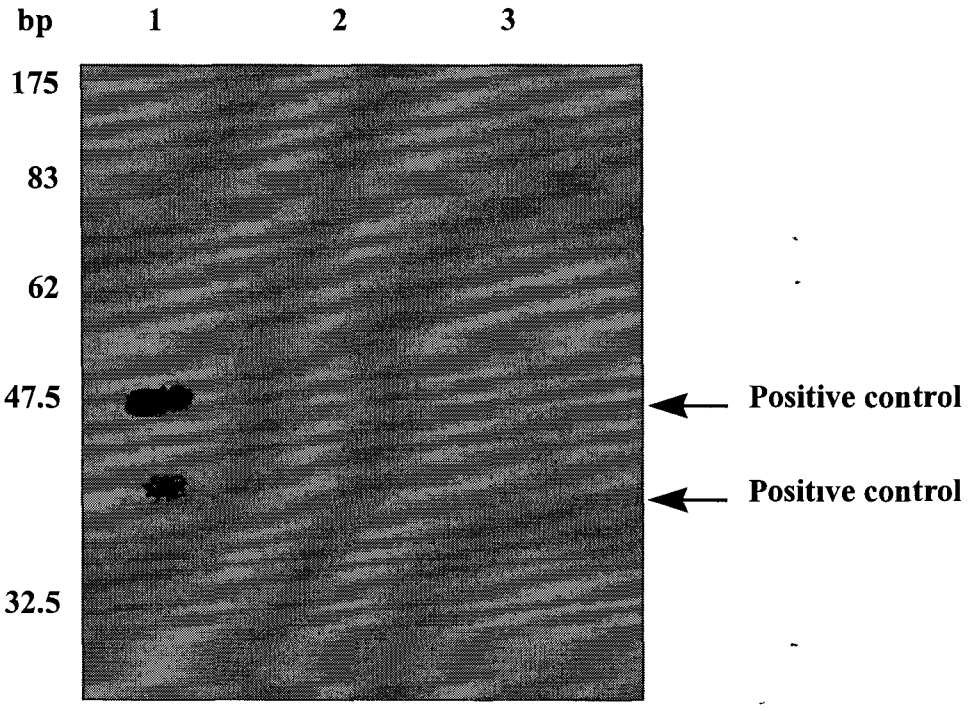
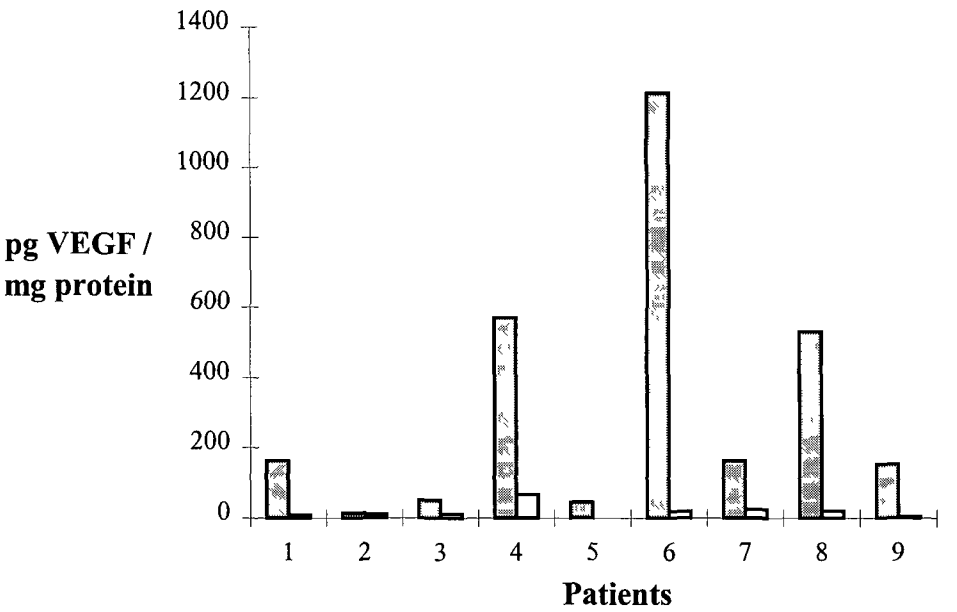


Fig 3.6: Western blot analysis of VEGFR-2 protein expression in BT474 and MDA-MB-231 cell lysates Cell lysates were fractionated on a 7 5% (w/v) SDS-PAGE, transferred to nitrocellulose membrane and incubated with an anti-VEGFR-2 antibody Bound antibody was detected by enhanced chemiluminescence Lane 1 shows antibody bound to a positive control, the carboxyl terminus of the VEGFR-2 protein Anti VEGFR-2 antibody did not bind to VEGFR-2 in lysates of BT474 and MDA-MB-231 cells, respectively (Lanes 2 and 3, respectively)

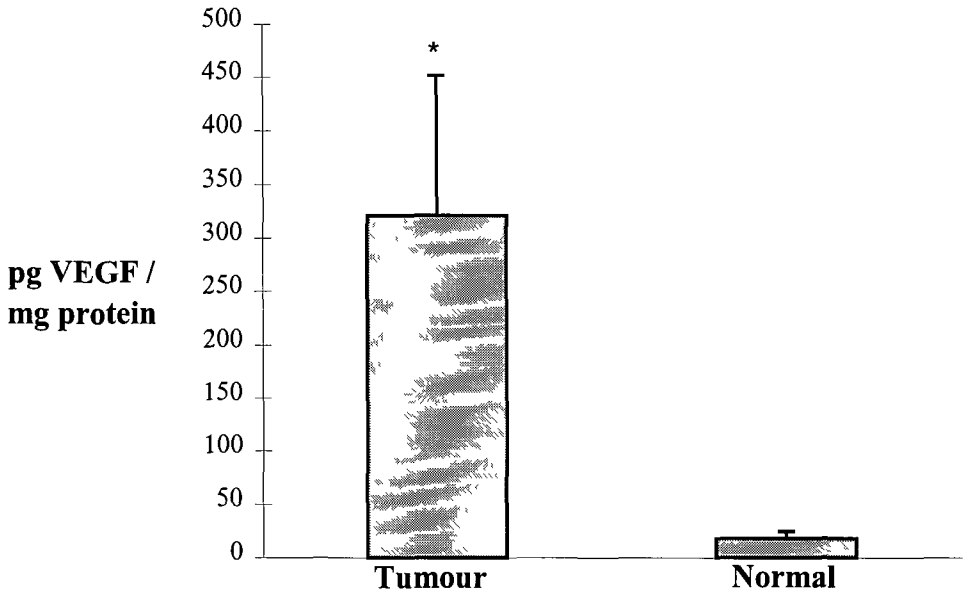
3.2.8 VEGF protein expression in breast tumour tissue as compared to matched normal tissue

As cultured breast cancer cells produced large quantities of VEGF, VEGF levels in tumour tissue were measured and compared to the levels found in a matched histologically normal piece of tissue (Fig 3 7A) Both normal and tumour tissue, with the exception of 1 normal sample, contained detectable levels of VEGF In every case there were greater levels of VEGF protein found in the tumour tissue as compared to the normal tissue The mean VEGF protein level in tumour tissue (321 3 ± 390 8 pg VEGF/ mg protein range 13 2 - 1212 pg/mg protein) was significantly greater than the mean VEGF level m normal tissue (18 01 ± 19 6 pg VEGF/ mg protein range 0 0 - 65 9 pg/mg protein) (Figure 3 7B) The mean increase in VEGF levels in tumour tissue was a 21 4 fold increase (range 1 3 - 64 8 fold increase)



A.

Fig. 3.7A: VEGF protein levels in tumour tissue (▨) and matched normal tissue (□) in 9 patients with malignant breast disease (**A**) Tissue was homogenised and VEGF measured by ELISA Total cell protein was measured by BCA assay and VEGF levels expressed per mg protein



B.

Fig. 3.7B: VEGF protein levels in tumour tissue and matched normal tissue in 9 patients with malignant breast disease Tissue was homogenised and VEGF measured by ELISA Total cell protein was measured by BCA assay and VEGF levels expressed per mg protein Data is expressed as mean VEGF levels ± SEM Statistical analysis was by Wilcoxon Signed Rank *, $p < 0.005$

3.2.9 VEGF is elevated in the serum of breast cancer patients

VEGF was measured in the serum of patients with malignant breast disease (n = 44) and from patients with benign fibroadenoma (n = 21). VEGF was also measured in a group of healthy controls (n = 15) who were age matched with the malignant group. Malignancy was later confirmed histologically. The patient series with malignant disease was comprised of 37 ductal, 6 lobular and 1 mucinous carcinoma. Tumours were staged according to the size definitions of the TNM staging system (TNM Atlas, 1992). Three of the patients had carcinoma *in situ* (Tis) and the remaining patients were as follows: T1, n = 13; T2, n = 18; T3, n = 5; T4, n = 5. Nodal status was also examined though there were sampling difficulties depending on the type of surgery performed. The nodal status for 16 patients was unknown, 13 patients were node positive and the remaining were node negative. Patient data is summarised in Table 3.1. The mean ages for the three groups was 59.1 ± 11.8 years, 36.5 ± 5.09 years and 50.33 ± 7.3 years for the malignant, benign and control groups respectively. There was no significant difference between the mean age of the malignant and control patients ($p > 0.05$) though both were significantly older than patients with benign breast disease (ANOVA, $p < 0.05$). Serum VEGF levels were significantly elevated ($p < 0.05$) in patients with malignant breast disease compared to both patients with benign fibroadenoma and age-matched controls as assessed by ANOVA (Fig. 3.8). The mean serum level in the malignant group was 393.07 ± 278.59 pg/ml, in the benign group 233.7 ± 184.21 pg/ml and in the control group was 230 ± 127.18 pg/ml. Serum VEGF levels in patients with malignant disease was also examined according to tumour stage (Fig 3.9). The levels of VEGF in patients with different stage of disease was Tis = 370.92 ± 127.93, T1 = 292.66 ± 159.78, T2 = 363.98 ± 212.51, T3 = 533.81 ± 490.59, T4 = 631.41 ± 384.07 pg/ml. Although there was no statistical significance in the serum VEGF levels between the different stages there is a very clear trend for increased serum VEGF with advancing stage. This is possibly due to the fact that there were a small number of T3 and T4 patients. It is unusual for patients to present with disease at these stages. Three patients who presented with known clinical metastatic disease had serum VEGF of greater than 1000 pg/ml (mean = 1157.26 ± 151.42). There was no significant difference in serum VEGF levels between patients who were node positive (295.07 ± 155.56 pg/ml) and node negative (358.51 ± 254.75 pg/ml)(Mann-Whitney $U > 0.05$).

There was also no significant difference between VEGF levels in patients who were ER positive (390 58 ± 348 8 pg/ml) and ER negative (336 65 ± 197 34 pg/ml)(Mann-Whitney U > 0 05)

Number	Stage	Age	Nodal status	ER Status	VEGF (pg/ml)
1	T2	74	n/a	pos	784.71
2	T2	66	neg	neg	498.27
3	T2	51	neg	n/a	417.67
4	T1	58	neg	n/a	489.17
5	T3	68	neg	pos	1092.48
6	T1	50	neg	pos	173.28
7	T2	44	pos	pos	43.40
8	T2	79	pos	neg	416.28
9	T2	74	n/a	pos	463.09
10	T2	43	pos	neg	249.92
11	T2	49	n/a	neg	768.59
12	T1	47	neg	neg	440.88
13	T4	58	pos	pos	277.38
14	T1	47	n/a	pos	88.31
15	Tis	44	pos	n/a	390.00
16	T2	62	neg	n/a	432.00
17	T2	43	neg	neg	46.00
18	T3	50	pos	neg	172.00
19	T1	32	neg	neg	360.00
20	T1	68	pos	pos	546.00
21	T3	60	n/a	n/a	1049.00
22	T2	75	pos	pos	252.84
23	T1	63	neg	pos	179.63
24	Tis	44	pos	n/a	234.52
25	T1	85	n/a	pos	245.94
26	Tis	51	n/a	n/a	488.23
27	T1	60	neg	pos	199.27
28	T3	54	pos	pos	197.53
29	T2	60	pos	neg	109.79
30	T2	65	neg	neg	313.93
31	T1	59	neg	neg	465.95
32	T1	76	neg	neg	219.25
33	T2	59	n/a	pos	318.28
34	T2	65	n/a	pos	364.30
35	T2	61	pos	neg	401.70
36	T3	74	n/a	neg	158.03
37	T4	53	n/a	pos	1330.29
38	T2	64	n/a	pos	126.36
39	T4	70	n/a	n/a	316.22
40	T1	59	neg	neg	49.81
41	T1	50	n/a	pos	347.27
42	T4	57	n/a	neg	508.18
43	T4	79	n/a	n/a	725.00
44	T2	52	pos	neg	544.55

Table 3.1 Patient characteristics. pos = positive, neg = negative, n/a = not available.

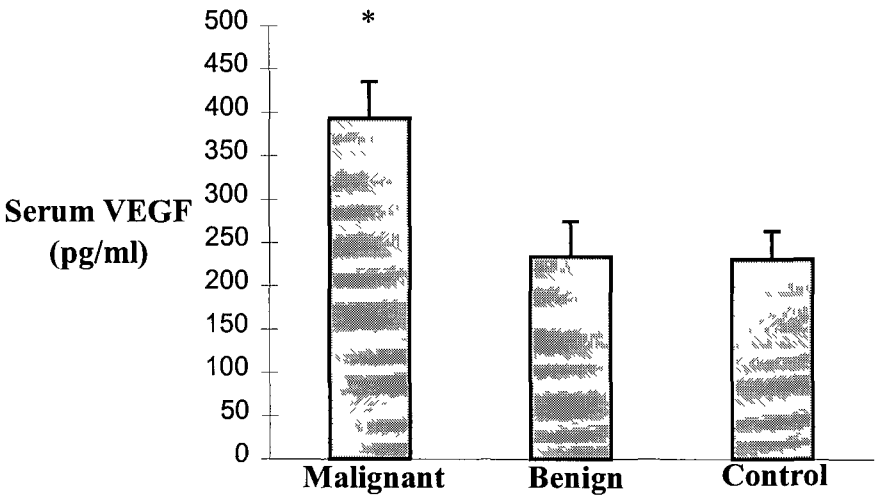


Fig. 3.8: Serum levels of VEGF m patients with malignant breast disease (n = 44), patients with benign fibroadenoma (n = 21) and healthy age-matched controls (n = 15) Data is expressed as the mean ± SEM Statistical analysis was by ANOVA with Scheffe post hoc correction *, p = 0 03, malignant vs benign and controls

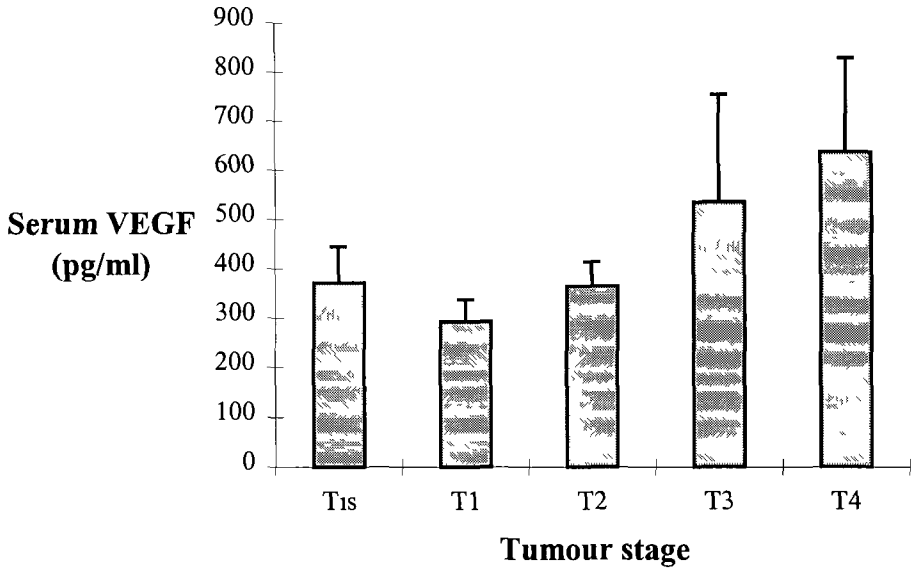


Fig. 3.9: Serum levels of VEGF according to stage in patients with breast cancer VEGF levels were measured preoperatively in the serum and tumour stage was assessed from the histology reports Tis, n = 3, T1, n = 13, T2, n = 18, T3, n = 5, T4, n = 5 Data is expressed as the mean ± SEM Statistical analysis was by ANOVA with Scheffe post hoc correction p = ns

3.3 Discussion

An ELISA method was developed to measure the amount of VEGF protein in cell culture supernatant. Cultured breast cancer cells produce high levels of VEGF protein and mRNA. MDA-MB-231 cells, which are metastatic in origin, produced significantly higher levels of both VEGF mRNA and protein than the primary BT474 cells. Isoform specific RT-PCR followed by southern blotting showed that the BT474 cell line produced the $VEGF_{121}$ and $VEGF_{165}$ isoforms while the MDA-MB-231 cells produced the $VEGF_{165}$ and $VEGF_{189}$ isoforms. The antibody used for measuring the levels of VEGF protein was raised against $VEGF_{165}$ and no information is available about its cross-reactivity with the other isoforms. If it does cross-react with the other isoforms then it is possible that the levels detected for MDA-MB-231 cells may be an under representation of total VEGF since this cell line produces the $VEGF_{189}$ that is not released into the conditioned medium. However, these cell lines are independently derived from different people so direct comparison of expression of VEGF and the origin of the cells is not possible. However, it has been shown that metastatic colon carcinoma cells produced more VEGF than did their primary counterparts (Takahashi et al., 1995). We also found higher levels of serum VEGF in patients with metastatic disease. Expression of VEGF has been shown to correlate with the level of metastases in colon carcinomas and in experimental melanomas (Takahashi et al., 1995; Claffey et al., 1996). VEGF causes hyperpermeability of blood vessels which may allow tumour cells to migrate through blood vessels and into the circulation. VEGF also increases expression of proteases, which are required for metastasis, including interstitial collagenase, gelatinase A, urokinase-like and tissue plasminogen activators and (Unemori et al., 1992) (Lamoreaux et al., 1998; Pepper et al., 1991).

There are currently no methods available to distinguish between the different isoforms so an RT-PCR method was developed to examine the isoform species that each cell line produced. RT-PCR analysis of mRNA using primers to exon 1 and exon 8 that span splice junctions and that yield different sized products depending on the isoform mRNA. This showed that the BT474 cell line produced the more highly diffusible $VEGF_{121}$ and the $VEGF_{165}$ isoforms while the MDA-MB-231 cell line produced the $VEGF_{165}$ and $VEGF_{189}$ isoforms. Although the BT474 cells appear to produce the more

immediately soluble isoforms, the $VEGF_{189}$ isoform that the MDA-MB-231 cells produce has a greater angiogenic potential once it is cleaved from the cell membrane (Keyt et al., 1996b). Breast tumours have been shown to produce a number of different proteases including urokinase, which has also been shown to be capable of cleaving $VEGF_{189}$ from the cell membrane (Plouët et al., 1997). Once released from the cell membrane $VEGF_{189}$ is capable of inducing a greater angiogenic response since it also has the ability to release bFGF, another angiogenic factor, from the extracellular matrix (Jonca et al., 1997). Production of the $VEGF_{165}$ and $VEGF_{189}$ isoforms could lead to a more co-ordinated angiogenic response. Release of $VEGF_{165}$ increases expression of proteases which can then release cell bound $VEGF_{189}$ so that the overall levels of VEGF are sustained, which may be important for mitogenesis and chemotaxis. The fact that the MDA-MB-231 cells produce more VEGF and also produces isoforms that are capable or eliciting a greater angiogenic response is in keeping with the more aggressive nature of these metastatic cells. The VEGFR-2 receptor was not detected on either the MDA-MB-231 or BT474 cell line, which indicates that VEGF is unlikely to act on these cells in an autocrine manner.

Examination of clinical material showed that malignant tumour tissue contained elevated levels of VEGF compared to the normal breast tissue from the same patient. Previous studies have shown that breast tumour tissue had increased levels of VEGF mRNA (Yoshiji et al., 1996) but our study showed that this was also true at the protein levels. We only looked at VEGF-A levels but both VEGF-B and VEGF-C have also been found to be expressed in tumours (Salven et al., 1998) and it is likely that the expression of these proteins is also increased in tumours. Together with evidence that breast cancer cell lines constitutively produce VEGF, the results presented here suggest that VEGF has a central role in breast cancer. There were also, in all but one case, detectable levels of VEGF within the normal tissue. Previous studies have shown VEGF mRNA expression in a variety of normal tissue (Berse et al., 1992) and VEGF mRNA was also found, at low levels, in normal breast (Brown et al., 1995). VEGF mRNA is regulated at several different stages and may not necessarily correlate with protein levels. However, in this study protein levels were measured. VEGF has been shown to bind to the extracellular matrix (Park et al., 1993) and small amounts of VEGF may be stored there for immediate use following injury, such as occurs with bFGF.

