EFFECT OF SURGERY AND ENDOTOXIN ON METASTATIC TUMOUR GROWTH AND REGULATION OF VEGF EXPRESSION AND ANGIOGENESIS BY ENDOTOXIN

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.

Signed: Graham Pidgeon ID No: 96971045

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Date: 29-01-'01

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Abstract

The surgical removal of primary tumours has been associated with the subsequent growth of previously dormant metastases. This accelerated growth has previously been ascribed to the removal of the primary tumour and the anti-angiogenic factors they produce, such as angiostatin and endostatin. Endotoxin or lipopolysaccharide (LPS) is a cell wall constituent of gram negative bacteria, ubiquitously present in air and endogenous gut bacteria, that may be introduced during surgery. LPS has been shown to be angiogenic and vascular endothelial growth factor (VEGF) is the most potent angiogenic cytokine identified to date. The role of endotoxin in surgically induced metastatic tumour growth, the therapeutic use of anti-endotoxin agents in the peri-operative period, the regulation of VEGF expression by endotoxin and the effect of VEGF and endotoxin on tumour cell survival was examined.

A murine model of experimental metastasis was established where no primary tumour was present. This allowed the effect of the surgical procedure on metastatic growth to be examined in the absence of any possible angiostatin / endostatin effect. Animals undergoing open surgery or laparoscopy with air sufflation of the peritoneum displayed increased metastatic tumour burden, reflected in higher proliferation and lower apoptosis within the metastases. Circulating levels of VEGF were also elevated in these groups and correlated with plasma levels of endotoxin. These changes were not observed in a group receiving laparoscopy with sterile CO_2. Endotoxin injection resulted in similar effects, with increased metastatic burden and significantly higher serum VEGF levels than controls. The anti-endotoxin agent, $rBPI_{21}$, reduced metastatic growth and serum VEGF levels in mice following LPS injection or open surgery, whereas the monoclonal antibody, E5, had no effect on tumour growth.

LPS increased tumour cell proliferation and VEGF production. Endotoxin also increased proliferation, decreased apoptosis and enhanced the production of VEGF by endothelial cells and resulted in increased angiogenesis *in vivo*. LPS, through the induction of VEGF, or VEGF alone increased Bcl-2 expression in tumour cells resulting in a significant decrease in tumour cell apoptosis. These results demonstrate that endotoxin plays a role in the enhanced growth of metastases following surgical trauma by altering the critical balances governing tumour growth. Treatment with the anti-endotoxin agent, $rBPI_{21}$, blocked post-operative growth of metastases. Endotoxin regulates immune and tumour cell production of VEGF. Furthermore, endotoxin, through the induction of VEGF, or VEGF alone act as survival factors for tumour cells.

Abbreviations

Ab	Antibody
Abs	Absorbance
aFGF	acidic fibroblast growth factor
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid assay
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bFGF	basic fibroblast growth factor
BPI	Bactericidal / permeability increasing protein
BrdU	Bromo-deoxy uridine
cDNA	complementary deoxyribonucleic acid
CSF	Colony stimulating factor
DEPC	Diethyl pyrocarbonate
DMEM	Dulbelco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease I
dNTP	Deoxyribonucleoside triphosphates
DTT	Dithiothreitol
EC	Endothelial cell
ECACC	European Collection of Animal Cell Cultures
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
EGM	Endothelial growth medium
ELISA	Enzyme linked immuno-sorbent assay
FCS	Foetal calf serum
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
IFN	Interferon

IGF	Insulin like growth factor
IPA	Isopropyl alcohol
IL	Interleukin
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide / endotoxin
MDMs	Monocyte-derived macrophages
MOPS	3-[*N*-morpholino]propanesulphonic acid
mRNA	messenger ribonuclueic acid
MTT	3-[4,5-Dimethylthiaxol-2-yl]-2,5-diphenyl-tetrazolium bromide
MVD	microvessel density
NBT	Nitro blue tetrazolium chloride
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-ECGF	Platelet derived endothelial cell growth factor
PDGF	Platelet derived growth factor
PMSF	Phenylmethylsulphonyl fluoride
rBPI	recombinant bactericidal / permeability increasing protein
rhVEGF	recombinant human vascular endothelial growth factor
rmVEGF	recombinant murine vascular endothelial growth factor
RNA	Ribonucleic acid
Rnase	Ribonuclease
RT	Reverse transcription
RT-PCR	Reverse transcription-polymerase chain reaction
RPMI	Roswell Park Memorial Institute medium
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
TAE	Tris-acetate
TBS	Tris-buffered saline
TBST	Tris-buffered saline with 0.1% Tween
TE	Tris EDTA
TEMED	*N,N,N',N'*-tetramethylethylenediamine