Small amounts of VEGF may also be required for the maintenance of normal vasculature (Alon et al , 1995)

The elevated VEGF found in tumour tissue was also reflected in serum measurements of breast cancer patients Patients with malignant breast disease had significantly higher levels of serum VEGF than did patients with benign fibroadenomas and also a group of healthy age-matched controls Serum VEGF levels in mice are significantly increased from undetectable levels by subcutaneous transplantation with solid tumour (Kondo et al , 1994) Other authors have found elevated VEGF in the sera of patients with various types of neoplasia including small cell lung carcinoma, ovarian and gastrointestinal carcinoma (Kraft et al , 1999, Salven et al , 1997) An elevation in the serum levels of VEGF was associated with poor outcome in urothelial cancer and in small-cell lung cancer (Miyake et al , 1999, Salven et al , 1998) We did not show that there was a statistically significant increase in serum VEGF with stage, however, there was a very clear trend indicating that VEGF levels increased with advancing stage Most patients present in the clinic with T1 or T2 tumours, relatively few present with advanced T3 or T4 disease Since there were smaller numbers of T3 and T4 tumours compared to lower stage tumours the variance is considerably greater and may account for the lack of significance One study, with over 500 patients, has shown that serum VEGF measurements are of high prognostic value in node negative breast cancer (Linderholm et al , 1998) Patients above the median value for serum VEGF measurements had a significantly shorter survival time Patients with metastatic lung cancer who had high levels of serum VEGF (greater than 500 pg/ml) had a poorer response to treatment than patients who had lower serum VEGF levels (Salven et al , 1998) This may be because metastases have already been established Preoperative serum levels of VEGF were significantly associated with tumour stage in colorectal cancer in that a high stage of disease was associated with higher levels of serum VEGF (Kumar et al , 1998) In colorectal cancer, patients with the highest tumour doubling time also had higher serum VEGF and bFGF levels than other patients (Dirix et al , 1996) In this study, patients with clinically confirmed metastatic disease had the highest levels of serum VEGF Together with the data in MDA-MB-231 and BT474 cells, showing that the metastatic MDA-MB-231 cells produced more VEGF than the

primary BT474 cells, this may suggest that VEGF with associated with presence of metastatic disease

VEGF was also detected in the serum of patients with benign fibroadenoma and in the age-matched controls In addition to its role in angiogenesis VEGF is also thought to play a role in maintaining the integrity of large blood vessels (Tsurumi et al , 1997) which would explain the presence of low levels of VEGF in people without evidence of cancer or other angiogenesis related disease pathologies This study was initiated prior to publication of data showing that platelets contain and release VEGF (Verheul et al , 1997) Degranulation of platelets, which may occur during serum preparation, would release VEGF into the serum The levels of serum VEGF measured may have a contribution from platelet release but the relative levels of VEGF between malignant patients and benign patients and controls would still exist as would the relative VEGF levels between different stages of cancer In future studies plasma preparation would be a better measure of VEGF within the circulation However, it is important to note that heparinised tubes should not be used since VEGF is a heparin binding growth factor and heparin in the tubes would deplete plasma VEGF

CHAPTER 4

REGULATION OF VEGF

4.1 Introduction

As VEGF plays a central role in tumour growth by promoting angiogenesis, factors regulating VEGF production are critical in control of angiogenesis One mechanism through which VEGF may be regulated is by hypoxia (low oxygen tension) Hypoxia within a tumour is a common feature of a number of malignancies including breast cancer (Vaupel et al , 1991) The obvious response to a hypoxic stress in any tissue is to increase the vascular supply to satisfy the metabolic needs of the tissue This response has been seen in the development of new capillaries in ischaemic tissue that would otherwise be quiescent for angiogenesis and with the angiogenesis associated with the healing of hypoxic wounds (Knighton et al 1983) We had previously shown that hypoxia increased VEGF expression by macrophages (Harmey et al 1998) Glioblastoma cells cultured *in vitro* showed increased VEGF mRNA levels when exposed to hypoxia and levels returned to normal following a return to normal oxygen levels (Shweiki et al , 1992) VEGF expression in glioblastoma tumours was increased beside necrotic regions which are presumed to have insufficient oxygen supply (Shweiki et al 1992) VEGF mRNA is more highly expressed within the inner regions of tumour spheroids which are presumed to be the areas with the lowest oxygen tension than in the outer regions which have easier access to oxygen (Shweiki et al , 1995) When these tumour spheroids were exposed to 95% oxygen for 16 hr, which would presumably allow oxygen to reach the inner core of the spheroid, the VEGF mRNA levels, within the inner regions, were reduced (Shweiki et al 1995) Hypoxia increase VEGF by increasing the levels of a transcription factor, hypoxia inducible factor-1 (HIF-1) that binds to a hypoxia response element (HRE) in the VEGF gene and initiates transcription (Forsythe et al , 1996) HIF-1 is constitutively produced but is rapidly broken down by ubiquitin-proteosomes in the presence of oxygen (Shih and Claffrey, 1998) Hypoxia inhibits this breakdown thereby increasing HIF-1 levels and binding to DNA The oxygen sensing mechanism of HIF-1 is believed to be controlled by a heme-protien (Jiang et al , 1996) In addition to a transcriptional increase, hypoxia may also increase VEGF mRNA levels by stabilising VEGF mRNA (Shima et al , 1995)

Insulin-like growth factor-I (IGF-I) is a single chain polypeptide that is a member of a large family of peptides that are structurally related to insulin It exerts its

cellular action by binding with high affinity to a heterotetrameric transmembrane tyrosine kinase receptor (IGF-1R) IGF-1 also binds with high affinity to a family of 6 soluble IGF-1 binding proteins (IGFBPs) that regulate IGF-1 bioavailability by binding to IGF-1 and thereby making it of incapable of binding to target cells (Ullrich et al , 1986) There is some significant evidence that IGF-1 promotes breast cancer *In vitro* IGF-1 is mitogenic for breast cancer cell lines, IGF-1R is present on cultured breast cancer cell lines and primary breast tumours and anti-IGF-1 antibodies inhibit breast tumour growth (Huynh et al , 1993) There is a positive association between circulating levels of IGF-1 and risk of breast cancer in premenopausal women (Hankinson et al , 1998) In a study of 44 patients with breast cancer, plasma levels of IGF-1 were significantly elevated in patients with breast cancer compared with healthy controls (Peyrat et al , 1993) Breast cancer patients show altered serum profiles of IGFBPs which may reflect IGF-1 bioavailability (Ng et al , 1998) A large number of cell lines express IGF-1 and IGF-1 mRNA has been detected in breast cancer cell lines which may indicate a possible autocrine loop (Quinn et al , 1996) In addition to the direct effects on epithelial cells IGF-1 promoted angiogenesis in the rabbit cornea assay (Grant et al 1993, Nicosia et al , 1994) IGF-1 increases VEGF mRNA and protein production in a number of colorectal carcinoma cell lines (Warren et al , 1996)

Another important molecule believed to be indirectly involved in angiogenesis is transforming growth factor β-1 (TGFβ-1) TGFβ-1 is one member of a family of growth factors that also include activin/inhibin, bone morphogenetic protein (BMP), Mullerian inhibiting substance and glial cell line-derived neurotrophic factor (Oft et al , 1996) There are 5 different forms of TGFβ which are TGFβ-1, 2, 3, 4, 5, though only β-1, β-2 and β-3 are expressed in mammals The three mammalian forms of TGFβ are coded by unique genes but share about 70 - 80% homology (Roberts et al , 1988) TGFβ is highly conserved amongst species suggesting an important biological role (Roberts et al 1988) TGFβ is synthesised as a precursor, the C-terminus is then cleaved and the molecule dimerises to give the mature 25 kDa protein TGFβ family members are secreted in an inactive form bound to 2 other proteins, non-covalently to a 75 kDa glycoprotein termed the latency associated protein (LAP) and covalently to the 135 kDa modulator/binding protein (Miyazono et al , 1988) This latent form is unable to bind to TGFβ receptors and can be activated enzymatically (plasmin, cathepsin),

thermally or by acid treatment (Miyazono et al. 1988). Hypoxia, which may be present in a tumour environment, activates TGFβ (Behzadian et al., 1998). The plasminogen activator system is also upregulated in a tumour environment and also activates TGFβ which can then further upregulate urokinase plasminogen activator (uPA) which is suggestive of autocrine activation of TGFβ (Arnoletti et al., 1995). TGFβ exerts its effects through the formation of heterotetrameric complexes between its 2 membrane serine-threonine kinases receptors TGFβRI and TGFβRII (Muñoz-Antonia et al., 1996). TGFβ binds to TGFβRII which then recruits TGFβRI. TGFβRII is constitutively phosphorylated and transphosphorylates TGFβRI to mediate cellular signal (Yamashita et al., 1994). TGFβ is involved in a great number of cellular activities including proliferation, differentiation, immune response, wound healing and tissue remodelling, embryogenesis and carcinogenesis (Roberts et al. 1988).

TGFβ-1 is recognised as one of the most potent inhibitors of epithelial cell proliferation (Satterwhite and Moses, 1995). It reversibly arrests cell cycle progression in late G1. In the early stages of breast cancer development TGFβ-1 has a tumour inhibiting effect by inhibiting proliferation of tumour cells (Reiss and Barcellos-Hoff, 1997). As a tumour progresses it produces increasing amounts of TGFβ-1 and it has been hypothesised that in advanced breast cancer TGFβ-1 may promote tumour development (Reiss and Barcellos-Hoff, 1997). Tumour cells can become resistant to the inhibitory effects of TGFβ-1 (Geiser et al., 1992). Absence of or mutations in the TGFβRII has been noted in cancer cells including breast cancer cell lines (Sun et al., 1994). Mutations in TGFβ receptors can result in loss of responsiveness to the growth inhibitory effects of TGFβ-1 while still regulating the expression of TGFβ-1 regulated genes, such as the induction of fibronectin expression (Geiser et al. 1992). A tumour promoting role for TGFβ-1 may be seen by the fact that it can act as an angiogenic factor (Roberts et al. 1986). Chinese hamster ovary (CHO) cells transfected with TGFβ-1 showed increased growth in nude mice with an associated increase in blood vessel formation (Ueki et al., 1992). Both of these effects were inhibited by the introduction of anti-TGFβ-1 antibodies (Ueki et al. 1992). There is a positive correlation between the plasma levels of TGFβ-1 and vascularity in patients with hepatocellular carcinoma (Ito et al., 1995). The manner in which TGFβ-1 stimulates angiogenesis would appear to be indirect since TGFβ-1 inhibits endothelial cell

proliferation, invasion and lumen formation (Pepper et al 1993) This indirect angiogenic response may be achieved by the induction of other growth factors such as VEGF TGFβ-1 has been shown to increase VEGF production by mouse embryo fibroblasts, a lung adenocarcinoma cell line, vascular smooth muscle cells, glial cells and macrophages (Pertovaara et al , 1994, Harmey et al 1998, Brogi et al , 1994)

The aim of this chapter was to establish factors that regulate VEGF expression in breast cancer cells A rational approach that involved the examination of factors that are known to be present in the breast tumour microenvironment and that have strong influences on VEGF expression and breast tumour growth was chosen The factors chosen therefore were hypoxia, IGF-1 and TGFβ-1 Also, the two latter factors, IGF-1 and TGFβ-1, may play a role in response of breast tumour cells to tamoxifen (Huynh et al 1993) and therefore had additional interest because of other work with tamoxifen (Butta et al , 1992)

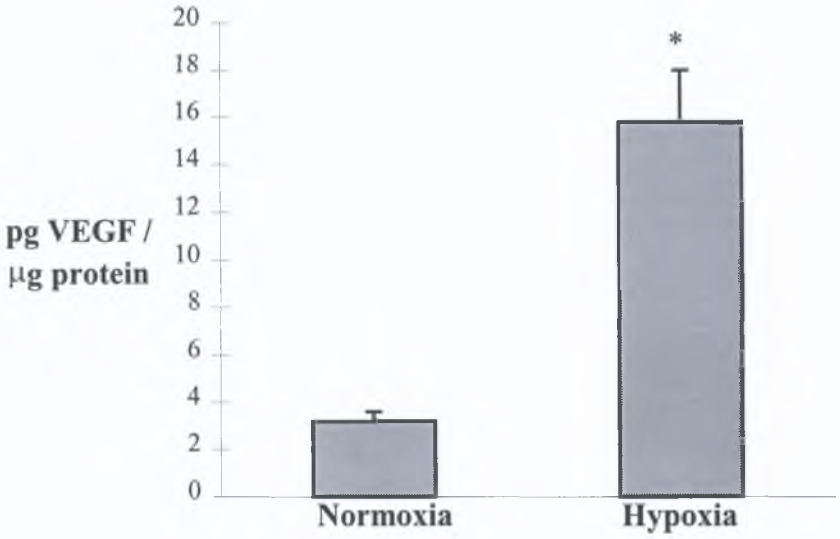
4.2 Results

4.2.1 Effect of hypoxia on VEGF protein production by BT474 and MDA-MB-231 cells

The effect of exposure to hypoxia on VEGF production by BT474 and MDA-MB-231 cells was examined Cells were exposed to a hypoxic environment (approximately 2% O_2 as measured using a dissolved oxygen probe) for 24 hr and the VEGF levels in the conditioned medium examined This is similar to the oxygen levels found within regions of solid tumour including breast tumours (Vaupel et al 1991) There was no FCS in the medium to avoid interference by agents in the FCS but VEGF produced was stabilised by the addition of 0 5% (w/v) BSA Hypoxia significantly ($p < 0\ 01$) increased VEGF expression by both the BT474 and MDA-MB-231 cell lines as measured by ELISA, figures 4 1A and 4 1B, respectively In the BT474 cells this was an increase from 3 18 ± 1 14 under normoxic conditions to 15 77 ± 3 16 pg/μg protein under hypoxic conditions This represents a 5-fold increase in VEGF protein production In the MDA-MB-231 cells the increase was from 15 54 ± 2 8 under normoxia to 52 17 ± 5 7 pg/μg protein under hypoxia, and is a 3 4 fold increase in VEGF production

4.2.2 Effect of IGF-1 on VEGF protein production by BT474 and MDA-MB-231 cells

As IGF-1 is associated with breast cancer progression and can promote angiogenesis, the effect of IGF-1 on VEGF protein production by BT474 and MDA-MB-231 cells was examined Cells were treated with IGF-1 (0, 100, 200, 300, 400, 500 ng/ml) in FCS free medium for 24 hr These were the same doses that have previously been shown to increase VEGF production by colorectal carcinoma cell lines (Warren et al 1996) VEGF produced was stabilised by the addition of 0 5% (w/v) BSA to the medium and was measured by ELISA Conditioned medium following 24 hr exposure to IGF-1 was examined for the levels of VEGF produced IGF-1 had no significant effect on VEGF production by either the BT474 (Fig 4 2A) or MDA-MB-231 cell line (Fig 4 2B)



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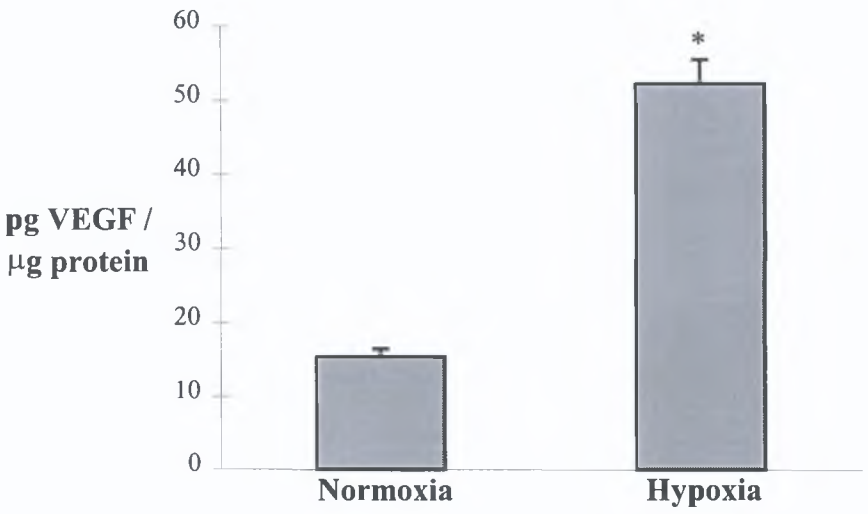
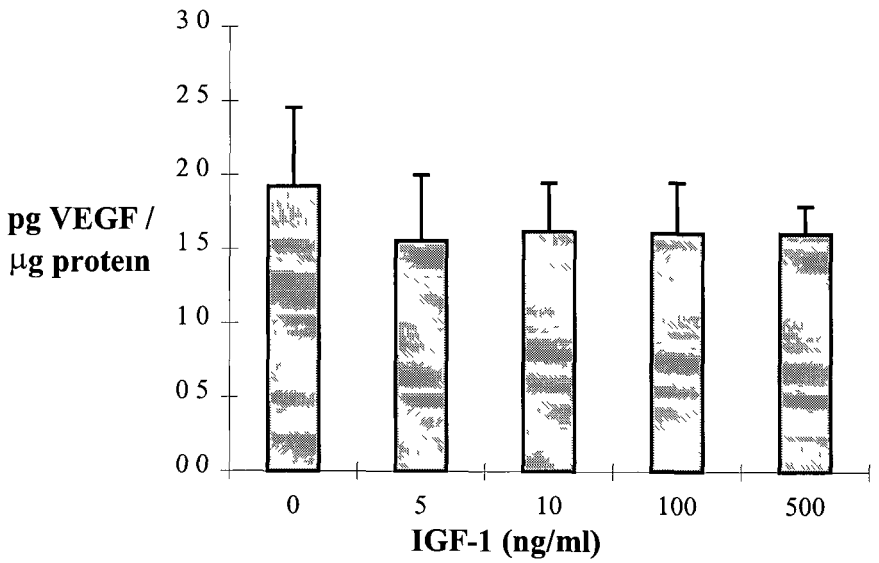
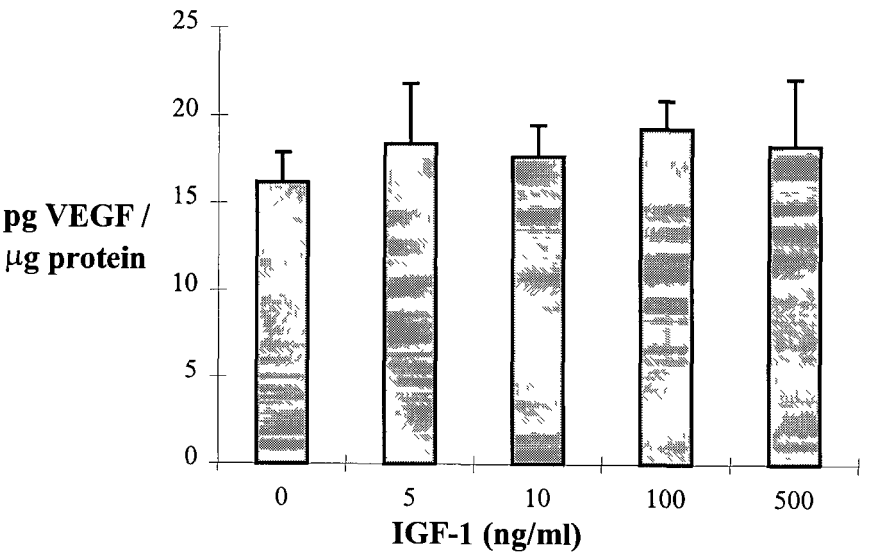


B.

Fig. 4.1: Effect of exposure to hypoxia for 24 hr on VEGF production by BT474 (**A**) and MDA-MB-231 (**B**) cell lines. Data is representative of 3 experiments carried out in triplicate and is expressed as the mean ± SEM. Statistical analysis was by students t-test. *, $p < 0.05$.



A.



B.

Fig. 4.2: Effect of IGF-1 on production of VEGF protein over 24 hr by BT474 (**A**) and MDA-MB-231 (**B**) cells Data is representative of 3 independent experiments carried out in triplicate and is expressed as mean ± SEM Statistical analysis was by ANOVA with Scheffe post hoc correction, p = non-significant

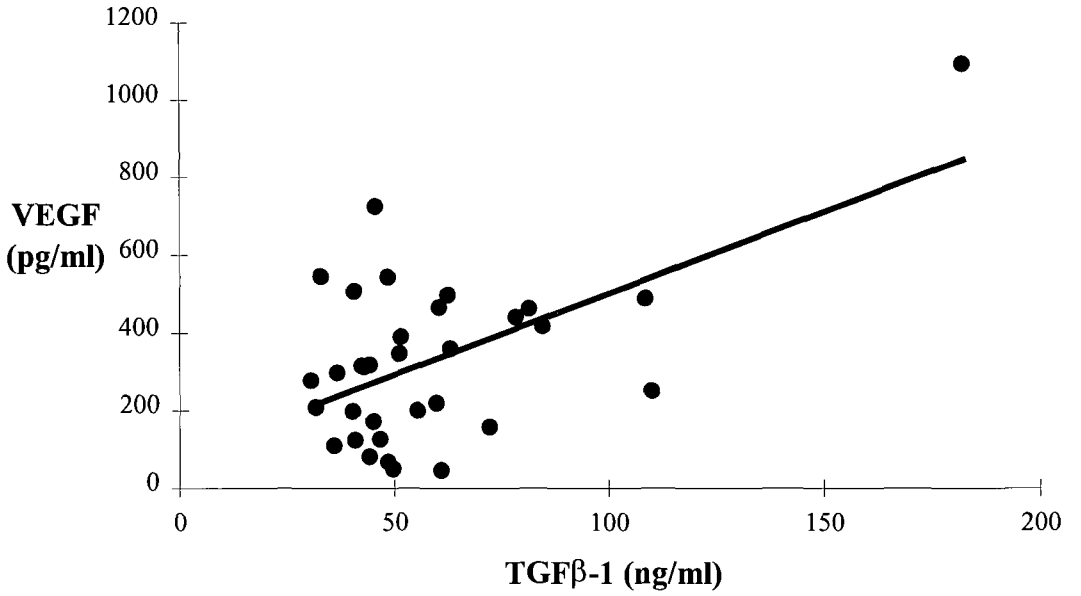
4.2.3 Correlation between serum and tumour VEGF and TGFβ-1

Having identified high levels of VEGF in breast cancer patients, levels of TGFβ-1, which has been shown to regulate VEGF in some cells types, were measured in a subset of breast cancer patients Measurements were made in both the serum and tumour tissue of these patients There was a moderate but statistically significant ($p < 0.05$) positive correlation (Pearson Product Moment Correlation coefficient = 0.57) between levels of TGFβ-1 and VEGF in the serum of breast cancer patients (n = 33) (Figure 4.3A) Table 4.1 outlines the patient characteristics The mean TGFβ-1 levels in the serum was 58.5 ± 26.9 ng/nl (range 30.68 – 110.07 ng/ml)

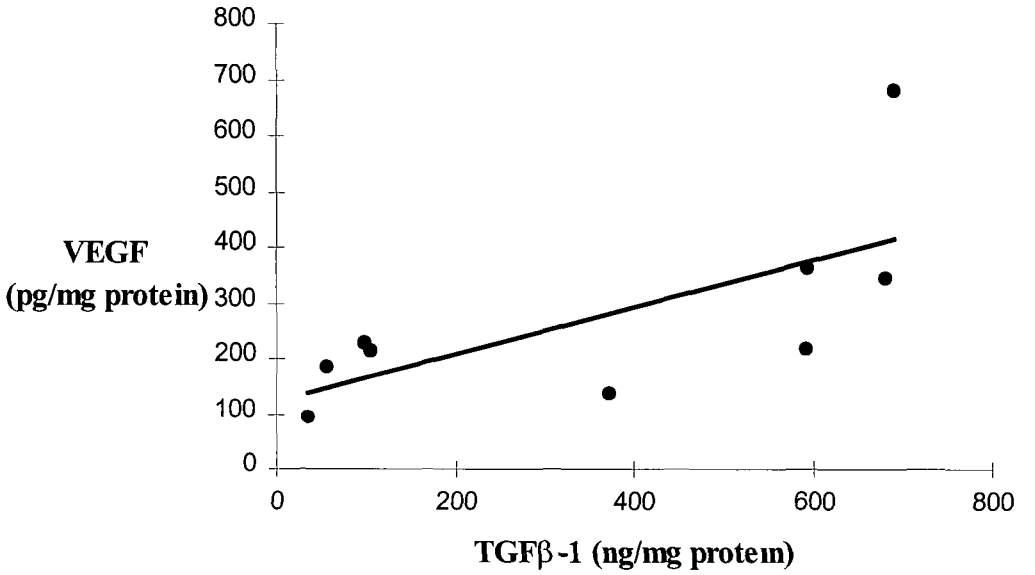
Tumour tissue was homogenised and the levels of both factors measured and expressed per mg of protein Tumour tissue from 9 patients was examined and there was a significant ($p < 0.05$) positive correlation between the levels of TGFβ-1 and VEGF (Pearson Product Moment Correlation coefficient = 0.69) (Figure 4.3B) The mean TGFβ-1 levels in tumour tissue was 358.7 ± 285.7 ng/mg protein (range 99.24 – 682.68 ng/mg protein)

No.	Stage	Age	Nodal status	ER Status	Type	TGFβ-1 (ng/ml)	VEGF (pg/ml)
1	T2	66	neg	neg	ductal	65 6	498 3
2	T2	51	neg	n/a	ductal	84 6	417 7
3	T1	58	neg	n/a	ductal	108 6	489 2
4	T3	68	neg	pos	ductal	182 2	1092 5
5	T2	74	n/a	pos	ductal	81 58	463 1
6	T2	43	pos	neg	ductal	110 1	249 9
7	T1	47	neg	neg	ductal	78 5	440 9
8	T4	58	pos	pos	ductal	30 7	277 4
9	Tis	44	pos	n/a	ductal	51 6	390 0
10	T2	43	neg	neg	ductal	61 0	46 0
11	T3	50	pos	neg	lobular	45 2	172 0
12	T1	32	neg	neg	ductal	63 1	360 0
13	T1	68	pos	pos	ductal	32 9	546 0
14	T1	76	neg	neg	ductal	31 6	208 1
15	n/a	69	n/a	n/a	n/a	36 7	297 4
16	T1	60	neg	pos	ductal	55 3	199 3
17	T3	54	pos	pos	lobular	40 3	197 5
18	T2	60	pos	neg	ductal	36 0	109 8
19	T2	65	neg	neg	ductal	42 9	313 9
20	T1	59	neg	neg	ductal	60 6	465 9
21	T1	76	neg	neg	ductal	59 8	219 2
22	T2	59	n/a	pos	ductal	44 3	318 3
23	T3	74	n/a	neg	ductal	72 3	158 0
24	n/a	63	n/a	n/a	n/a	40 9	124 5
25	T2	64	n/a	pos	ductal	46 7	126 4
26	T4	70	n/a	n/a	ductal	42 5	316 2
27	n/a	66	n/a	neg	ductal	48 6	68 3
28	T1	59	neg	neg	ductal	49 8	49 8
29	n/a	54	n/a	n/a	n/a	44 3	81 4
30	T1	50	n/a	pos	ductal	51 2	347 3
31	T4	57	n/a	neg	ductal	40 7	508 2
32	T4	79	n/a	n/a	ductal	45 7	725 0
33	T2	52	pos	neg	ductal	48 5	544 5

Table 4.1 Patient characteristics for correlation of serum TGFβ-1 with VEGF pos = positive, neg = negative, n/a = not available



A.

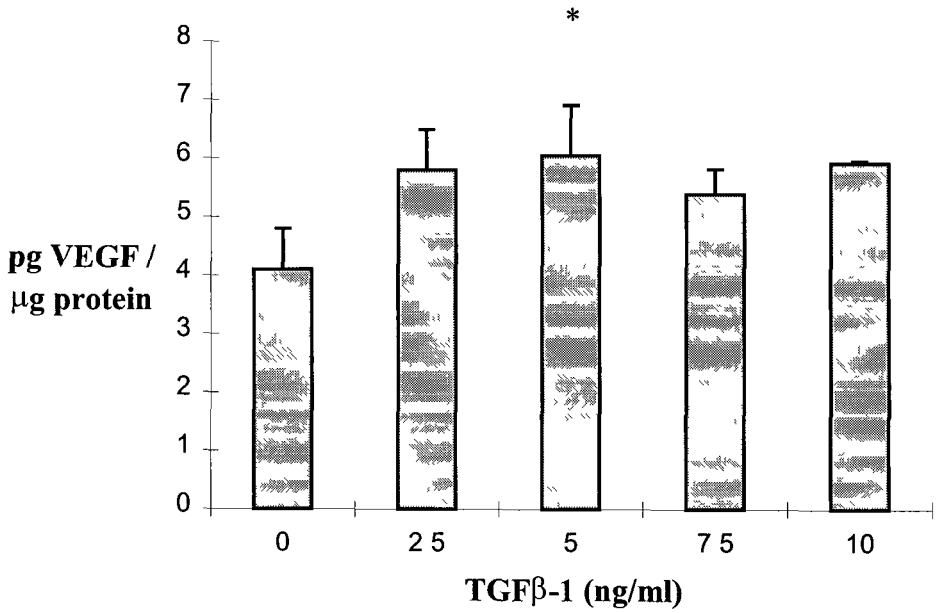


B.

Fig 4.3: Association between serum (**A**) and tumour (**B**) TGFβ-1 and VEGF levels Statistical analysis was by Pearson Product Moment Correlation Correlation coefficient = 0 57, serum and 0 69, tumour tissue, $p < 0\ 05$

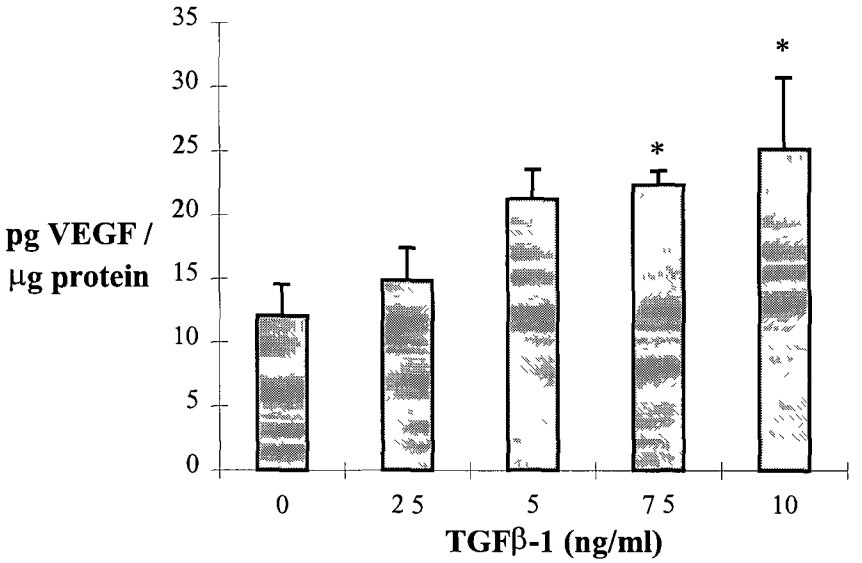
4.2.4 Effect of TGFβ-1 on VEGF protein production by BT474 and MDA-MB-231 cells

Having found a positive correlation between the serum and tumour levels of TGFβ-1 and VEGF in breast cancer patients, the effect of TGFβ-1 on VEGF production by cultured breast cancer cells was examined BT474 and MDA-MB-231 cells were exposed to TGFβ-1 (0, 2 5, 5, 7 5 10 ng/ml) for a 24 hr period and VEGF levels in the conditioned medium measured by ELISA The medium did not contain FCS so that there would be no interference from agents m the FCS and 0 5% (w/v) BSA was included to stabilise secreted VEGF The BT474 cell line produced significantly ($p = 0\ 03$) more VEGF protein following 5 ng/ml TGFβ-1 compared to controls (6 04 ± 0 68 vs 4 09 ± 0 69 pg/μg protein) (Figure 4 4A) The MDA-MB-231 cell line showed a concentration dependent increase in VEGF production following TGFβ-1 exposure (Figure 4 4B) This was statistically significant at 7 5 and 10 ng/ml TGFβ-1 compared to untreated controls (21 24 ± 2 26 and 25 25 ± 5 5 vs 12 1 ± 2 4 pg/μg protein, $p = 0\ 03$) The correlation between TGFβ-1 and VEGF in the serum and tumour tissue only shows there is an association between these two factors but gives no information of the effect of each factor on the other A reciprocal experiment, examining the effect of VEGF on TGFβ-1 expression by BT474 and MDA-MB-231 cells was also performed The BT474 cells did not produce any TGFβ-1 as measured by ELISA The MDA-MB-231 cell line produced very low levels of TGFβ-1 (3 39 ± 2 58 pg/ml in untreated controls), that were at the limits of detection of the ELISA used, but VEGF (0, 1, 5, 10, 20, 100 ng/ml) did not significantly alter ($p > 0\ 05$) TGFβ-1 protein production over 24 hr (Figure 4 5)



A.

Fig. 4.4A: Effect of TGFβ-1 on VEGF production by BT474 cells Cells were exposed to TGFβ-1 for 24 hr and VEGF in conditioned medium examined by ELISA Data is representative of 3 independent experiments and is expressed as mean ± SD Statistical analysis is by ANOVA with Scheffe post hoc correction *, $p < 0.05$ vs 0 ng/ml TGFβ-1



B.

Fig. 4.4B: Effect of TGFβ-1 on VEGF production by MDA-MB-231 cells Cells were exposed to TGFβ-1 for 24 hr and VEGF in conditioned medium examined by ELISA Data is representative of 3 independent experiments and is expressed as mean ± SD Statistical analysis is by ANOVA with Scheffe post hoc correction *, $p < 0.05$ vs 0 ng/ml TGFβ-1

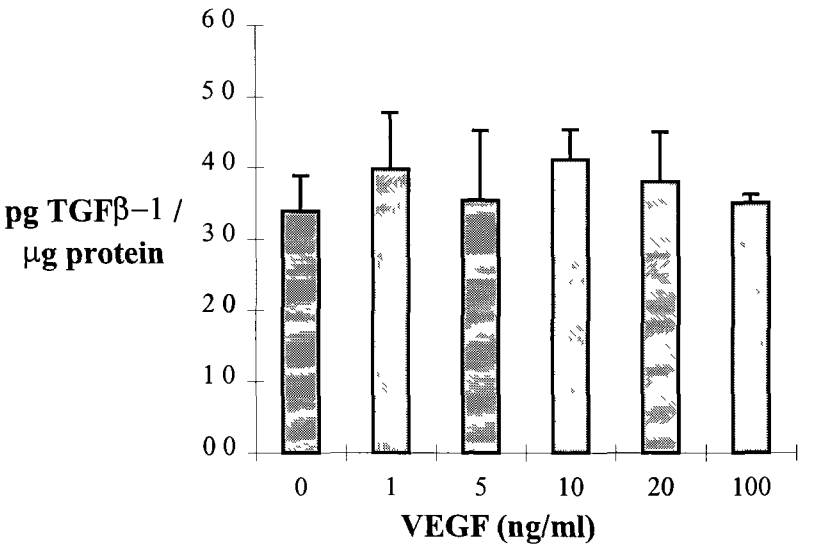
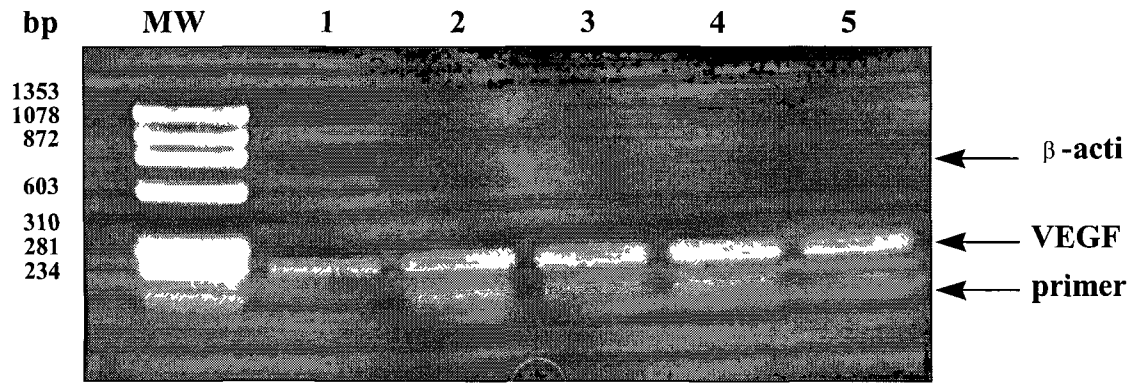


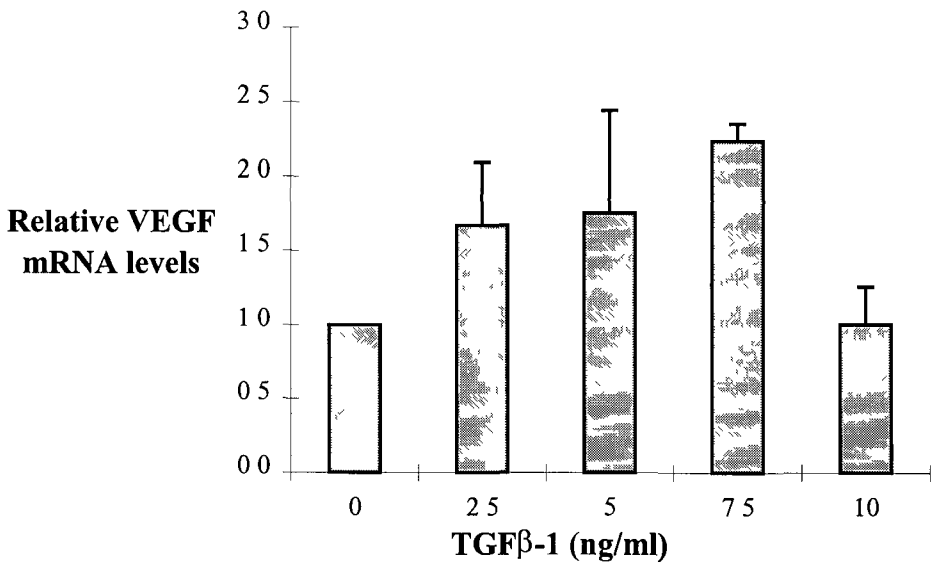
Fig. 4.5: Effect of VEGF on TGFβ-1 production by MDA-MB-231 cells Cells were exposed to VEGF for 24 hr and TGFβ-1 m the conditioned medium was examined by ELISA Data is representative of 3 independent experiments and is expressed as mean ± SEM Statistics is by ANOVA with Scheffe post hoc correction p = ns

4.2.5 Effect of TGFβ-1 on VEGF mRNA in BT474 and MDA-MB-231 cells

Having found that TGFβ-1 increased VEGF protein production by BT474 and MDA-MB-231 cells the effect of TGFβ-1 on VEGF mRNA levels in these cells was examined Cell lines were treated with TGFβ-1 (0, 2 5, 5, 7 5, 10 ng/ml) for 12 hr in FCS free medium Total RNA was isolated, reverse transcribed and VEGF mRNA amplified by PCR The mRNA for β-actm, a constitutively expressed gene, was also amplified and the ratio of VEGF/β-actin intensity in an agarose gel was assessed by densitometry Figure 4 6A shows a representative agarose gel of PCR products amplified from the BT474 cells Figure 4 6B represents the relative VEGF/β-actm ratio from 3 independent experiments A representative photograph of VEGF and β-actin PCR products m the MDA-MB-231 cell line following exposure to TGFβ-1 is shown in figure 4 7A and the VEGF/β-actin ratio from 3 experiments can be seen in figure 4 7B For the graphs in both figures the ratio of VEGF/β-actin in the control sample is taken as 1 and the other samples are graphed relative to the control Though it was not significant, TGFβ-1 at 2 5,5 and 7 5 ng/ml appears to increase VEGF mRNA levels in BT474 cells while TGFβ-1 at 7 5 and 10 ng/ml increases VEGF mRNA levels m MDA-MB-231 cells

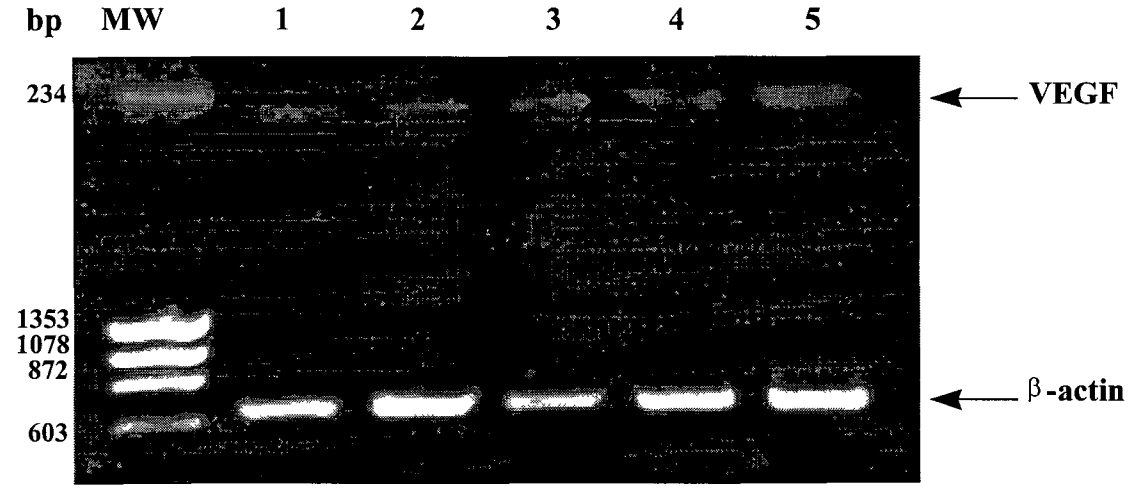


A.

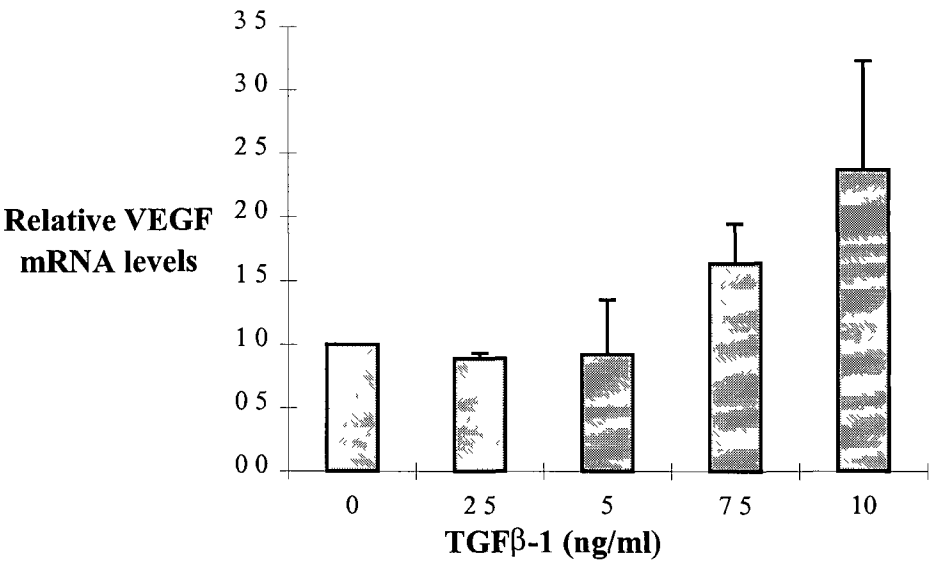


B.

Fig. 4.6: Effect of TGFβ-1 on BT474 VEGF mRNA over 12 hr **(A)** shows a 1 5% agarose gel of VEGF and β-actin PCR products generated by RT-PCR from total RNA Position of molecular weight markers (MW), ϕX174 digested with *Hae*III, are shown (bp) (Lane 1) Lanes 2 - 5 are samples treated with 0, 2 5, 5, 7 5 and 10 ng/ml TGFβ-1, respectively Position of VEGF and β-actin PCR products is indicated **(B)** Densitometric analysis of the relative ratio of VEGF/β-actin Data is expressed as mean VEGF/β-actin relative to the untreated sample ± SEM and is from 3 independent experiments Control value is taken as 1



A.

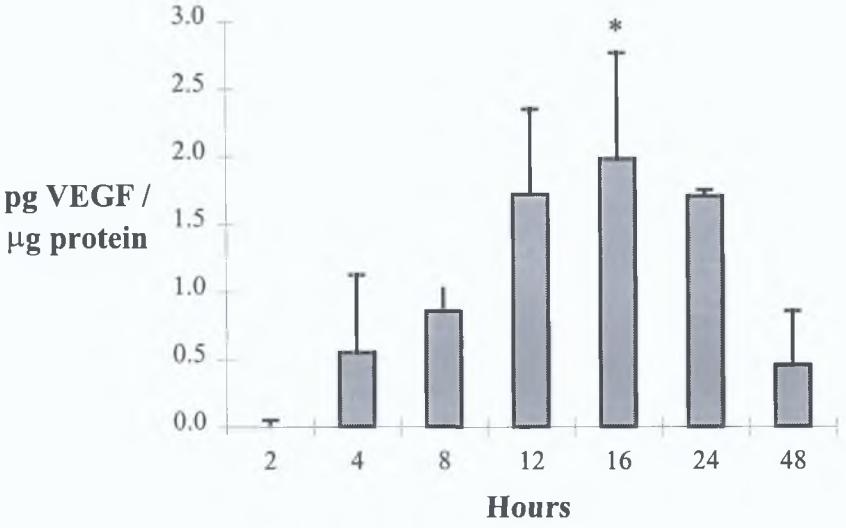


B.

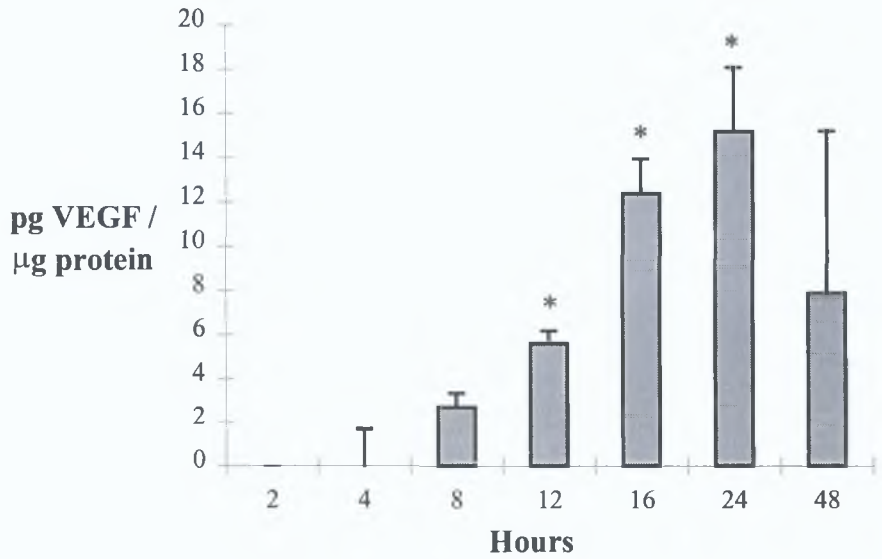
Fig. 4.7: Effect of TGFβ-1 on MDA-MB-231 VEGF mRNA over 12 hr (**A**) shows a 1 5% agarose gel of VEGF and β-actm PCR products generated by RT-PCR from total RNA Positıon of molecular weıght markers (MW), ϕX174 dıgested wıth *Hae*III, are shown (bp) (Lane 1) Lanes 2 - 5 are samples treated wıth 0, 2 5, 5, 7 5 and 10 ng/ml TGFβ-1, respectıvely Posıtıon of VEGF and β-actın PCR products ıs ındıcated (**B**) Densıtometrıc analysıs of the relatıve ratıo of VEGF/β-actın Data ıs expressed as mean VEGF/β-actın relatıve to the untreated sample ± SEM and ıs from 3 ındependent experıments Control value ıs taken as 1

4.2.6 Time course of TGFβ-1 induction of VEGF protein production by BT474 and MDA-MB-231 cells

As TGFβ-1 increased breast cancer cell line production of VEGF, a time course of TGFβ-1 induction of VEGF was carried out The BT474 and MDA-MB-231 cell lines were treated for various time periods (2, 4, 8, 12, 16, 24 and 48 hr) with TGFβ-1 at the dose previously shown to result m maximal induction of VEGF (5 ng/ml for BT474 cells and 10 ng/ml for MDA-MB-231 cells, Fig 4 4A and Fig 4 4B respectively) As before, the medium did not contain FCS so that there would be no interference from agents in FCS and VEGF produced was stabilised by the addition of 0 5% (w/v) BSA In order to account for VEGF that would have accumulated under basal conditions the same experiment was carried out without the addition of TGFβ-1 and subtracted from TGFβ-1 stimulated production The BT474 cell line showed increased VEGF production from 4 - 48 hr (Figure 4 8A) This induction was maximal at 16 hr (1 98 pg/μg protein increase over untreated cells) The MDA-MB-231 cell line showed increased VEGF production from 8 - 48 hr (Figure 4 8B) The maximal induction for MDA-MB-231 cells was at 24 hr (15 13 pg/μg protein increase over untreated cells)



A.