TGFα	Transforming growth factor α
TGFβ	Transforming growth factor β
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor α
TUNEL	TdT-mediated dUTP nick-end labelling
VEGF	Vascular endothelial growth factor
UV	Ultraviolet

Units

bp	base pairs
°C	degrees celsius
g	grams
h	hours
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar
M	molar
mA	milliamp
mg	milligram
min	minutes
ml	millilitre
mM	millimolar
ng	nanogram
nm	nanometre
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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Oral Presentations

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Surgical Research Society, Royal College of Surgeons Ireland, Dublin, 6-7 June, 1998.

RCSI Annual Research Day, Dublin, 15 April, 1998.

Surgical Research Society, St Thomas Hospital, London UK, 6-7 January, 1998

The Association of Endoscopic Surgeons of Great Britain and Ireland, Whipps Cross Hospital, London UK, 31st October, 1997.

22nd Sir Peter Freyer Surgical Symposium, Galway, 5-6 September, 1997.

Prizes

Sheppard's Prize for research, Beaumont Hospital, Dublin, 12 Jan, 2000. 1st prize for oral presentation.

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Chapter 1

Introduction

1.1 Cancer epidemiology

Cancer is recognised as the second leading cause of death in modern society (21%), with only cardiovascular disease killing more people world-wide annually (35%) (WHO, 1998). Statistics from the World Health Organisation (WHO) report an estimated 10 million new cases yearly and 6.3 million world-wide deaths from cancer in 1996 alone (WHO, 1997). In Ireland there were over 20,800 cases of cancer with approximately 7,500 deaths in 1996 (Irish Cancer Registry, 1999). Within Europe, Ireland has a relatively low incidence of male cancers, being 11th of the 20 countries assessed. Ireland ranked higher in the incidence of female cancers, being 4th of the 20. In Ireland, the risk of developing cancer before the age of 75 is 40% in men and 37% in women, while the risk of dying from the disease before the age of 75 is 17% in men and 12% in women.

World-wide, breast cancer is the most common malignancy in women with an incidence of 910,000 new cases in 1996, whereas lung cancer is the most common cancer in men with an incidence of 988,000 in 1996 (WHO). In Ireland, non-melanoma skin cancer is the most common cancer accounting for 30% of all cases. In women, breast cancer is the second most common accounting for 15%, whereas prostate is the second most common in men accounting for 11% of all cancers. With regard to breast cancer in Ireland, there were 1695 new cases and 641 deaths in 1996 alone (Irish Cancer Registry, 1999). The risk of developing breast cancer before the age of 75 is 7.9% with an associated risk of death before the age of 75 at 2.8%. The mortality rate in patients diagnosed with breast cancer is currently 38%.

1.2 Development of cancer

In every multi-cellular organism the cell numbers are tightly regulated by complex mechanisms that govern cellular proliferation, differentiation and the survival of individual cells. Deregulation of any of these processes results in alterations to the finite balances controlling cell numbers and consequently results in altered cellular growth (Holmgren *et al.*, 1995). Cancer cells exhibit characteristics that indicate they have undergone extreme failure in the control of cellular growth at the genetic level. In such cases, changes in gene expression result in uncontrolled and disorganised cell growth. Numerous factors regulate the growth and development of cells. These include growth factors and growth factor inhibitors, their receptors, signal transduction proteins and nuclear proteins regulating cellular processes (Cox and Goding, 1991). Alterations in these growth control mechanisms have been attributed, at the genetic level, to changes in a subset of genes, oncogenes or tumour suppressor genes.

The development of cancer has been described as a two step process involving initiation and promotion. Initiation is described as mutations that occur facilitating cancerous growth at a later stage. Many factors such as ultraviolet (UV) radiation, cigarette smoke and free radical species are carcinogens that act as initiators causing DNA damage (Goldstein and Witz, 1990). Promotion occurs when the cell is triggered to grow in an uncontrolled fashion. Promotors can be factors causing similar DNA damage to that occurring in the initiation phase, or they may provide an environment that causes transformed cells to progress to form a tumour (Moustacchi, 1998). Either way the overall effect is for a single cell to be altered and proliferate rapidly. Over time, a hyperplastic lesion is formed which may or may not be further altered until its proliferative rate, state of differentiation and micro-environmental influences result in the development of a neoplasm

which is capable of metastasising to distant organs. Uncoordinated growth of a heterogenous population of cancer cells results in organ destruction, loss of function and ultimately death.