B.

Fig. 4.8: Effect of TGFβ-1 over time on VEGF production by BT474 (**A**) and MDA-MB-231 cells (**B**). Data is representative of 3 independent experiments and is expressed as the increase in VEGF compared to untreated cells at the same time period ± SEM. Statistical analysis was by ANOVA with Scheffe post hoc correction. *, p 0.05 vs 2 hr.

4.3 Discussion

Hypoxia upregulated VEGF production by the breast cancer cell lines, BT474 and MDA-MB-231 In the BT474 cell line there was a 5 fold increase while in the MDA-MB-231 cells there was a 3 4 fold increase This is a similar to the increase found by another group that showed an increase of 1 4 to 6 9 fold in VEGF expression levels in a panel of different breast cancer cell lines (Scott et al , 1998) Other authors have found VEGF levels to be highest near necrotic regions of breast tumours, which are believed to be the most hypoxic regions of a tumour (Wright et al , 1997) The exact mechanism through which hypoxia regulated VEGF was not examined but other studies have highlighted a number of mechanisms that may be employed by these cells Hypoxia increases VEGF mRNA transcription rate in C6 glioma cells (Levy et al , 1995) A post-transcriptional mechanism whereby hypoxia stabilises VEGF mRNA has also been described in pheochromocytoma cell line and in C6 glioma cells (Levy et al , 1996, Levy et al 1995) Increasing the half life of mRNA means that there is increased translation of transcripts and therefore more protein produced Hypoxia increased the half life of VEGF mRNA by 43 mins to 106 min by a mechanism that involved the binding of a hypoxia-inducible factor to the 3'- untranslated region (UTR) of the VEGF gene (Levy et al 1996) The inclusion of a binding site for hypoxia-inducible factor 1 (HIF-1) in the promotor region of the VEGF gene and the requirement for the 3'-regulatory region, that conforms under certain conditions to stabilise VEGF mRNA, further supports the hypothesis that hypoxic induction is by a combination of transcription and post-transcriptional mechanisms (Damert et al , 1997) The mRNA levels of VEGFR-1 (flt-1) were upregulated threefold in the liver of rats exposed to low oxygen levels or injection with cobalt, which mimics the effects of hypoxia (Sandner et al , 1997) The levels of the other VEGF receptors remained unchanged Endothelial cells do not normally express VEGF but exposure to hypoxia allows both human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMEC) to express VEGF (Namiki et al , 1995) It would seem that the extreme stress of hypoxia may allow VEGF to act on endothelial cells in an autocrine manner Hypoxia did not upregulate the expression of VEGF-B or VEGF-C in human fibroblasts (Enholm et al , 1997) and downregulated PlGF expression in a trophoblast

choriocarcinoma cell line (Khaliq et al , 1999) which demonstrates that the response to hypoxia is not conserved amongst the VEGF family members

IGF-1 did not increase BT474 or MDA-MB-231 cell line production of VEGF protein Concomitant research within the lab had previously demonstrated that these cell lines do express the IGF-1 receptor, however, activation of the IGF-1 receptor results in the stimulation of diverse intracellular pathways and it is possible that upon binding of IGF-1 the growth related genes are activated without activation of the VEGF gene (Rubin and Baserga, 1995) In colorectal carcinoma cell lines IGF-1, at the same doses, increased VEGF protein by both transcriptional and post-transcriptional mechanisms (Warren et al 1996) IGF-1 also increased VEGF expression by colon carcinoma cell lines by a transcriptional mechanism (Akagi et al , 1998) In this case the increase of VEGF by IGF-1 occurred regardless of the presence of IGFBPs IGF-1 also increased VEGF mRNA, in a concentration dependent manner in retinal epithelial cells (Punglia et al , 1997) Interestingly, IGF-1 has been found to increase expression of target genes, the glucose transporters GLUT-1 and GLUT-3, by increasing HIF-1 levels (Zelzer et al , 1998) The association of IGF-1with the progression of breast cancer may be solely related to the mitogenic and anti-apoptotic effects of IGF-1 (Winder et al , 1989)

A positive association was found between the levels of TGFβ-1 and VEGF in the serum of patients with breast cancer and in the breast tumour tissue of breast cancer patients A high level of TGFβ-1 was associated with a high level of VEGF in both serum and tumour tissue The levels of TGFβ-1 found in the serum (mean concentration approximately 60 ng/ml) are slightly higher than previously reported circulating levels in a study of 26 breast cancer patients (20 ng/ml) and considerably higher than the reported levels in normal healthy controls (4 3 ng/ml) (Kong et al , 1995) In that study the levels of TGFβ-1 were reduced after removal of the tumour indicating that elevated TGFβ-1 was most probably produced by the tumour (Kong et al 1995) This is supported by the finding that TGFβ-1 was elevated in the tumour tissue compared to the matched normal tissue of breast cancer patients (Toomey, 1998) Although we did not look at TGFβ-1 levels m healthy controls or in normal tissue there does appear to be very high levels of TGFβ-1 within the tumour tissue BT474 and MDA-MB-231 cells in this study produced negligible amounts of TGFβ-1 which raise the question of which

cells within the tumour are producing TGFβ-1 Previous studies have shown that breast tumour cells do produce TGFβ-1 (Zajchowski et al , 1988) TGFβ-1 in tumours may also be produced by the stromal cells Northern blot analysis has demonstrated that fibroblasts from benign and malignant breast lesions produce TGFβ-1 mRNA (Cullen et al , 1991) TGFβ-1 is chemotactic for macrophages which may also produce more TGFβ-1 (Assoian et al , 1987) VEGF upregulates endothelial cell expression of plasminogen activators (Pepper et al 1991) which may release matrix bound TGFβ-1 resulting m an increase in circulating and tumour levels of TGFβ-1 TGFβ-1 stimulates macrophage production of urokinase which can release more TGFβ-1 and other matrix bound angiogenic factors such as bFGF and some VEGF isoforms (Falcone et al , 1993)

TGFβ-1 increased VEGF protein production by BT474 and MDA-MB-231 breast cancer cell lines In a reciprocal experiment both cell lines were treated with varying concentration of VEGF (0 – 100 pg/ml) Neither control nor TGFβ-1 treated BT474 cells produced detectable TGFβ-1 while the MDA-MB-231 cell line produced low levels of TGFβ-1 that were at the limit of detection of the assay used and did not vary with exposure to VEGF Though it was not significant, TGFβ-1 at 2 5,5 and 7 5 ng/ml appears to increase VEGF mRNA levels in BT474 cells while TGFβ-1 at 7 5 and 10 ng/ml increases VEGF mRNA levels in MDA-MB-231 cells The response of BT474 and MDA-MB-231 cell lines to TGFβ-1 is slightly different The upregulation of VEGF in BT474 cells was somewhat irregular while the increase in MDA-MB-231 cells was clearly concentration dependent TGFβ-1 induction of VEGF mRNA was at the same doses that increased VEGF protein production and suggest that the increase in VEGF protein is directly due to increased VEGF mRNA Induction of VEGF by TGFβ-1 in the BT474 cells was more rapid than in the MDA-MB-231 cells, increasing within 4 hr in BT474 cells and 8 hr in MDA-MB-231 cells However, levels began to decrease earlier m the BT474 cells (within 16 hr) compared to in the MDA-MB-231 cells (within 24 hr) TGFβ-1 does not universally upregulate VEGF production as it has been found to inhibit VEGF expression in both undifferentiated and differentiated colon cancer cells (Hsu et al , 1995) TGFβ-1 increases expression of VEGF-C, the VEGF species associated with lymphatic vessels, but did not upregulate expression of VEGF-B which shares the most homology to VEGF and can be expressed in tumours (Enholm et al

1997). TGFβ-1 downregulates levels of the VEGF receptor VEGFR-2 (Flk-1) and also decreases the binding of VEGF to this receptor (Mandriota et al., 1996). Hypoxia is known to activate TGFβ-1 and hypoxia with TGFβ-1 synergistically increases VEGF expression in breast cancer cells and macrophages (Berse et al., 1999; Harmey et al. 1998). In macrophages, TGFβ-1 increased VEGF protein but not mRNA suggesting in addition to transcriptional mechanisms there is also post transcriptional mechanisms involved (Harmey et al. 1998).

The TGFβ-1 levels used *in vitro* for this study are lower than the levels that were found in the tumour tissue but are similar to the levels that have previously been shown to increase VEGF expression within cells (Brogi et al. 1994). The effects of TGFβ-1 appear to be dependent on the concentration and probably also on the presence of other factors. Levels that increased VEGF expression in this study have been found to inhibit endothelial cell migration and capillary lumen formation (Pepper et al. 1993). Lower doses of TGFβ-1 (0.2 - 0.5 ng/ml) potentiated the angiogenic effects of both bFGF and VEGF (Pepper et al. 1993) but at this concentration in this study did not effect VEGF production by BT474 or MDA-MB-231 cells. In a separate study, TGFβ-1 at 0.5 ng/ml induced tube-like formation of microvascular endothelial cells *in vitro* (Merwin et al., 1990) and at 1 ng/ml enhanced maturation and lumen formation of endothelial cells exposed to smooth muscle cell conditioned medium (Sakuda et al., 1992). TGFβ-1 at 2 ng/ml caused apoptosis of endothelial cells and development of capillary morphogenesis of microvascular endothelial cells (Choi and Ballermann, 1995). Transfection of cells with a mutant TGFRII inhibited this effect confirming the role of TGFβ. Apoptosis of endothelial cells may be an important event in capillary morphogenesis since it allows for homeostasis of endothelial cells and allows these cells to align correctly and form functional endothelium. TGFβ-1 also inhibits the formation of the anti-angiogenic molecule angiostatin by human pancreatic cancer cells (O'Mahony et al., 1998) which is yet another part in the complex role of this molecule with regard to angiogenesis.

The data presented in this chapter suggests a complex, self regulating association between TGFβ-1 and VEGF and also with several other steps of the angiogenic cascade. The mechanism through which TGFβ-1 aids tumour progression and angiogenesis is complex. We have confirmed that amongst other factors, TGFβ-1 is a regulator of VEGF expression and with other evidence suggest that this molecule will play an

important role in breast cancer This data supports a recent hypothesis by Reiss *et al* (1997) which suggests that while TGFβ-1 initially inhibits breast tumour growth it may begin to promote tumour growth as the disease advances

CHAPTER 5

ANTI-ANGIOGENIC MECHANISMS OF TAMOXIFEN

5.1 Introduction

Tamoxifen has been used as the primary hormonal therapy for breast cancer for almost 30 years Structurally, tamoxifen is a nonsteroidal triphenylethylene although it is capable of adopting a structure resembling that of steroids such as 17β-oestradiol (Buckley and Goa, 1989) Tamoxifen was originally designed as an anti-oestrogen oral contraceptive in the late 1950s but it was found to increase ovulation Later it was found to have very complex pharmacological actions including those of a pure oestrogen agonist, a partial oestrogen agonist and an oestrogen antagonist depending on the tissue type and species In humans tamoxifen acts as an oestrogen antagonist in the breast and an agonist in the uterus (Boothe et al , 1994) Tamoxifen has been widely used as an anti-oestrogen in breast cancer Oestrogen is mitogenic for epithelial cells of the breast and oestrogen receptors are expressed by approximately 60% of breast tumours (Lippman and Swain, 1992) Many tumours escape oestrogen dependency but it is believed that oestrogen is essential for tumour growth at the early stages of all breast tumours and remains the primary mitogenic signal m many tumours (King, 1991) The binding of oestradiol, the most soluble and freely available form of oestrogen, to the oestrogen receptor (ER) triggers a number of events Upon binding the ER is activated, dimerises, moves to the nucleus and binds to an oestrogen response element (ERE) in the promotor region of oestrogen responsive genes The oestrogen receptor then forms a transcription unit with RNA polymerase by binding various transcription activating factors and initiates transcription The exact anti-oestrogenic mechanisms of tamoxifen are unknown The probable mechanism, however, is that by competitively binding to the ER, tamoxifen blocks oestradiol binding and induction of gene expression and in turn only induces the expression of a limited number of genes (Jordan, 1994)

Traditionally, tamoxifen has been used as an oestrogen antagonist in the treatment of ER positive breast cancer as adjuvant therapy following surgical removal of tumour However, in recent years, there has been considerable interest in the potential benefits of tamoxifen therapy for ER negative breast cancer A report from the Early Breast Cancer Trialist's Collaborative Group, involving 75,000 women in 133 randomised trials, showed that tamoxifen was of benefit m the treatment of some ER

negative breast cancer Tamoxifen therapy significantly increased the recurrence-free survival by 13% in breast cancer patients of all ages with ER negative disease and by 16% in those over 50 years Although this increase in recurrence free survival is less than in ER positive breast cancer patients (32% increase in recurrence-free survival) it indicates that tamoxifen has additional anti-tumour effects that are independent of the oestrogen receptor Overall survival was also improved in these patients, following tamoxifen therapy, by 11% in patients of all ages and by 16% m patients over 50 years (Early Breast Cancer Trialist's Collaborative Group, 1992) There was no significant difference in the overall survival between ER negative and ER positive patients though the trend suggested better survival in ER positive patients (Early Breast Cancer Trialist's Collaborative Group, 1992)

In addition to its use in the treatment of breast cancer, tamoxifen has been used in the treatment of some other malignancies Tamoxifen in conjunction with dacarbazme was found to be more effective than dacarbazine alone in the treatment of metastatic melanoma (Cocconi et al , 1992) In a separate study, tamoxifen reduced the size of metastatic melanoma lesions by approximately 50% in male patients (Hirota, 1997) The use of high dose tamoxifen treatment (100 mg/day) in patients with malignant gliomas resulted in a favourable response, reduction m tumour volume, in a minority (approximately a quarter) of these patients (Couldwell et al , 1993) m a manner that is independent of the ER The results of the EBCTCG trials and the benefit of tamoxifen therapy in the treatment of melanoma and gliomas suggests that while the principal mode of action of tamoxifen is mediated through the ER it has other lesser but significant anti-tumour activities that do not depend on the ER

At a cellular level there is further evidence of ER independent actions of tamoxifen Inhibition of ER positive breast cancer cell growth by tamoxifen is not completely reversed by a large excess of oestradiol (Sutherland et al , 1983), suggesting that tamoxifen may be acting at sites other than the oestrogen receptor Tamoxifen binds with low affinity to a high capacity microsomal protein termed the anti-oestrogen binding site (AEBS) (Sutherland et al , 1980) though the relevance of this protein in breast cancer is still unknown Tamoxifen inhibits the action of protein kinase C (PKC) though it does not seem to do so through any interaction with the active site (O'Brian et al , 1985) Protein kinase C forms a part of the second messenger system for many

growth factors and mutations in PKC have been shown to promote tumour growth (Guillem et al , 1987) Calmodulin is an intracellular calcium receptor involved in the cell cycle and its inhibition with the specific antagonist calmidazohum blocks progress of MCF-7 cells through the cell cycle (Musgrove et al , 1989) Calmodulin-dependent cAMP phosphodiesterase (CDP) is also inhibited by tamoxifen (Lam, 1984) Tamoxifen binds to and interferes with the action of calmodulin rather than the phosphodiesterase Tamoxifen retards glycosphingolipid metabolism in human cancer cells by inhibiting the glucosylceramide synthase (Cabot et al , 1996) Glycosphingolipids accumulate in multi-drug resistant (MDR) cancer cells (Lavie et al , 1996) and tamoxifen reverses MDR in an ER independent manner (Ramu et al , 1984) Tamoxifen regulates the expression of a number of cytokines through an ER-mdependent mechanism Tamoxifen increased TGFβ-1 production by fibroblasts that were ER negative (Colletta et al , 1990) Immunohistochemical analysis of breast tissue before and after 3 months of tamoxifen treatment demonstrated increased expression of TGFβ-1, this increase was not associated with ER expression (Butta et al 1992) Interestingly, TGFβ-1 staining was predominantly associated with stromal tissue rather than the tumour cells themselves Tamoxifen reduces the levels of the positive growth regulator insulin-like growth factors I in breast cancer patients (Pollak, 1990) Further work in rats led the authors to suggest that this may be through a mechanism independent of the interaction between tamoxifen and the ER since the effect was observed in variety of tissue types including those that would that have minimal levels ER expression (Huynh et al 1993)

There are a number of reports suggesting that tamoxifen is capable of inhibiting angiogenesis A sulphated polysaccharide peptidoglycan complex (SP-PG) isolated from *Arthrobacter* species inhibits angiogenesis induced by variety of breast cancer cell lines in female mice (Inoue et al , 1988) Tamoxifen in combination with SP-PG suppressed the number of capillaries in MCF-7 and U-2 induced tumours in mice (Tanaka et al , 1991) Tamoxifen treatment reduced blood vessel counts in MCF-7 mammary tumours in Swiss mice by approximately 70% (Lindner and Borden, 1993) In a separate study tamoxifen therapy reduced microvessel density of MCF-7 tumours implanted in mice by 50%, as measured by magnetic resonance imaging and histochemical staining (Furman Haran et al , 1994) Tamoxifen inhibited angiogenesis

in the chick egg chorioallantoic membrane assay (CAM)(Gagliardi and Collins, 1993) As this effect was not reversed by the addition of excess oestrogen it suggests that anti-angiogenic activity is by an ER independent mechanism One potential mechanism by which tamoxifen may reduce angiogenesis is by inhibiting the proliferation of endothelial cells and a number of studies have shown that this does occur Tamoxifen reduced H^3-thymidine uptake by HUVECs by 70%, an effect that was potentiated by the addition of shark cartilage (McGuire et al , 1994) Exposure of porcine pulmonary artery and human dermal microvascular endothelial cells to VEGF and bFGF, separately, increased the proliferation of these cells, but exposure with tamoxifen abolished this induction in proliferation (Gagliardi et al , 1996) The effect was not reversed by an addition of excess 17β-oestradiol and provides further evidence that this effect is independent of the ER

The aim of this chapter was to examine potential anti-angiogenic effects of tamoxifen VEGF expression had already been found to be elevated in breast cancer and most likely plays a central role in the angiogenic response mounted by tumours Since tamoxifen has previously been shown to have anti-angiogemc activity and alters cytokine expression the effect of tamoxifen on VEGF levels in cancer patients was examined The effect of tamoxifen on breast cancer cell and macrophage production of VEGF was examined to determine the anti-angiogemc effect of tamoxifen on these cells Furthermore, the effect of tamoxifen on endothelial cell migration and the transendothehal migration of monocytes was assessed since these are important aspects in the development of angiogenesis

5.2 Results

5.2.1 Tamoxifen decreases serum and ascites fluid levels of VEGF

The accumulation of ascites fluid is a frequent complication of advanced cancer and guinea pig ascites fluid contains high levels of VEGF (greater than approximately 400 pg/ml)(Yeo et al , 1993) Figure 5 1 shows the levels of VEGF m the serum and ascites fluid of a 67-year-old male patient with malignant ascites secondary to a gastric tumour during tamoxifen treatment (20 mg/day) Levels of VEGF m serum and ascites fluid, prior to tamoxifen therapy, were very high (732 29 and 1999 36 pg/ml, respectively) Following 2 weeks of tamoxifen treatment the levels of VEGF were notably reduced in both the serum and ascites fluid (408 24 and 361 35 pg/ml, respectively) This represents a reduction of 44% m the serum and 82% in the ascites fluid Continued tamoxifen treatment, for a further 2 weeks, maintained these reduced VEGF levels

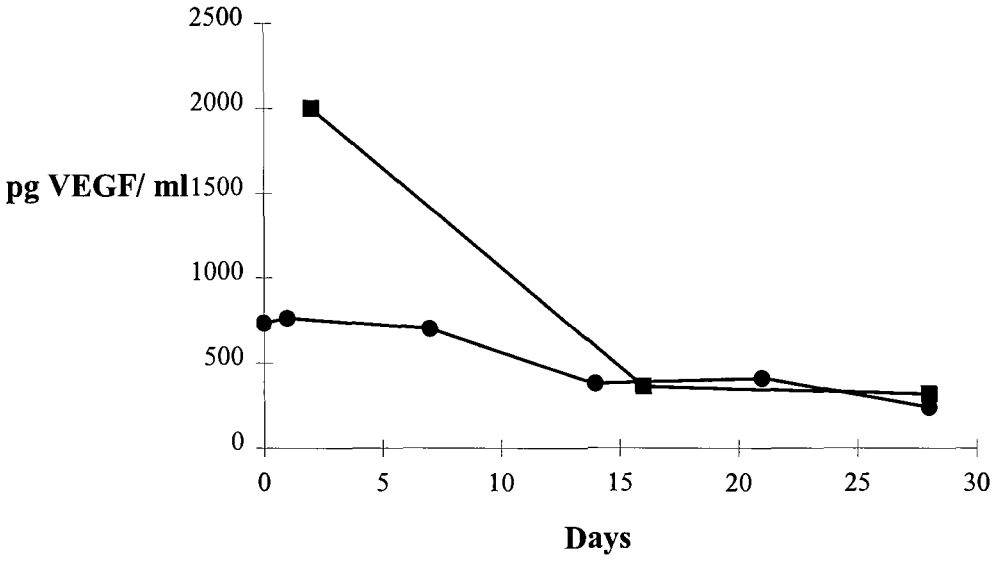


Fig. 5.1: Effect of tamoxifen treatment (20 mg/day) on both serum (—●—) and (—■—) ascites fluid levels of VEGF in a male patient with ascites associated with a gastric tumour

5.2.2 Preoperative tamoxifen decreased serum VEGF levels in a subpopulation breast cancer patients

Patients presenting at the outpatients clinic with primary breast cancer were treated with 20 mg of tamoxifen a day for 2 weeks prior to surgery Only patients with no previous history of cancer and who were not receiving chemotherapy or radiotherapy were recruited All patients were post-menopausal Approval from the Hospital Ethics Board had been sought and granted before patients were recruited A sample of serum was taken before (day 0) and after (day 14) tamoxifen treatment A 2 week treatment period was chosen since in a male patient treated with tamoxifen the maximum reduction m serum and ascites VEGF occurred within 2 weeks In addition, treatment for 2 weeks did not delay surgery, which is usually scheduled 2 – 3 weeks after initial clinical visit Patient details are summarised in Table 5 1 Presence of tumour was confirmed from cytopathology or histology reports Tumour type, stage and presence of ER was determined from histology reports For some patients, who did not undergo curative surgery, this data was not available Tumours were staged using the size component of the TNM staging system (TNM Atlas, 1992) Tamoxifen treatment decreased serum levels of VEGF in 75% (15/20) of patients (Fig 5 2) Patient 8 had a massive increase m serum VEGF levels (318 3 to 1721 2 pg/ml) following tamoxifen treatment The basis of this atypical response is unknown The change in this patient skewed the mean so that there was an average increase in serum VEGF following tamoxifen therapy However, when patient 8 is excluded from analysis there was a significant ($p < 0\ 01$) decrease of 29 6% in the median serum VEGF levels following tamoxifen treatment (Figure 5 3) The median was plotted since the data was not normally distributed and a non-parametric test was carried to examine statistical significance For the 15 patients who showed a decrease in serum VEGF levels the mean decrease was a 28 6% decrease Of the 15 patients that showed decreased serum VEGF levels 8 were ER negative, 4 were ER positive, 1 of which was weakly positive, and for the remaining 3 the ER status was unknown This indicates that the decrease in serum VEGF is independent of the ER Of the patients that demonstrated a decrease in serum VEGF in response to tamoxifen therapy disease stages were as follows 3 wereT1, 4 were T2, 2 were T3, 4 were T4 and the stage of remaining 2 tumours was unknown

In the same patient cohort serum TGFβ-1 was measured prior to and 14 days after tamoxifen treatment Serum TGFβ-1 levels were decreased in 65% (13/20) of patients (Figure 5 4) There was a mean decrease of serum TGFβ-1 of 9 98% though this did not prove to be statistically significant (Figure 5 5) The 13 patients that demonstrated a decrease in serum TGFβ-1 following tamoxifen therapy had a mean decrease of 22 15% Of the 13 patients in whom tamoxifen therapy decreased TGFβ-1, 12 also showed a decrease in serum VEGF levels Patient 8 that demonstrated a very large atypical increase in serum VEGF following tamoxifen treatment had a slight decrease of 7 53% (44 35 to 41 01 ng/ml) There was a significant ($p < 0\ 01$) positive correlation between the change in serum VEGF and TGFβ-1 levels (Spearman Rank Correlation coefficient = 0 75) (Figure 5 6)

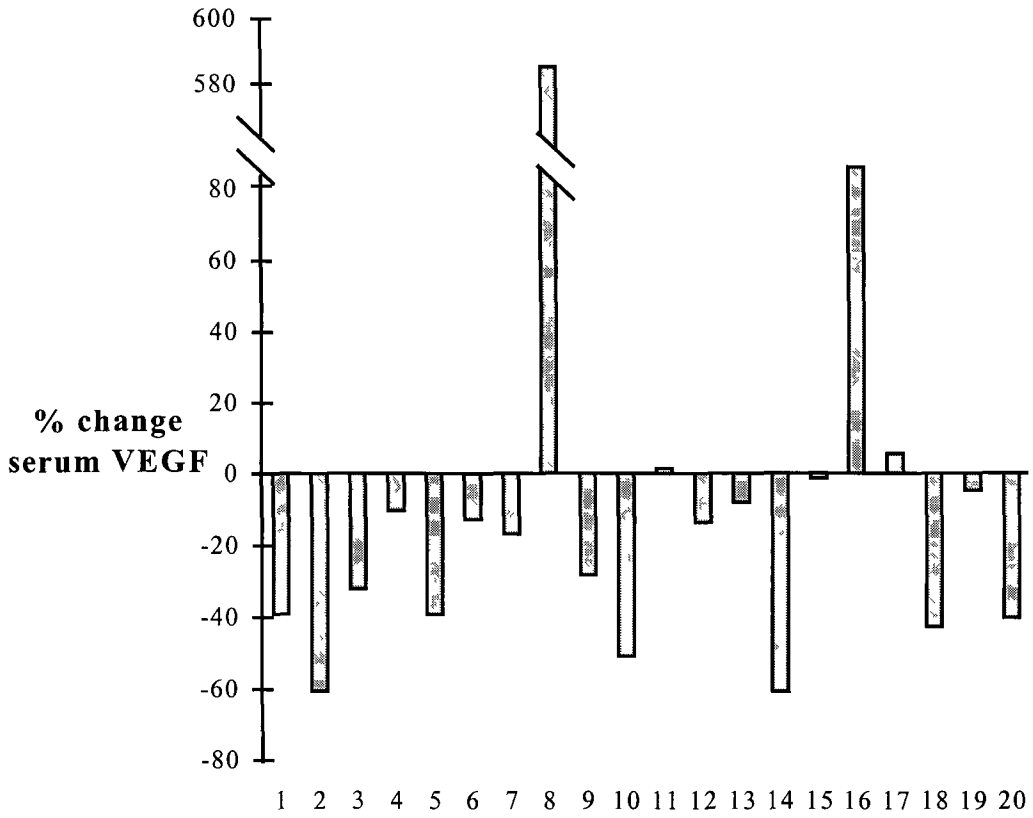


Fig. 5.2: The effect of 2 weeks of tamoxifen treatment (20 mg/day) on the serum levels of VEGF in breast cancer patients Serum was obtained prior to, and 14 days after, tamoxifen treatment and the change in serum VEGF between the two samples plotted Bars below the x-axis are representative of a decrease in serum VEGF while the bars above the x-axis represent an increase in serum VEGF The y-axis is broken to order to graph patient no 8

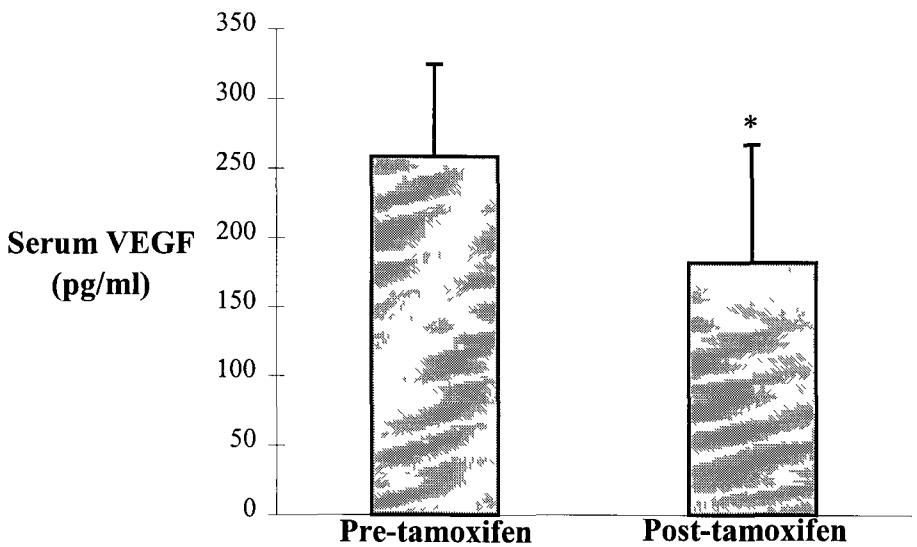


Fig. 5.3: Effect of 2 weeks tamoxifen treatment (20 mg/day) on serum VEGF levels in 20 breast cancer patients Data is expressed as median ± SEM Statistical analysis is by Wilcoxon Signed Rank *, $p < 0.01$

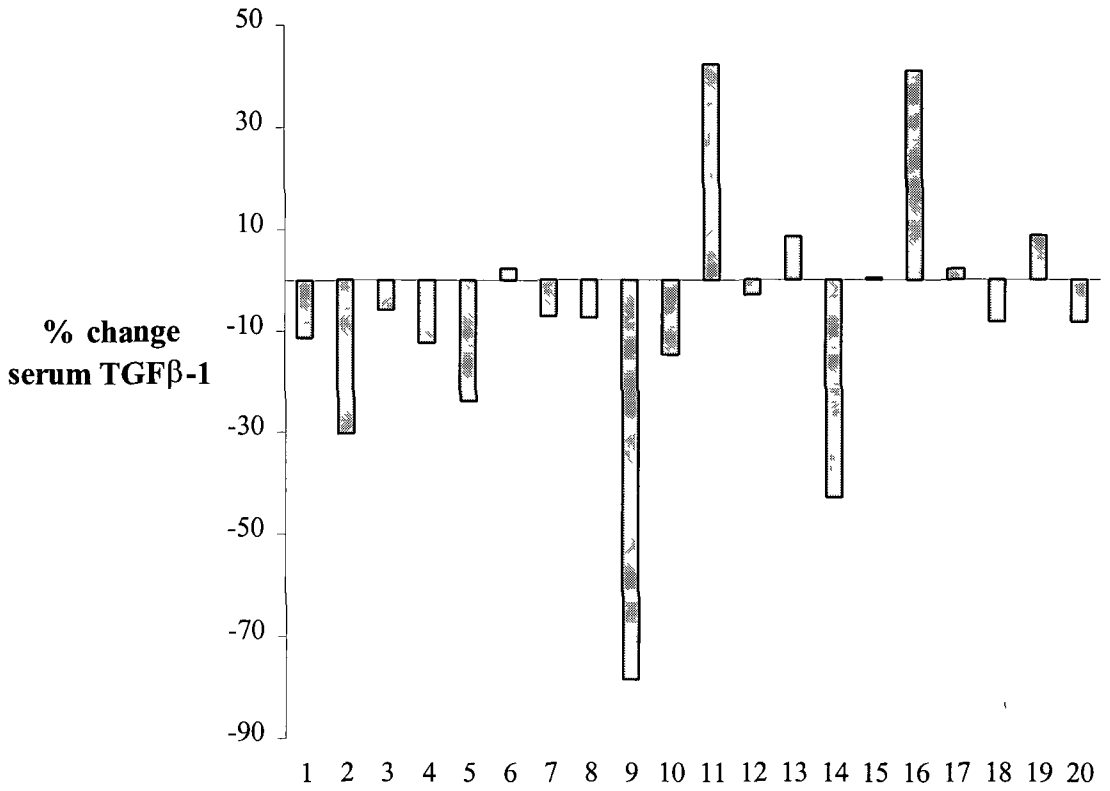


Fig. 5.4: The effect of 2 weeks of tamoxifen treatment (20 mg/day) on the serum levels of TGFβ-1 in breast cancer patients Serum was obtained prior to, and 14 days after, tamoxifen treatment and the change in serum TGFβ-1 between the two samples plotted Bars below the x-axis are representative of a decrease in serum VEGF while the bars above the x-axis represent an increase in serum TGFβ-1

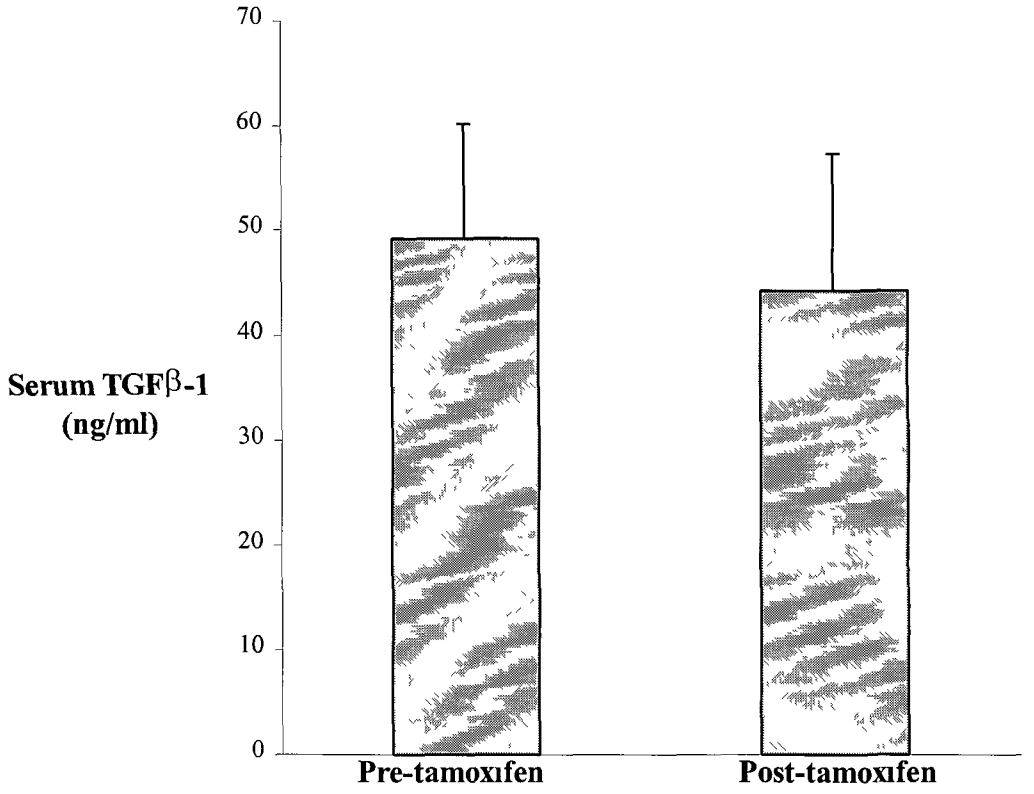


Fig. 5.5: Effect of 2 weeks tamoxifen treatment (20 mg/day) on serum TGFβ-1 levels in 20 breast cancer patients Data is expressed as median ± SEM Statistical analysis is by Wilcoxon Signed Rank, p = ns

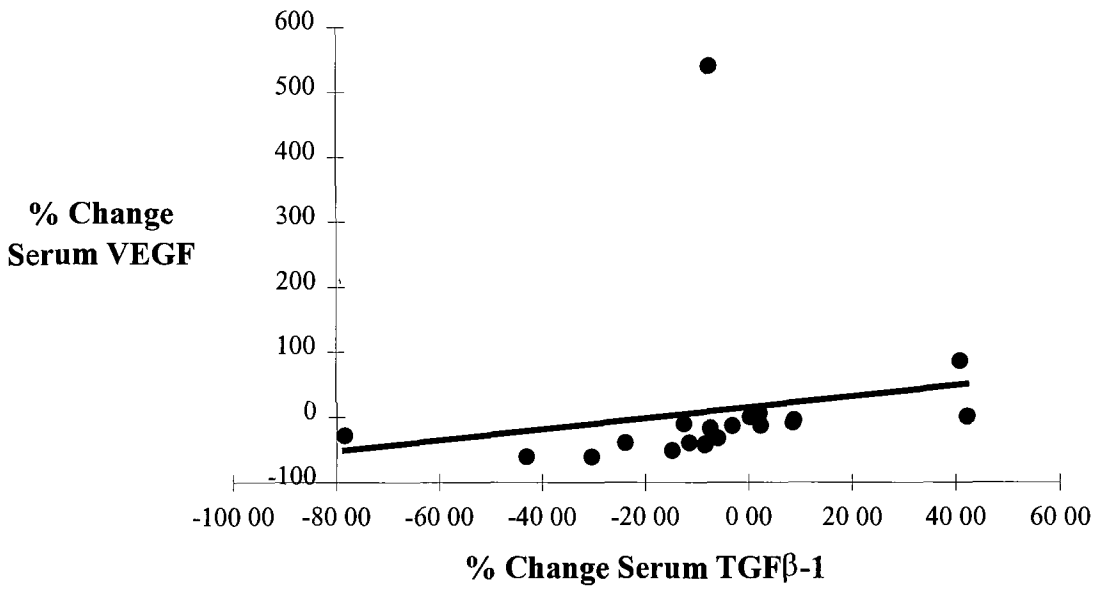


Fig. 5.6: Correlation between the % change in serum TGFβ-1 with the % change in serum VEGF There is a significant ($p < 0.05$) positive correlation (Spearman Rank Correlation coefficient = 0.75) between % change TGFβ-1 and VEGF The outlying point is patient 8 who showed a massive increase in VEGF following tamoxifen therapy

Number	% Change VEGF	% Change TGFβ-1	Age	Type	Stage	ER Status
1	-39 14	-11 47	69	n/a	n/a	n/a
2	-60 60	-30 33	60	ductal	T1	weak pos
3	-32 10	-5 95	54	lobular	T3	pos
4	-10 29	-12 51	60	ductal	T2	neg
5	-39 29	-23 84	65	ductal	T2	neg
6	-12 68	2 28	59	ductal	T1	neg
7	-16 64	-7 42	76	ductal	T1	neg
8	540 79	-7 53	59	ductal	T2	pos
9	-28 24	-78 39	74	ductal	T3	neg
10	-50 80	-14 76	53	ductal	T4	pos
11	1 05	42 24	63	n/a	n/a	neg
12	-13 30	-3 13	64	ductal	T2	pos
13	-7 99	8 54	70	ductal	T4	n/a
14	-60 65	-43 00	66	ductal	n/a	neg
15	0 26	0 26	59	ductal	T1	neg
16	86 03	41 07	54	n/a	n/a	n/a
17	5 75	2 05	50	ductal	T1	pos
18	-42 75	-8 38	57	ductal	T4	neg
19	-4 57	8 73	79	n/a	T4	n/a
20	-40 15	-8 37	52	ductal	T2	neg

Table 5.1: Patient characteristics The table shows the % change in VEGF and TGFβ-1, tumour type, tumour stage, ER status and age of patient n/a indicates that the data was not available A table showing the values of serum VEGF and TGFβ-1 prior to and after tamoxifen therapy is included in appendix 3

5.2.3 BT474 cells and MDA-MB-231 cells do not express the oestrogen receptor

To examine the oestrogen receptor independent activities of tamoxifen, *in vitro,* BT474 and MDA-MB-231 breast cancer cells and human microvascular endothelial cells (HMEC) were used ER negativity was established by immunocytochemistry Cytospms of these cells were stained by immunocytochemistry for presence of ER in the routine pathology lab in Beaumont Hospital Figure 5 7A represents a section of endometrial tissue and is used as a positive control that demonstrates positive nuclear staining for the ER Figure 5 7B, 5 7C and 5 7D, respectively, demonstrate that there was no positive nuclear staining for either the BT474, MDA-MB-231 or HMEC cells There is some non-specific cytoplasmic staining m Fig 5 7D In addition to immunocytochemistry results, a functional assay was carried out to determine whether growth of BT474 and MDA-MB-231 cells was oestrogen independent Cells were treated with 20 nM oestradiol, which has been shown to increase the proliferation of oestrogen receptor positive breast cancer cell lines (Iacobelli et al , 1984), and the proliferation of each cell line was examined Figure 5 8 shows that neither the BT474 or MDA-MB-231 cell lines showed a significant (unpaired students t-test, $p > 0\ 05$) increase in proliferation relative to control (100% vs 105 ± 6% and 100% vs 97 ± 5% proliferation relative to control, respectively) with the addition of oestradiol

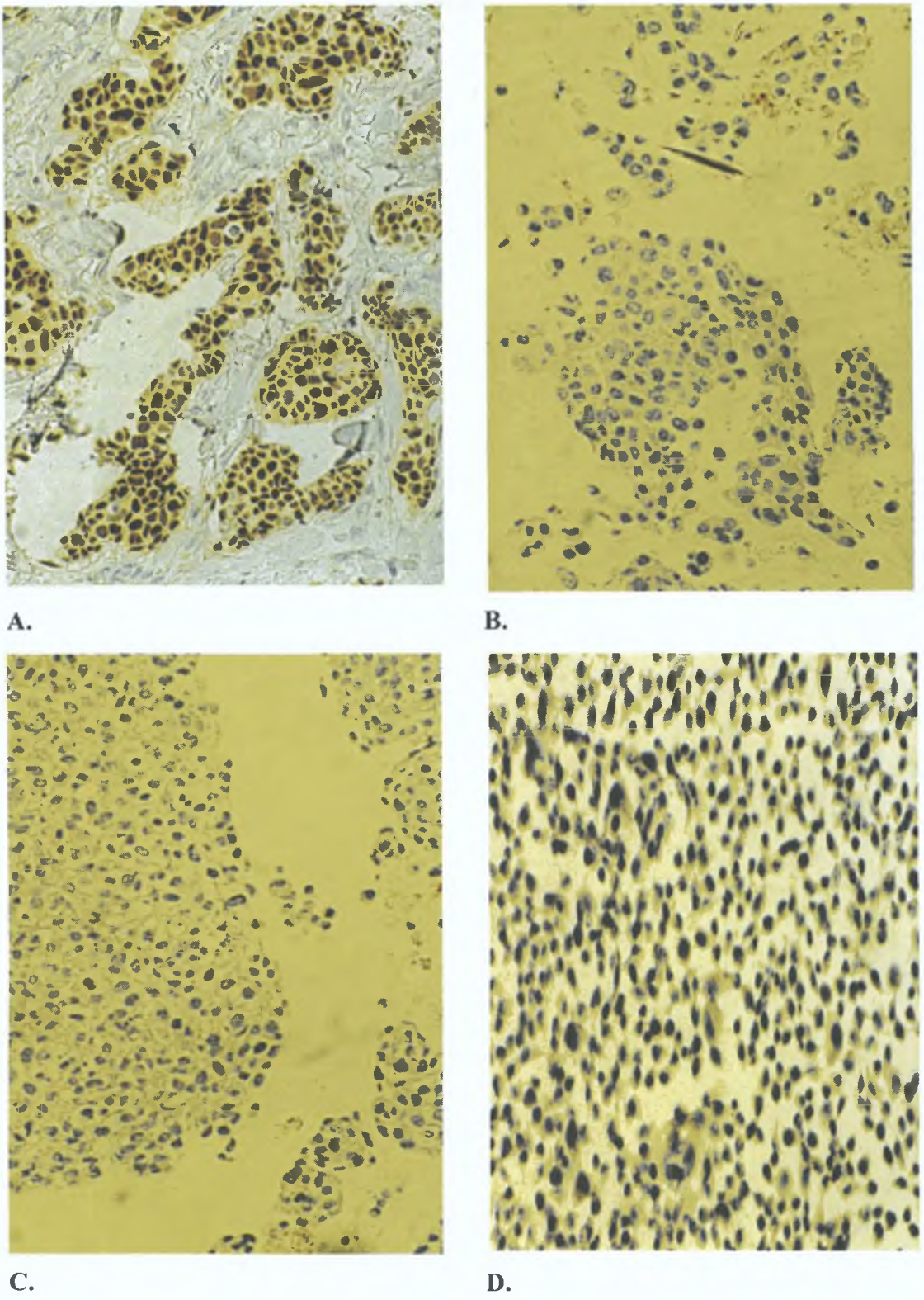
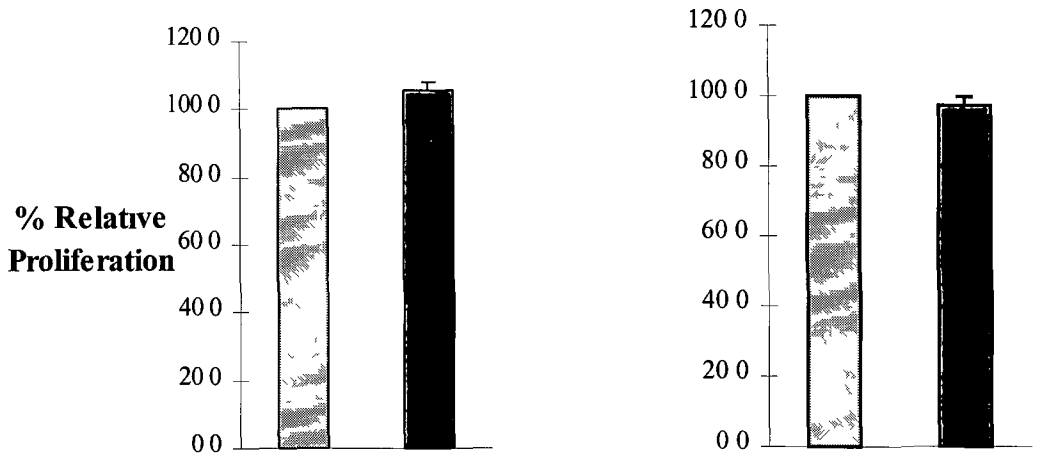


Fig. 5.7: Examination of cells for ER status. The positive control (**A**) is a section of endometrial tissue and demonstrates positive staining. Cytocentrifuge preparations of BT474 (**B**), MDA-MB-231s (**C**) and HMEC (**D**) cells were stained immunohistochemically for ER. There is some non-specific cytoplasmic staining in panel (**D**). Original magnification × 400.



A. **B.**

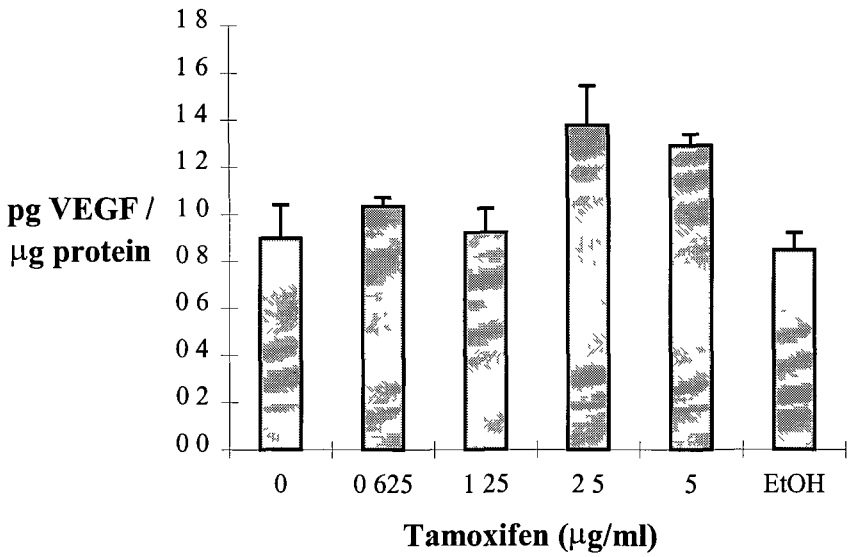
Fig. 5.8: Effect of 17β-oestradiol on the proliferation of BT474 (**A**) and MDA-MB-231 (**B**) cells The bars represent control cells with no treatment (□) and oestradiol treated cells (20 nM for 48 hr) (■) Data is expressed as the mean proliferation relative to control cells ± SEM and is representative of 3 individual experiment carried out in triplicate Statistical analysis was by unpaired students t-test, p = ns

5.2.4 Effect of tamoxifen on breast cancer cell line production of VEGF

As tamoxifen decreased the serum levels of VEGF in a subpopulation of breast cancer patients, independently of ER status, the effect of tamoxifen on the production of VEGF by ER negative breast cancer cell lines, BT474 and MDA-MB-231, was examined Both cell lines were treated with base tamoxifen (0, 0 625, 1 25, 2 5, 5 μg/ml) for 24 hr The tamoxifen doses chosen are similar to levels that have been found within breast tissue in patients receiving steady-state tamoxifen treatment (Lien et al , 1991) The levels in tissue are higher than reported levels of tamoxifen in the serum indicating that the majority of tamoxifen is found in tissue (Lien et al 1991) This is most likely due to the poor solubility of tamoxifen in aqueous solution and its highly lipophilic nature As tamoxifen was originally solubilised in ethanol, cells were also treated with 0 5% (v/v) ethanol that is equivalent to the amount of ethanol present in the 5 μg/ml dose of tamoxifen Tamoxifen base at 2 5 and 5 μg/ml significantly ($p < 0\ 05$) increased VEGF levels in the conditioned medium of the BT474 cell line (1 37 and 1 29 pg/μg protein vs 0 9 pg/μg protein, respectively) (Fig 5 9A) Tamoxifen did appear to be cytotoxic to the BT474 cells at these concentrations as observed by a more rounded morphology and increased presence of detached cells floating in the medium The apparent increase of VEGF in the conditioned medium may be due to release by cell lysis Tamoxifen had no significant ($p > 0\ 05$) effect on the production of VEGF by the MDA-MB-231 cell line (Fig 5 9B)

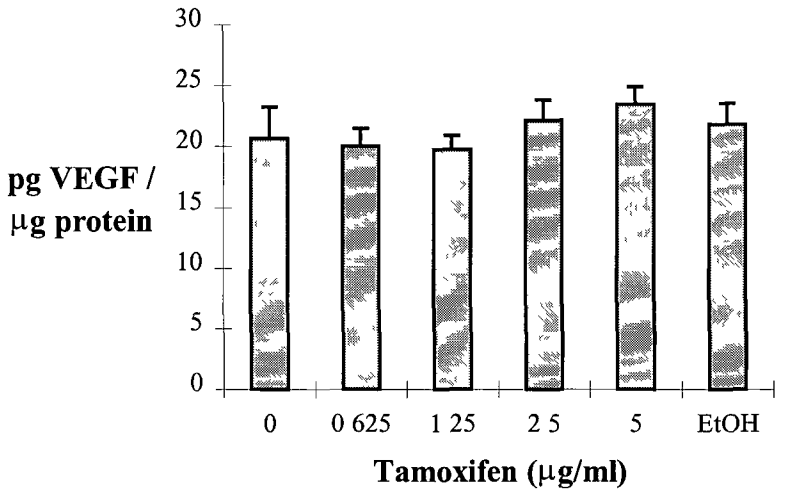
5.2.5 Effect of 4-hydroxytamoxifen on VEGF production by breast cancer cell lines

Tamoxifen is readily metabolised to form a number of different metabolites including 4-hydroxytamoxifen This metabolite is only produced in small quantities but it binds with greater affinity to the ER and is more potently anti-oestrogenic than tamoxifen (Buckley and Goa, 1989) We examined whether this more potent metabolite, 4-hydroxytamoxifen, had any effect on the VEGF production by BT474 and MDA-MB-231 cell lines The doses chosen were similar to tissue levels m breast cancer patients (Lien et al 1991) Fig 5 10 shows that 4-hydroxytamoxifen (0 - 100 ng/ml) for 24 hr did not significantly ($p > 0\ 05$) alter the production of VEGF by either of these cell lines



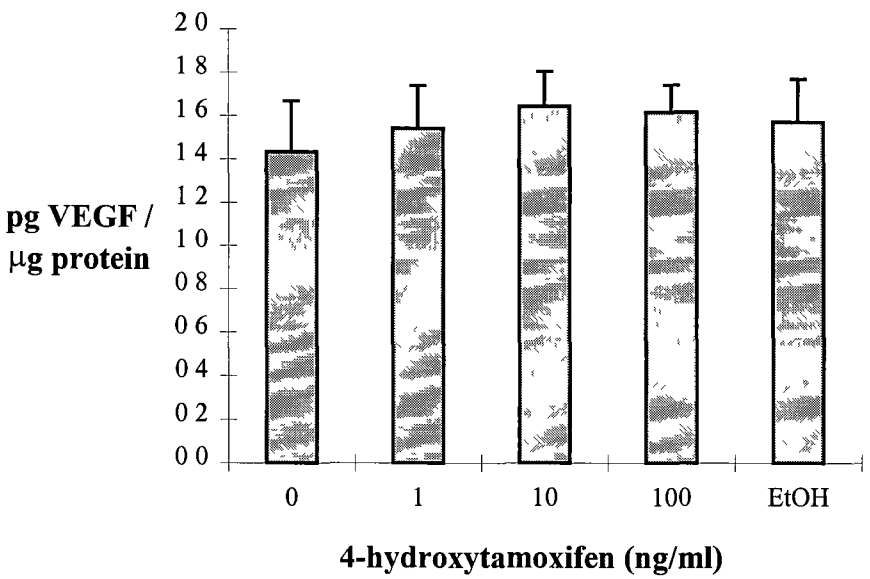
A.

Fig. 5.9A: Effect of tamoxifen on VEGF production by the BT474 cell line Cells were exposed to tamoxifen or 0 5% (v/v) ethanol (EtOH) for 24 hr Data is representative of 3 experiments done in duplicate and is expressed as mean ± SEM Statistical analysis is by ANOVA with a Scheffe post hoc *, $p < 0\ 05$ vs 0 µg/ml tamoxifen



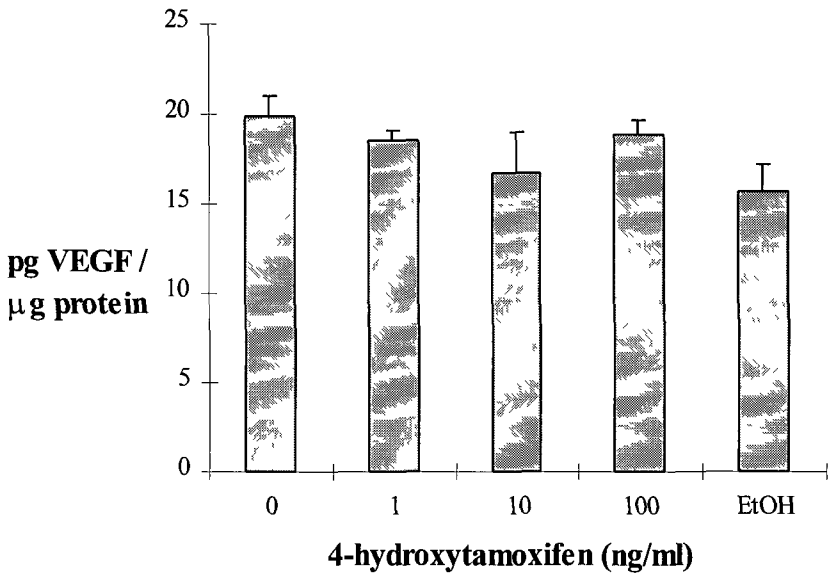
B.

Fig. 5.9B: Effect of tamoxifen on VEGF production by the MDA-MB-231 cell line Cells were exposed to tamoxifen or 0 5% (v/v) ethanol (EtOH) for 24 hr Data is representative of 3 experiments done in duplicate and is expressed as mean ± SEM Statistical analysis is by ANOVA with a Scheffe post hoc *, $p < 0\ 05$ vs 0 µg/ml tamoxifen



A

Fig 5.10A: Effect of 4-hydroxytamoxifen on VEGF production by the BT474 cell line Cells were exposed to 4-hydroxytamoxifen or 0 1% (v/v) ethanol (EtOH) for 24 hr Data is representative of 3 individual experiment carried out in duplicate and is expressed as mean ± SEM Statistical analysis was by ANOVA with Scheffe post hoc correction p = ns



B.

Fig 5.10B: Effect of 4-hydroxytamoxifen on VEGF production by the MDA-MB-231 cell line Cells were exposed to 4-hydroxytamoxifen or 0 1% (v/v) ethanol (EtOH) for 24 hr Data is representative of 3 individual experiment carried out in duplicate and is expressed as mean ± SEM Statistical analysis was by ANOVA with Scheffe post hoc correction p = ns

5.2.6 Effect of tamoxifen on the production of monocyte derived macrophage VEGF

Since tamoxifen did not decrease breast cancer cell line production of VEGF, the effect of tamoxifen on monocyte derived macrophage production of VEGF was examined Breast tumours can contain very high numbers of macrophages and macrophages have been shown to produce angiogenic factors Monocyte were isolated (2 2 8), matured to to macrophages and activated with 100 ng/ml IFNγ for 24 hr Over this period monocytes increased in size and assumed a more macrophage like morphology (Figure 5 11A and B) Monocyte derived macrophages were treated with various doses of base tamoxifen (0 - 5 μg/ml) while in the presence of tumour conditioned medium and hypoxia to simulate tumour environment, conditions previously shown to upregulate macrophage production of VEGF (Harmey et al 1998) Figure 5 12 shows that there was no decrease in VEGF production by monocyte derived macrophages under these conditions Since macrophages isolated from different individuals express different amounts of VEGF the data was calculated as the change in VEGF production relative to the untreated controls for each individual Mean VEGF production in the untreated controls under these conditions was 10 5 ± 8 5 pg/μg protein which is roughly equivalent to that produced constitutively by the breast cancer cell lines The amount of VEGF produced by monocyte derived macrophages was adjusted for the presence of VEGF in the tumour conditioned medium

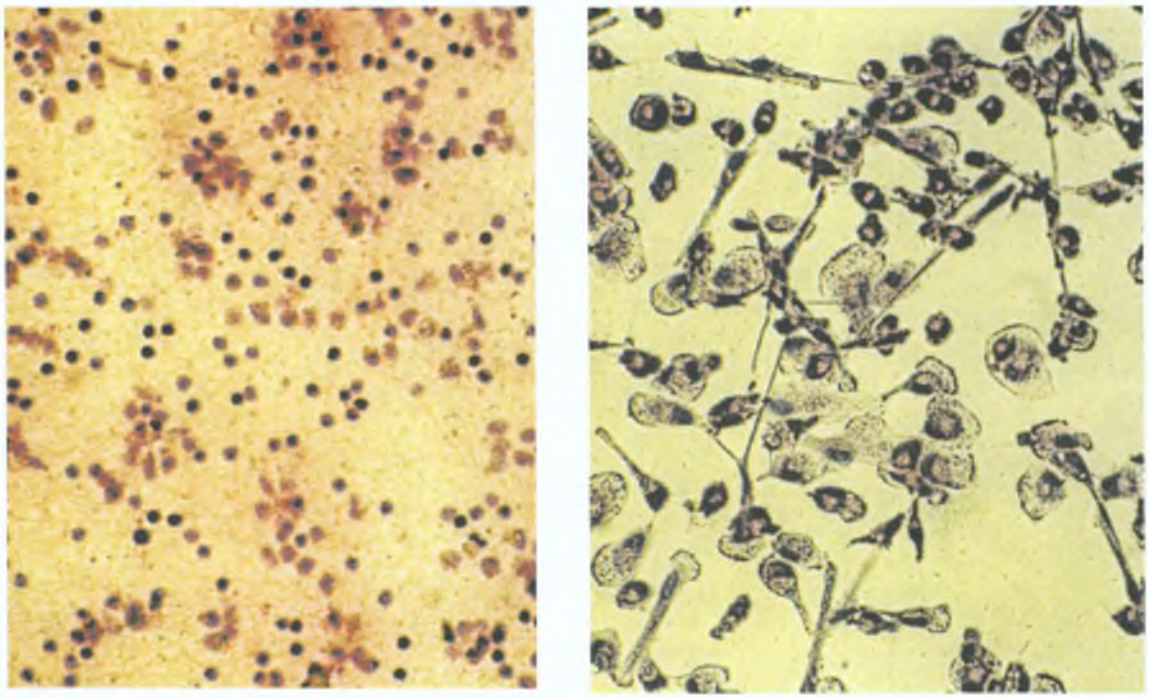


Fig. 5.11: Maturation and activation of monocyte derived macorphages. Monocytes 24 hr after isolation (**A**). Mature monocytes (5 days) activated for 24 hr with 1,000U of rh-IFNγ. Original magnification × 400.

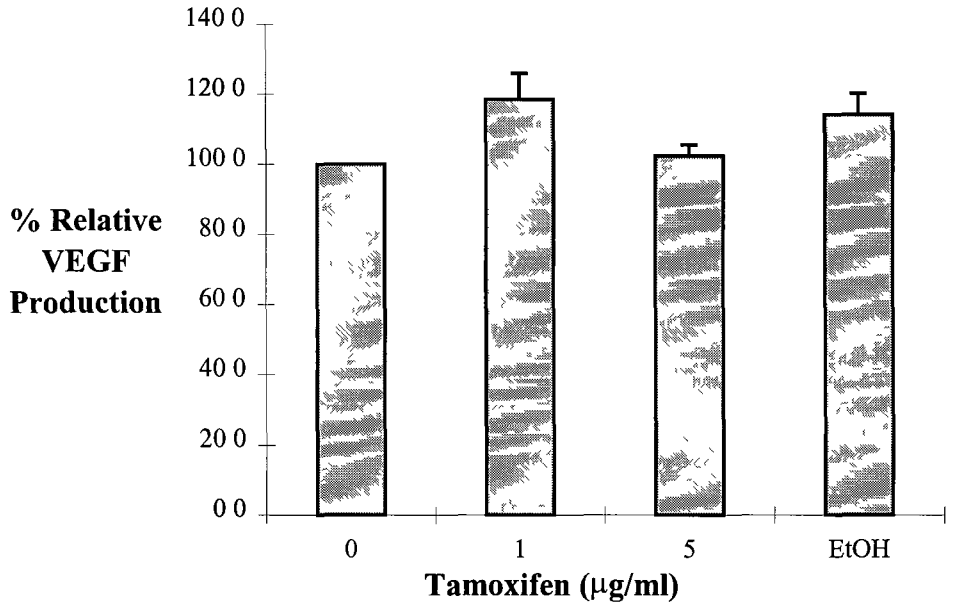


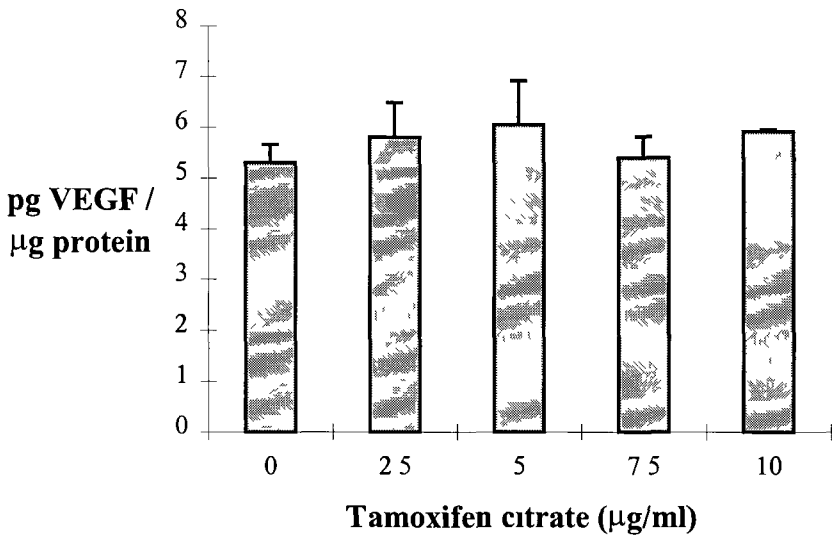
Fig 5.12: Effect of tamoxifen on monocyte derived macrophages production of VEGF in the presence of tumour conditioned medium and hypoxia Cells were exposed to 24 hr of tamoxifen or 0 5% (v/v) ethanol (EtOH) Data is representative of experiments, carried out in triplicate, using monocytes from 3 different male individuals Statistical analysis was by ANOVA with Scheffe post hoc correction p = ns

5.2.7 Effect of tamoxifen on TGFβ-1 stimulated production of VEGF by breast cancer cells

TGFβ-1 increased VEGF production by both BT474 and MDA-MB-231 cells (4 2 4) High levels of TGFβ-1 were found in the serum and tumour tissue of breast cancer patients and there was a correlation between the levels of these two factors Therefore, the effect of tamoxifen on TGFβ-1 stimulated breast cancer cell line production of VEGF was examined The dose of TGFβ-1 that maximally increased VEGF production was 5 ng/ml m the BT474 cells and 10 ng/ml in the MDA-MB-231 cells The effect of tamoxifen citrate (0, 2 5, 5, 7 5, 10 μg/ml) on TGFβ-1 stimulated production of VEGF was examined Tamoxifen citrate is the salt form of tamoxifen and a slightly higher concentration of tamoxifen citrate is used so that it is stoichiometrically equivalent to the doses of base tamoxifen used The stoichiometric ratio for base tamoxifen to tamoxifen citrate is 1 1 2 Cells were treated for 24 hr with tamoxifen Tamoxifen did not significantly ($p > 0\ 05$) alter the TGFβ-1 induced production of VEGF by either cell line (Fig 5 13)

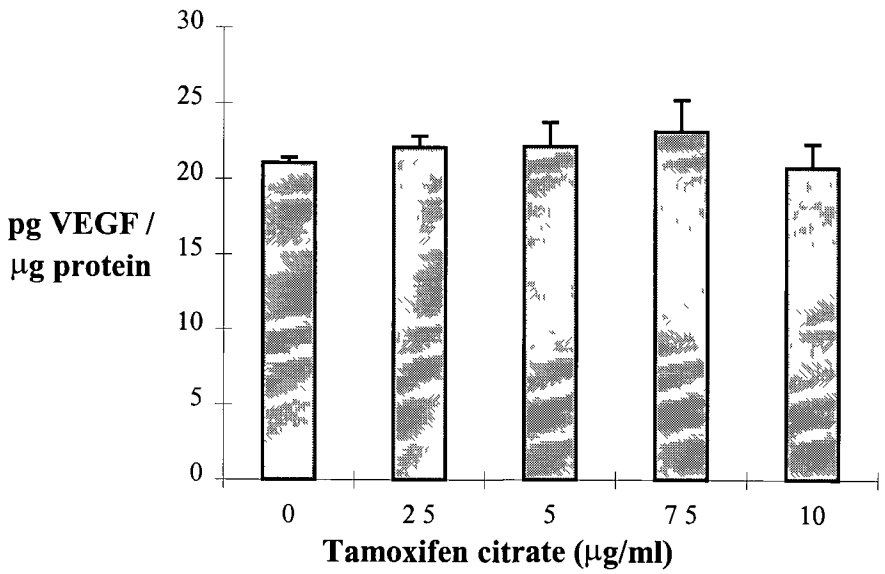
5.2.8 Effect of tamoxifen on macrophage production of TGFβ-1

Tamoxifen decreased serum VEGF levels in a subpopulation of breast cancer patients but did not decrease VEGF production by breast cancer cells *in vitro* indicating that tamoxifen may decrease VEGF by an indirect mechanism TGFβ-1 was found to increase breast cancer cell production of VEGF Neither the BT474 nor the MDA-MB-231 cell line produced detectable levels of TGFβ-1 Macrophages, however, can produce TGFβ-1 and it is possible that infiltrating macrophages produce TGFβ-1 which may then stimulate breast cancer cell production of VEGF The effect of tamoxifen citrate (0 - 10 μg/ml) for 24 hr on the production of TGFβ-1 by monocyte derived macrophages was examined Macrophages produced detectable levels of TGFβ-1, mean production of TGFβ-1 produced by untreated macrophages was 520 ± 17 pg/μg protein Tamoxifen did not significantly ($p > 0\ 05$) decrease TGFβ-1 production (Figure 5 14) Data is plotted as the relative change in TGFβ-1 production compared to the untreated control for each individual experiment



A.

Fig. 5.13A: Effect of tamoxifen on VEGF production by BT474 cells stimulated with 5 ng/ml and 10 ng/ml TGFβ-1, respectively Cells were exposed to tamoxifen for 24 hr Data is representative of 3 experiments carried out m duplicate and is expressed as mean ± SEM Statistical analysis was by ANOVA with Scheffe post hoc correction p = ns



B.

Fig. 5.13B: Effect of tamoxifen on VEGF production by MDA-MB-231 cells stimulated with 5 ng/ml and 10 ng/ml TGFβ-1, respectively Cells were exposed to tamoxifen for 24 hr Data is representative of 3 experiments carried out in duplicate and is expressed as mean ± SEM Statistical analysis was by ANOVA with Scheffe post hoc correction p = ns

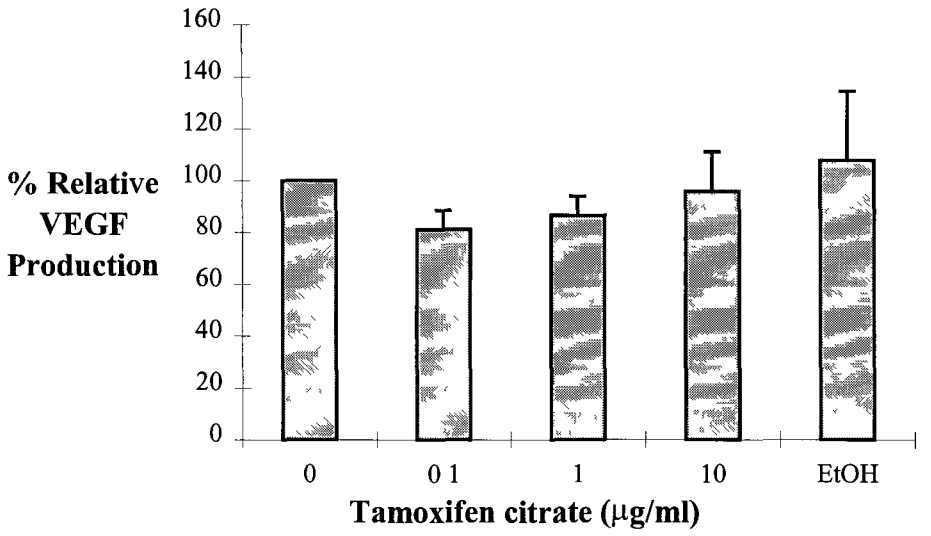
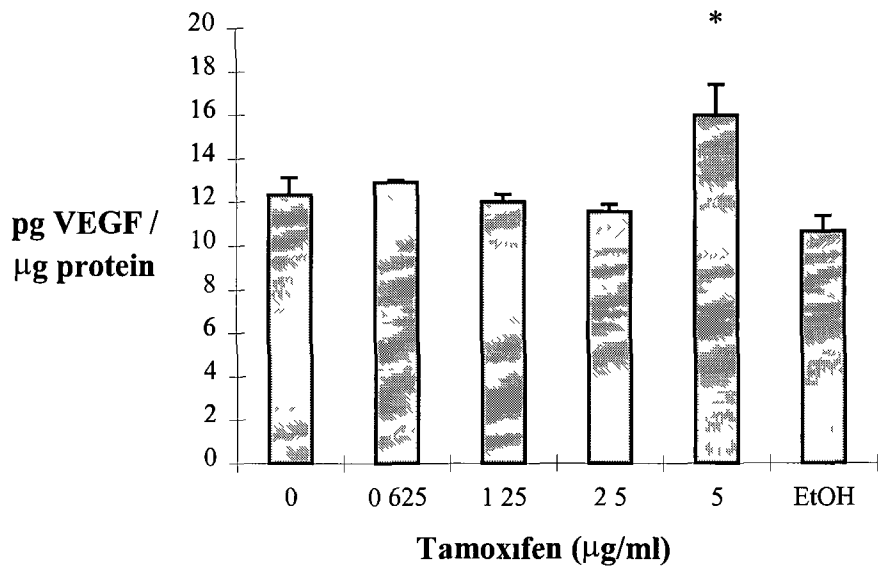


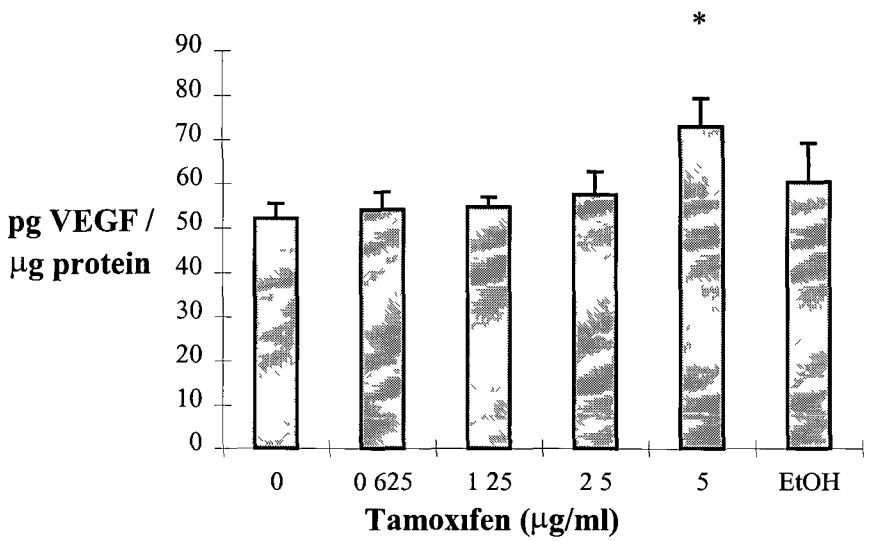
Fig 5.14: Effect of tamoxifen on monocyte derived macrophage production of TGFβ-1 Cells were exposed to tamoxifen or 1% (v/v) ethanol (EtOH) for 24 hr Data is representative of 3 experiments, carried out in triplicate, using monocytes from 3 different male individuals and is plotted as the % VEGF production relative to untreated control for each individual experiment ± SEM Statistical analysis was by ANOVA with Scheffe post hoc correction p = ns

5.2.9 Effect of tamoxifen on VEGF production by breast cancer cells in hypoxia

It was established that breast cancer cell lines produce elevated levels of VEGF under hypoxic conditions (4 2 1) The effect of base tamoxifen (0, 0 625, 1 25, 2 5, 5 μg/ml) for 24 hr on hypoxia induced production of VEGF by BT474 and MDA-MB-231 cells was examined Both cell lines produced elevated levels of VEGF m hypoxia than in normoxia (BT474 cells, 12 32 vs 3 19 pg/μg protein and MDA-MB-231 cells, 52 17 vs 15 64 pg/μg protein) Tamoxifen at 5 μg/ml significantly ($p < 0\ 05$) increased VEGF production in both cell lines compared to the untreated control (BT474s, 15 99 vs 12 32 pg/μg protein and MDA-MB-231s, 72 8 vs 52 17 pg/μg protein) (Figure 5 15) This represents an increase of 29 8% and a 39 5% in the conditioned medium of the BT474 and MDA-MB-231 cells, respectively Under these conditions however, tamoxifen appeared to be partially cytotoxic to these cells at these concentrations as seen by morphological changes and the detachment of cells from the surface of the flask This may account for the apparent increase in VEGF in the conditioned medium since dead cells will release their intracellular levels of VEGF



A.



B.

Fig 5.15: Effect of tamoxifen on hypoxia-induced VEGF production by BT474 (**A**) and MDA-MB-231 (**B**) cells Cells were exposed to tamoxifen or 0 5% (v/v) ethanol (EtOH) for 24 hr under hypoxic conditions Data is representative of 3 individual experiments performed in duplicate and are expressed as the mean ± SEM Statistical analysis is by ANOVA with Scheffe post hoc correction *, $p < 0\ 05$

5.2.10 Effect of tamoxifen on microvascular endothelial cell migration

For angiogenesis to occur endothelial cells must migrate, into and within the tumour, in response to binding of VEGF Human microvascular endothelial cells (HMEC) are endothelial cells derived from small dermal blood vessels and are more representative of the type of endothelial cells involved in capillary angiogenesis than other endothelial cells that are cultured *in vitro* Figure 5 16 shows a photograph of these cells m which the typical 'cobblestone' appearance of endothelial cells in culture is clearly visible The effect of base tamoxifen (0 - 5 μg/ml) on HMEC migration was assessed Migration was assessed by counting HMEC cells that moved from the upper layer of a membrane containing 8 μm pores to the lower layer in response to VEGF over 48 hr 20 ng/ml m the lower chamber increased HMEC migration (15 4 vs 2 7 counts/per field of view (pfv) in controls without VEGF) (Figure 5 17) Tamoxifen at 5μg/ml significantly ($p < 0\ 05$) reduced the number of cells that migrated to the underside of the membrane (3 2 vs 15 4 cells/pfv untreated control) This may have been partially due to a non-specific effect since there was a visible change in the morphology of these cells at this dose of tamoxifen Figure 5 18 shows representative photographs of cells that have migrated through the membrane into the lower compartment

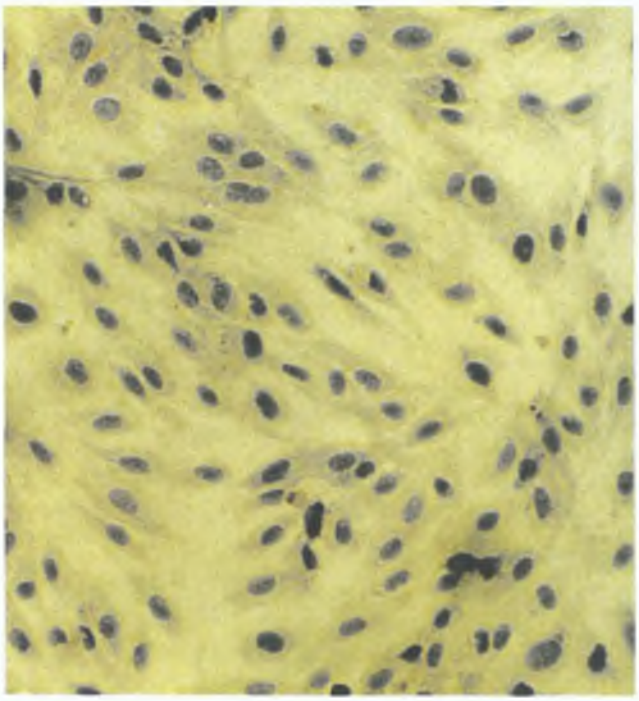


Fig. 5.16: Monolayer culture of HMEC cells. The cells can clearly be seen to grow with a cobblestone appearance that is typical of cultured endothelial cells.

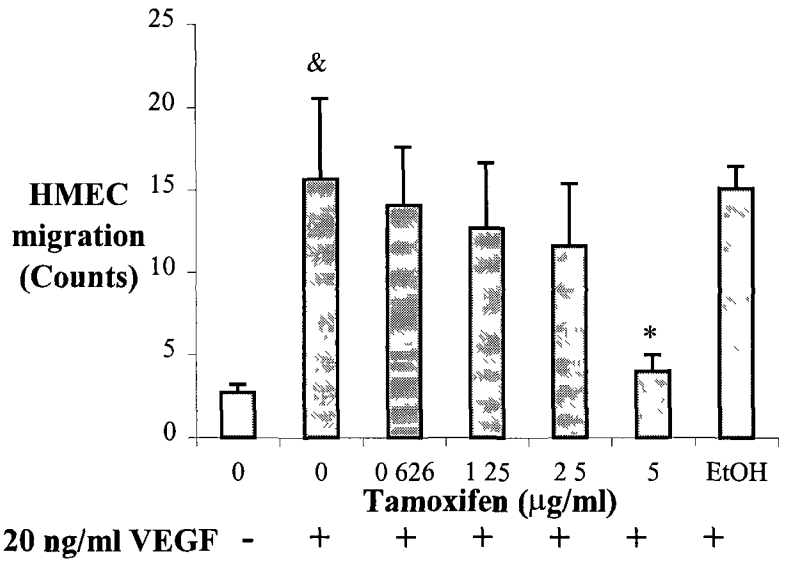


Fig 5.17: Effect of tamoxifen on HMEC migration Cells were exposed to tamoxifen or 0 5% (v/v) ethanol for 48 hr Data is representative of 3 independent experiments performed in duplicate and is expressed as the mean count ± SEM Statistical analysis is by ANOVA with Scheffe post hoc correction &, $p < 0\ 05$ vs control with no VEGF *, $p < 0\ 05$ vs untreated control with VEGF

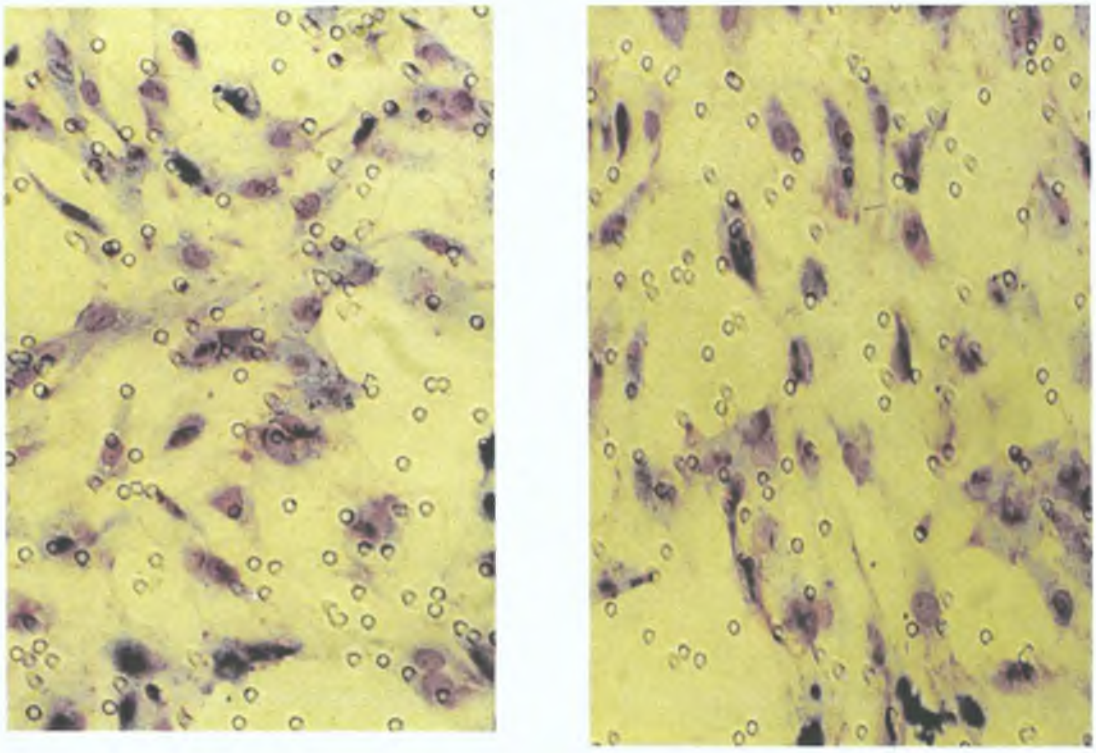


Fig. 5.18: Representative photographs of migrated endothelial cells. Positive control **(A)** is untreated cells with 20 ng/ml VEGF used as a chemoattractant while **(B)** represents migration of cells exposed to 2.5 μg/ml tamoxifen. The pores through which the cells migrate are clearly visible.

5.2.10 Effect of tamoxifen on monocyte transendothelial migration

Macrophages constitute an important source of breast tumour VEGF In order for tumours to recruit macrophages, monocytes must first cross the endothelial barrier The effect of base tamoxifen (2 5 μg/ml) on monocyte transendothelial migration was assessed (2 2 12) A single dose of tamoxifen was chosen because of restrictions in the number of samples that could be run, the dose chosen was the highest dose that could be used without cytotoxicity to the cells VEGF at 20 ng/ml is chemotactic for monocytes (Clauss et al 1996) Monocyte transendothelial migration was simulated *in vitro* by culturing a monolayer of HMEC cells on a collagen coated membrane containing 3 μm pores and adding VEGF (20 ng/ml) as a chemoattractant for monocytes to the medium in the bottom compartment A smaller pore size was used than that used for endothelial cell migration since monocytes are considerably smaller Monocytes are added to the top compartment and those that have migrated through the endothelial monolayer over 4 hr are collected and counted Monocyte migration is considerably faster than endothelial cell migration Using 20 ng/ml VEGF as a chemoattractant in the bottom compartment significantly ($p < 0\ 05$) increased, by 4 fold, monocyte transmigration Tamoxifen at 2 5 μg/ml significantly ($p < 0\ 05$) inhibited the VEGF induced migration of monocytes (70 4% migration vs 100% in untreated controls) (Figure 5 19) The ethanol control had no effect on VEGF induced migration

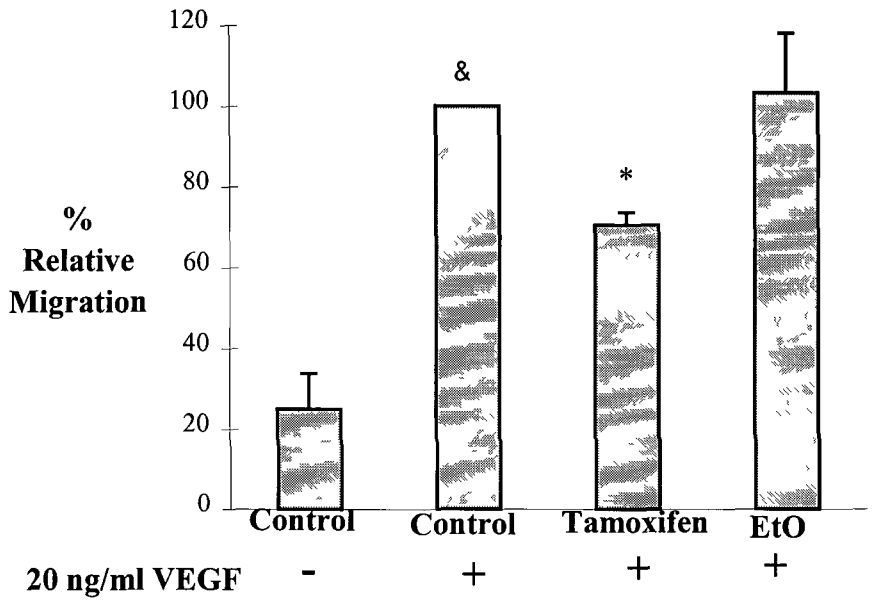


Fig 5.19: Effect of tamoxifen on monocyte transendothelial migration Cells were exposed to tamoxifen or 0 25% (v/v) ethanol (ETOH) for 4 hr and the numbers of monocytes that migrated was counted 20 ng/ml VEGF was used as a chemoattractant, a plus sign indicates its use while a minus sign indicates that VEGF was not used Data is representative of 3 individual experiments performed in duplicate and is expressed as the relative migration compared to the control, that received no tamoxifen but did have VEGF as a chemoattractant, for each individual experiment ± SEM Statistics is by ANOVA with Scheffe post-hoc correction &, $p < 0\ 05$ vs no VEGF control *, $p < 0\ 05$ vs VEGF control

5.3 Discussion

The data presented in this chapter show that tamoxifen therapy (20 mg/day for 2 weeks) reduces serum levels of VEGF in a subset of cancer patients. Tamoxifen therapy reduced VEGF in serum and ascites fluid of a male patient with malignant ascites secondary to gastric cancer (Donovan *et al.*, 1997). Tamoxifen reduced serum VEGF in 15 of 20 breast cancer patients – of these 53% (8/15) had ER negative tumours. These results suggest that tamoxifen reduces VEGF independently of the oestrogen receptor. This effect was achieved within 2 weeks of preoperative tamoxifen. Most patients are scheduled for surgery 2 – 3 weeks after initial clinic visit and it would have been unethical to delay surgery for a longer period. It is possible that if treatment was extended for a longer time period, and a steady state level of tamoxifen formed in the tissue, a greater decrease in serum VEGF levels may have been seen. However, in the male ascites patient tamoxifen therapy was for 4 weeks but the reduction in serum and ascites VEGF was observed within 2 weeks. Although reduced levels were maintained, there was little further reduction after 2 weeks.

Tamoxifen has been traditionally used as an adjuvant therapy following surgery. The reduction in serum VEGF observed in patients receiving tamoxifen therapy pre-operatively suggest that tamoxifen may be a useful perioperative therapy. As angiogenesis is critical for both tumour growth and metastases any agent capable of reducing VEGF levels should decrease tumour growth and metastasis by reducing angiogenesis. Antibodies against VEGF have already been shown to decrease tumour growth in animal models (Kim et al., 1993; Borgström et al., 1996). There is as yet no other pharmacological agent shown to decrease VEGF production. Reducing VEGF levels before surgery may be useful for temporarily halting tumour growth before commencing surgery. Surgery has been found to dislodge tumour cells and tumour cells have been found in the circulation following or during surgery (McCulloch et al., 1995). VEGF causes hyperpermeability of blood vessels and tumour microvessels are known to be highly permeable. In addition, tumour blood vessels are known to be in a constant state of flux and are rarely properly formed vessels such as would be found in normal tissue. This may be in part due to the continual stimulation of endothelial cells by the many angiogenic factors that tumours produce. It is possible that the leaky, ill-

formed, blood vessels within tumours facilitate dissemination of tumour cells into the bloodstream during surgery Reducing VEGF levels before surgery may be useful for decreasing the permeability of blood vessels and therefore deny tumour cells access to the circulation before or during the surgical procedure A possible adverse effect of tamoxifen therapy could be impaired wound healing as angiogenesis is essential for wound healing In our study group no adverse effects on wound healing were observed and other data from this lab demonstrated increased anastamotic strength in rats treated with tamoxifen (McNamara, unpublished observations) Other studies have demonstrated reduced scar formation in rats treated with tamoxifen and this effect was ascribed to tamoxifen induction of TGFβ-3

In the healthy adult VEGF is primarily produced for wound healing Surgical procedures involve extensive wounding of the tissue and under these circumstance VEGF is highly upregulated (Nissen et al , 1998) Many surgical procedures do not remove every single tumour cell Cells that are left behind may be capable of utilising the abundant growth and angiogenic factors present at the site of the wound Tumours have been often been found to occur at porthole sites following laparoscopic surgery (Neuhaus et al , 1998) Reducing VEGF levels perioperatively may inhibit the formation of stable metastasis following surgery Up to 50% of cancer patients already have metastases at time of diagnosis (Fidler and Ellis, 1994) Surgery has been found to alter the net balance of angiogenic signals for metastases that are present at the time of surgery Rapid growth of previously dormant metastases following surgical removal of a primary tumour is well documented (Holmgren et al , 1995) Primary tumours produce angiostatin and endostatm that inhibit the growth of secondary tumours (Quinn et al , 1993) By removing the primary tumour this may reduce anti-angiogenic signals and allow secondary tumour growth In an animal model where lung metastases were established by tail vein injection, surgical trauma has been found to increase metastatic growth (Pidgeon *et al* , in press) As no primary was present the increase in growth cannot be ascribed to removal of the primary tumour derived angiostatm, thus surgery creates a permissive environment for tumour growth The increase of VEGF following surgery may provide an additional mechanism allowing growth of secondary tumours Pre-operative tamoxifen therapy may reduce this advantage by decreasing circulating

VEGF and thereby reduce angiogenesis in the primary tumour before surgery and in secondary tumours after surgery.

The potential benefits of short term preoperative tamoxifen, as discussed above, are based purely on the effect we have noticed on serum levels of VEGF. The effect on serum VEGF is a quite modest decrease but could be of clinical significance. The use of perioperative tamoxifen, purely to reduce oestrogenic effects, has already been proposed (Jordan, 1994). If patients receiving preoperative tamoxifen also expressed the ER the additive benefits may be even greater and may possibly lead to revised surgery from a mastectomy to a wide local excision (Robinson et al., 1996). VEGF is thought to be regulated in some cases by oestrogen (McClure et al., 1994). Tamoxifen acts as an oestrogen agonist in the uterus (Boothe et al. 1994) and has been found to increase VEGF production in this tissue. Although we did not find any association with the ER in this study, the oestrogen antagonist activity of tamoxifen may have the reverse effect in the breast and decrease VEGF. Short-term preoperative tamoxifen has been shown to a be a safe use of this agent as it does not adversely affect tumour grade over this period (Robinson et al. 1996). We did not have the opportunity to look at long term effects of preoperative tamoxifen but it would be of great interest to see if this could extend survival figures for this disease.

We also found that tamoxifen reduced serum levels of TGFβ-1 in a large proportion of patients in this study. This is in contrast with other results which have tended to show that tamoxifen increased TGFβ-1 levels (Butta et al., 1992; MacCallum et al., 1996). These measurements were made within the tissue while ours were measured in the circulation, however the primary source of plasma TGFβ-1 in cancer patients is though to be from the tumour (Kong et al., 1995) so we are still in effect measuring tumour TGFβ-1. TGFβ-1 mRNA levels have been found to be reduced in patients who responded to tamoxifen treatment (Thompson et al., 1991). Patients on tamoxifen have been found to have a better quality of scar tissue which is a feature associated with reduced TGFβ-1 levels and increased TGFβ-3 levels (Ashcroft et al., 1997). We also found there was a strong correlation between the reduction in TGFβ-1 and VEGF, a reduction in TGFβ-1was associated with a reduction in VEGF levels. Since we had shown before that TGFβ-1 increased tumour cell production of VEGF it is possible that a reduction in serum VEGF is secondary to reduced TGFβ-1 production.

Neither the BT474 nor MDA-MB-231 cell line produced readily detectable amounts of TGFβ-1 Macrophages are a known source of TGFβ-1 (Assoian et al , 1987) so the effect of tamoxifen on macrophage production of this factor was examined Tamoxifen did not reduce macrophage TGFβ-1 It is possible that tamoxifen is reducing stromal cell production of TGFβ-1 though tamoxifen was found to increase TGFβ-1 production in stromal cells (Butta et al , 1992)

As tamoxifen decreased circulating VEGF in a subpopulation of breast cancer patients, the direct effect of tamoxifen on the *in vitro* constitutive production of VEGF was assessed Tamoxifen did not reduce VEGF production by BT474 or MDA-MB-231 breast cancer cells or macrophage VEGF production There was a slight elevation in VEGF production by the BT474 cell line in response to tamoxifen This may due to the fact that tamoxifen appeared to be cytotoxic to this cell line at lower doses (5 μg/ml base tamoxifen) than any other cell line tested in the lab At these doses there is some cytotoxicity, as seen by morphological changes and detachment of cells, which probably results in lysis of the cell and release of intracellular VEGF into the conditioned medium We also looked at the effect of tamoxifen on the production of VEGF by BT474 and MDA-MB-231 cells exposed to TGFβ-1 and hypoxia, both of which increase VEGF Tamoxifen did not inhibit VEGF production in stimulated cells There was again a slight increase in VEGF production in both the BT474 and MDA-MB-231 cell line treated with hypoxia and tamoxifen This is most probably due to cytotoxicity when the cells are exposed to a double stress, namely tamoxifen and hypoxia, and intracellular VEGF is released into the conditioned medium

Tamoxifen apparently reduced endothelial migration Although tamoxifen at 5 μg/ml was not cytotoxic to HMECs m a proliferation assay, there may have been a non-specific effect during the migration assay since there was a visible morphological change in these cells at this dose While it was not statistically significant there was a visible trend for reduced migration at the lower doses of tamoxifen Tamoxifen significantly reduced monocyte transendothelial migration This experiment was carried out at a single dose of 2 5 μg/ml but it would be of interest to examine this further using a greater range of doses. This result could explain, to some extent, the decrease of serum VEGF observed in patients in response to tamoxifen therapy Macrophages constitute a large proportion of the breast tumour mass, up to 50%, and produce large

amounts of VEGF (O'Sullivan and Lewis, 1994). In an *in vivo* situation tamoxifen may stop monocytes from migrating from the circulation, through the endothelium, and into the tumour. By reducing macrophage recruitment into the tumour tamoxifen may reduce macrophage derived VEGF. Linomide has been found to reduce tumour growth by a similar mechanism, that is by reducing macrophage infiltration into the tumour (Leek et al., 1996). The mechanism by which tamoxifen reduced monocyte transendothelial migration is unknown. By inhibiting macrophage recruitment by the tumour other macrophage derived factors that aid angiogenesis, such as urokinase, EGF, TNFα, and TGFβ-1 would also be reduced. Tamoxifen inhibited the adhesion of tumour cells to endothelium and invasion into basement membrane (Bliss et al., 1996). Tamoxifen has also been found to inhibit the binding of melanona cells and MCF-7 breast cancer cells to components of the extracellular matrix (MacNeil et al., 1993). In this case the authors believe this effect was mediated through the effect of tamoxifen on intracellular calcium. It is possible that the effect of tamoxifen on calmodulin inhibits monocyte adhesion to components of the HMEC monolayer. Calmodulin antagonists inhibited the binding of B16 melanoma cells to the extracellular matrix (MacNeil et al., 1992). Another mechanism through which tamoxifen may inhibit migration is by interfering with the VEGF signal by interacting with the VEGF receptors. VEGF is chemotactic for monocytes and binds to the VEGFR-1 receptor on these cells (Clauss et al., 1990). Tamoxifen inhibited VEGF stimulated proliferation of endothelial cells which could be explained by the interaction of tamoxifen with the VEGF receptor thereby reducing its mitogenic signal. The tamoxifen metabolite 4-hydroxytamoxifen inhibits the proliferation of EGF stimulated cells and the mechanism proposed was by alteration of the receptor functionality (Freiss et al., 1990). The EGF receptor is similar to the VEGF receptors in that it is also a tyrosine kinase molecule. Tamoxifen inhibits an autocrine IGF-I loop by interfering with a tyrosine kinase activity (Howe et al., 1996; Guvakova and Surmacz, 1997).

Tamoxifen has recently been found to reduce the risk of development of breast cancer in high risk women by nearly 50% (National Cancer Institute (NCI), 1998). There are probably multiple mechanisms involved in this effect but reducing tumour derived VEGF could possibly be one effect. Reducing tumour VEGF may maintain the tumour in an avascular state and chronically manageable. Tamoxifen is a well tolerated

drug that millions of patients throughout the world have taken for many years Its mechanisms of actions including its non-oestrogen mechanisms, however, are still poorly understood and are still under intense investigation This is evidenced by the fact that a great number of trials involving tamoxifen are still running From these results we suggest that a novel mechanism of action of tamoxifen is the reduction of tumour derived VEGF This may be achieved by the reduction of macrophage infiltrate into the tumour and reduced endothelial cell migration Further investigation of this property of tamoxifen may reveal exact mechanisms lead to the development of therapeutic agents that have a greater effect Until that time the use of preoperative tamoxifen should be further investigated

CHAPTER 6

CONCLUDING DISCUSSION

This thesis has demonstrated that VEGF is overexpressed in breast cancer Breast tumour cell lines were found to produce abundant VEGF protein and mRNA A breast cancer cell line derived from a pleural effusion, MDA-MB-231 cells, produced more VEGF protein and mRNA than a breast cancer cell line that was derived from a primary lesion, BT474 cells The metastatic MDA-MB-231 cells also produce a VEGF isoform, $VEGF_{189}$, that is not produced by the primary BT474 cell line and is capable of inducing a greater angiogenic response than the other VEGF isoforms Serum VEGF levels were increased in patients with malignant breast disease as compared to patients with benign fibroadenomas or healthy age-matched controls Though it was not statistically significant, most likely due to the low number of patients with disease of a higher stage, there was a visual trend for increased serum VEGF levels with increasing stage in breast cancer patients In addition, patients with metastatic disease had the highest levels of serum VEGF, commonly over 1000 pg/ml Together with the data from the cell lines indicating that a metastatic cell line produced more VEGF this may indicate a role for VEGF in the development of metastases VEGF levels within tumour tissue were substantially increased compared to normal breast tissue from the same patient Data from other labs has demonstrated that other breast cancer cell lines, including T47-D, MDA-MB-468 and MCF-7 cells, have also been found to produce VEGF protein (Bianco et al , 1997, Hyder et al , 1998, McLeskey et al , 1998) These results indicate that VEGF is overexpressed by breast tumours

Since VEGF expression is elevated in patients with breast cancer it is important to know factors that regulate the expression of VEGF Several factors were examined for their effect on VEGF expression Factors were chosen based on evidence in the literature that they were relevant to breast cancer progression and had an association with the angiogenic phenotype Thus, the factors chosen were hypoxia, IGF-1 and TGFβ-1 IGF-1 did not affect the expression of VEGF by BT474 or MDA-MB-231 cells Hypoxia induced a several fold increase in VEGF expression in both cell lines A positive association was found between the levels of expression of TGFβ-1 and VEGF in both tumour tissue and in pre-operative serum samples TGFβ-1 increased VEGF expression in both the BT474 and MDA-MB-231 cell lines Exogenous VEGF had no effect on the expression of TGFβ-1 in either cell

line Using a semi-quantitative assay for VEGF mRNA levels in cells it was demonstrated that TGFβ-1 increased VEGF protein, in part, by increasing levels of VEGF mRNA TGFβ-1 increased VEGF production within 12 hr and increase in expression was still detectable at 48 hr which is likely sufficient time to have an effect on angiogenesis Future work could focus on the exact mechanisms whereby hypoxia and TGFβ-1 increase expression of VEGF in order to design strategies aimed at disrupting the elevation of VEGF caused by these factors We examined a small subset of factors that play an important role in breast cancer and examined their effect on VEGF expression This was by no means a comprehensive study of all the factors that may be involved in the regulation of VEGF expression in breast cancer and it would be of great interest to examine other factors associated with the progression of breast cancer on the regulation of VEGF

Since it has been demonstrated that angiogenesis is a requirement for tumour growth and the development of metastases, the inhibition of angiogenesis has been recognised as a potential therapeutic target Tamoxifen, though it is traditionally used as an anti-oestrogen, has displayed several effects which are independent of the oestrogen receptor including the inhibition of angiogenesis in the chick egg chorioallanotoic membrane assay No mechanism has yet been described for the anti-angiogenic effects of tamoxifen As VEGF is a key angiogenic factor, the effect of tamoxifen on VEGF levels was examined Patients treated with 20 mg/day pre-operative tamoxifen displayed a significant reduction m serum VEGF levels This reduction was independent of expression of the oestrogen receptor since there was no significant difference in change of serum VEGF levels in patients with ER positive or ER negative tumours Downregulation of VEGF is a potential mechanism whereby tamoxifen may inhibit angiogenesis Furthermore, since tamoxifen is a well tolerated and well studied drug, there exist opportunities for the expansion of it's uses to other forms of cancer that express high levels of VEGF Since there was no association with reduction in VEGF and ER status tamoxifen may be of value in male patients to reduce VEGF levels, as demonstrated in a single male patient with high levels of serum and ascites fluid VEGF

In many of the patients that displayed a reduction in serum VEGF there was also a reduction in serum TGFβ-1 and indeed there was a strong correlation between

the changes in serum TGFβ-1 and VEGF While this correlation demonstrates that there is an association between the changes in the levels of these two factors it does not indicate the possible influence of one factor on the other Since we had previously demonstrated that TGFβ-1 increased breast cancer cell line production of VEGF it is possible that the reduction of serum TGFβ-1 precedes the reduction of VEGF It would be of interest to monitor levels of both of these factors over shorter time intervals in order to determine the kinetics of this response to tamoxifen While the reduction in VEGF alone is of importance in breast cancer, the concomitant reduction of TGFβ-1 could also be of great benefit There is considerable evidence to suggest that TGFβ-1 expression in advanced tumours is associated with tumour growth By reducing both of these factors tamoxifen may play a dual role in inhibiting breast tumour growth

Various *in vitro* experiments were carried out to determine the mechanism through which tamoxifen reduced serum levels of VEGF Initially, the effect of tamoxifen on breast tumour cell line and monocyte derived macrophage VEGF production was examined, as these cells are the key producers of VEGF Tamoxifen did not directly lower VEGF production by either the BT474 or MDA-MB-231 breast cancer cell line or by macrophages Tamoxifen also did not reduce the production of VEGF by these cell lines when they were stimulated with TGFβ-1 and hypoxia This indicated that the mechanism through which tamoxifen reduced serum VEGF was an indirect mechanism Since TGFβ-1 increases breast cancer cell line production of VEGF the effect of tamoxifen on production of TGFβ-1 by cell lines and macrophages was examined In contrast to other breast cancer cell lines the BT474 and MDA-MB-231 cell lines did not produce significant amounts of TGFβ-1 which indicates that there was no autocrine stimulation of these cells Monocyte derived macrophages did produce large quantities of VEGF but this was not reduced by tamoxifen Concomitant research in the lab had indicated that the use of tamoxifen in rats may reduce endothelial migration Migration of endothelial cells is an important aspect of angiogenesis In addition, since there is a high macrophage content in breast tumours these cells must transmigrate from the circulation into the tumour stroma The migration of endothelial cells and the transendothelial migration of monocytes following exposure to tamoxifen was examined There was a visible

trend for reduced migration of endothelial cells following exposure to tamoxifen and tamoxifen significantly inhibited the transendothelial migration of monocytes The inhibition of migration of these cells could explain the reduction of serum VEGF following tamoxifen treatment By reducing endothelial cell migration the overall level of angiogenesis would be reduced so that fewer VEGF producing tumour cells would be supported and the levels of VEGF would decline The inhibition of migration of monocytes would mean that there would be a decrease in the number of tumour associated macrophages within the tumour and therefore a reduction in VEGF producing cells The precise mechanism through which tamoxifen inhibits migration is unknown but should be the focus of further study Tamoxifen has been used as an anti-metastatic drug and it may reduce metastasis by reducing the migration of tumour cells which is an essential component of the metastatic cascade

This thesis has clearly indicated that VEGF is elevated in breast cancer, several regulatory factors have been elucidated and a tamoxifen mediated inhibition of VEGF production *in vivo* has been illustrated Further study is warranted to discover other factors regulating the expression of VEGF The mechamsms through which tamoxifen reduces serum VEGF should also be further studied with the ultimate goal of producing a more potent therapeutic agent that further reduces VEGF production by tumours and further decreases tumour growth

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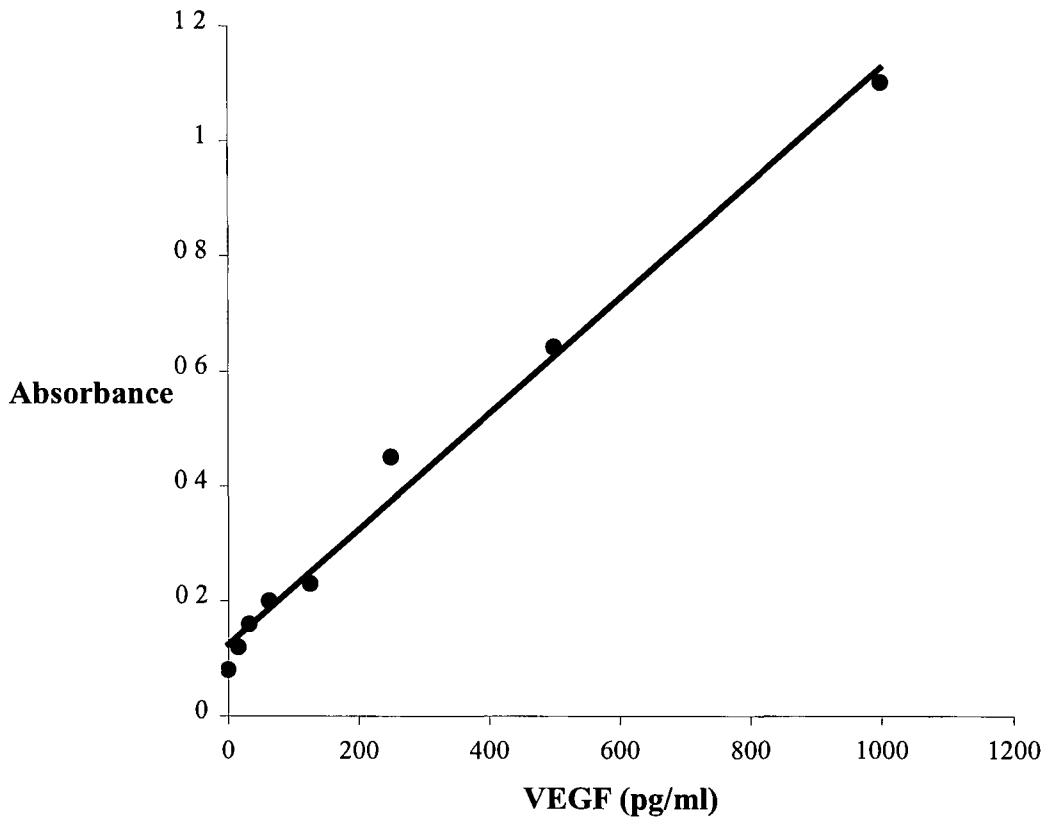
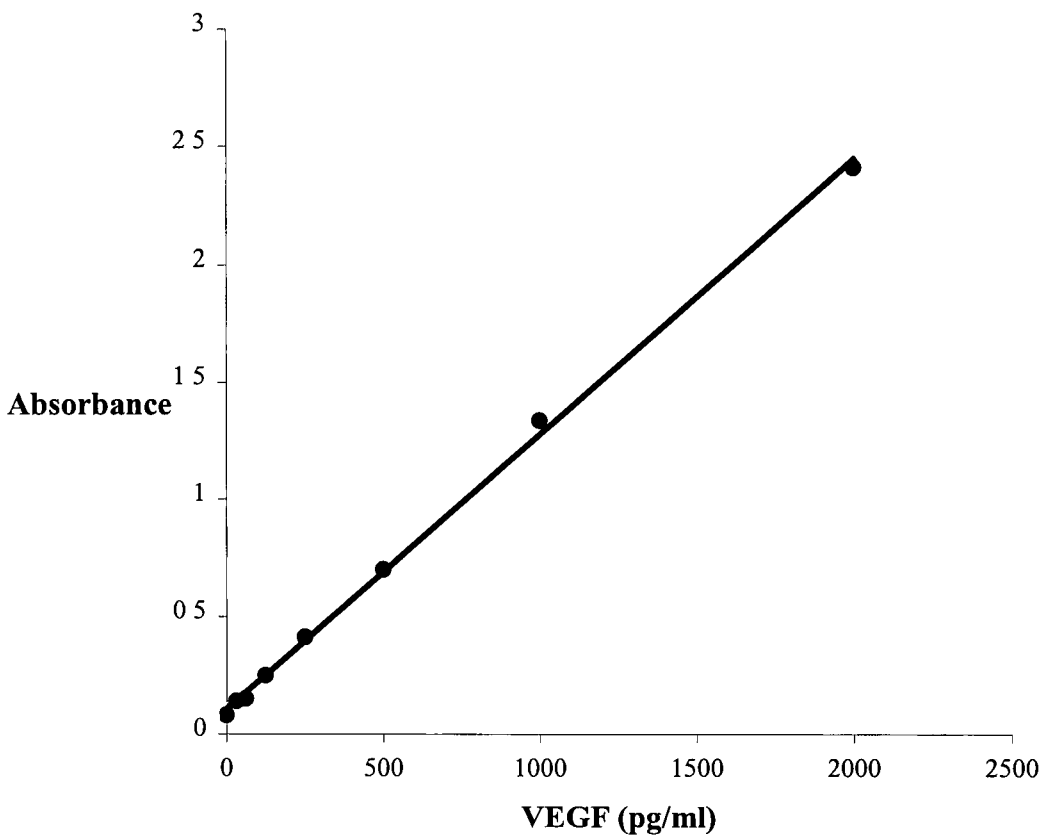
Appendix

Appendix I - Representative standard curves.



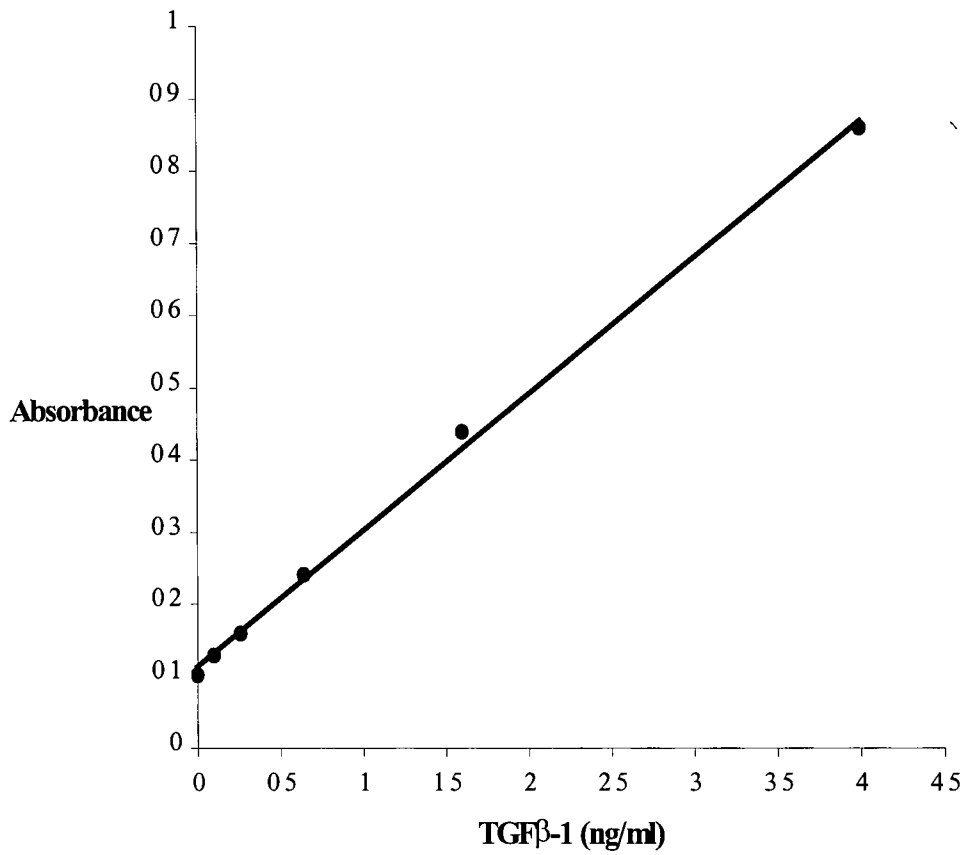
A.

Representative standard curve from developed ELISA used for VEGF measurements in conditioned medium Regression coefficient, $r^2 = 0\ 988$



B

Representative standard curve from commercial ELISA used for VEGF measurements m serum and tumour homogenates Regression coefficient, $r^2 = 0\ 998$



C.

Representative standard curve from commercial ELISA used for TGFβ-1 measurements m serum, tumour homogenates and conditioned medium Regression coefficient, r^2 = 0 998

Appendix 2 – Table of pre and post-tamoxifen serum VEGF and TGFβ-1 levels in patients receiving 2 weeks pre-operative tamoxifen (5.2.2).

No.	VEGF (pre)	VEGF (post)	TGFβ-1 (pre)	TGFβ-1 (post)	ER	Stage	Age	Type
1	297 43	181 02	36 73	32 51	n/a	n/a	69	n/a
2	199 27	78 52	55 32	38 54	pos	T1	60	ductal
3	197 53	134 12	40 29	37 89	pos	T3	54	lobular
4	109 79	98 50	36 00	31 49	neg	T2	60	ductal
5	313 93	190 58	42 97	32 73	neg	T2	65	ductal
6	465 95	406 88	60 55	61 94	neg	T1	59	ductal
7	219 25	182 76	59 76	55 32	neg	T1	76	ductal
8	318 28	1721 21	44 35	41 01	pos	T2	59	ductal
9	158 03	113 40	72 30	15 63	neg	T3	74	ductal
10	1330 29	654 39	76 90	65 55	pos	T4	53	ductal
11	124 47	125 78	40 90	58 18	neg	N/A	63	n/a
12	126 36	109 55	46 75	45 29	pos	T2	64	ductal
13	316 22	290 94	42 52	46 15	n/a	T4	70	ductal
14	68 26	26 86	48 60	27 70	neg	N/A	66	ductal
15	49 81	49 94	49 81	49 94	neg	T1	59	ductal
16	81 36	151 36	44 30	62 49	n/a	N/A	54	n/a
17	347 27	367 27	51 24	52 29	pos	T1	50	ductal
18	508 18	290 91	40 70	37 29	neg	T4	57	ductal
19	725 00	691 82	45 72	49 71	n/a	T4	79	n/a
20	544 55	325 91	48 50	44 44	neg	T2	52	ductal

Pre = pre-tamoxifen, post = post-tamoxifen, n/a = not available, neg = negative, pos = positive