Progression of the tumour is not controlled by genetic expression in the tumour cells alone. Solid tumours contain a variety of normal host cells including fibroblasts, smooth muscle cells, immune cells and endothelial cells (Foidart *et al.*, 1994). Survival of the tumour is reliant on the support of these host cells, which the tumour can utilise to aid its growth, differentiation and subsequent spread.

1.3 Metastasis

The spread of tumours from the primary site to distant organs is the most clinically important property of malignant tumours and is termed metastasis. This process allows the cancer to survive surgical excision of the primary tumour, and forms the basis for geometric increases in tumour burden along with increasing the difficulty in its clinical management. Recurrence, as a consequence of metastases, is an alarming problem in all forms of cancer. Up to 50% of patients presenting with primary cancer are estimated to have established, but often clinically undetectable, metastatic deposits (Fidler and Ellis, 1994).

The formation of secondary deposits at distant sites is a complex multistep process. It begins with the emergence of a subpopulation of tumour cells within the primary tumour with increased metastatic potential which cross tissue boundaries and infiltrate neighbouring cellular populations. As the tumour grows, it requires a blood supply to provide fresh nutrients and remove harmful waste products, as diffusion alone becomes insufficient. Infiltrating blood vessels provide tumour cells with access to the circulation and thus a route to distant sites. Metastasising cells must leave the primary site, invading

the extra-cellular matrix of adjacent tissues and migrate until they reach capillary or lymphatic linings. Then cells with the ability to penetrate basement membranes and insinuate between endothelial cells, intravasate and enter the circulation. At this point they are vulnerable to mechanical damage by shear forces and collisions as well as to immunesurveillance by the body's defence mechanisms. Surviving cells must reattach to the interior of the blood vessel, extravasate, proliferate and develop a new vascular supply in order to form secondary tumours. Tumour cells unable to mobilise the appropriate biochemical and physiological responses at the correct moment in the metastatic process fail to progress any further in tumour dissemination. An overview of the main events in the metastatic spread of tumours is outlined in Fig. 1.1. The number of checkpoints illustrated here suggests why the majority of cells which are shed die in transit and how the process is highly selective for the cells which are most robust and suited to its demands. The lack of cells surviving the process is termed metastatic inefficiency. The evidence suggests that metastasis and metastatic inefficiency are determined by a series of random events, and that the presence of metastatic variants may alter the rate or efficiency with which metastasis occurs, but are not expected to determine whether they occur or not (Weiss, 1983). One example of the process of metastatic inefficiency was reported by Luzzi and colleagues after the intraportal injection of B16 melanoma cells in mice. Only 1 in 5,000 cells detected in the circulation after intra-portal injection progressed to form a macroscopic deposit (Luzzi *et al.*, 1998). They reported that dormancy of solitary cells following successful extravasation, and limited survival of early micrometastases were responsible for the metastatic inefficiency observed in their study.

Studies on the pattern of secondary tumour growth have shown that the growth and development of metastases relies not only on the intrinsic properties of the disseminating

tumour cells described previously (i.e. the 'seed'), but also on whether they arrive in a congenial tissue or organ (i.e. the 'soil'). This 'seed and soil' hypothesis was originally proposed by Stephen Paget in 1889 following his observations on the distribution of metastases in breast cancer patients (Paget, 1889). The underlying mechanisms responsible for the distribution of metastases are not properly understood, however some evidence from clinical studies indicates that vascular drainage patterns and organ-specific factors may also be involved (Willis, 1973). The frequency of metastases in the lungs of patients with primary tumours draining into the pulmonary veins (incidence of approximately 40%) supports the vascular drainage mechanism. However, other patterns of metastases cannot be explained purely by circulatory anatomy. The high incidence of brain metastases in patients with carcinoma of the lung (approximately 30% compared to an overall incidence of approximately 5% in carcinomas generally) (Willis, 1973) and of bone deposits in breast cancer patients (approximately 70% compared to 20% for malignant tumours overall) (Paget, 1889; Lee, 1983) cannot be explained by vascular drainage patterns. These observations indicate that organ-specific factors, perhaps local paracrine hormones or other micro-environmental conditions at the secondary site, affect the survival and growth of metastatic cells and ultimately the pattern of metastases seen clinically.

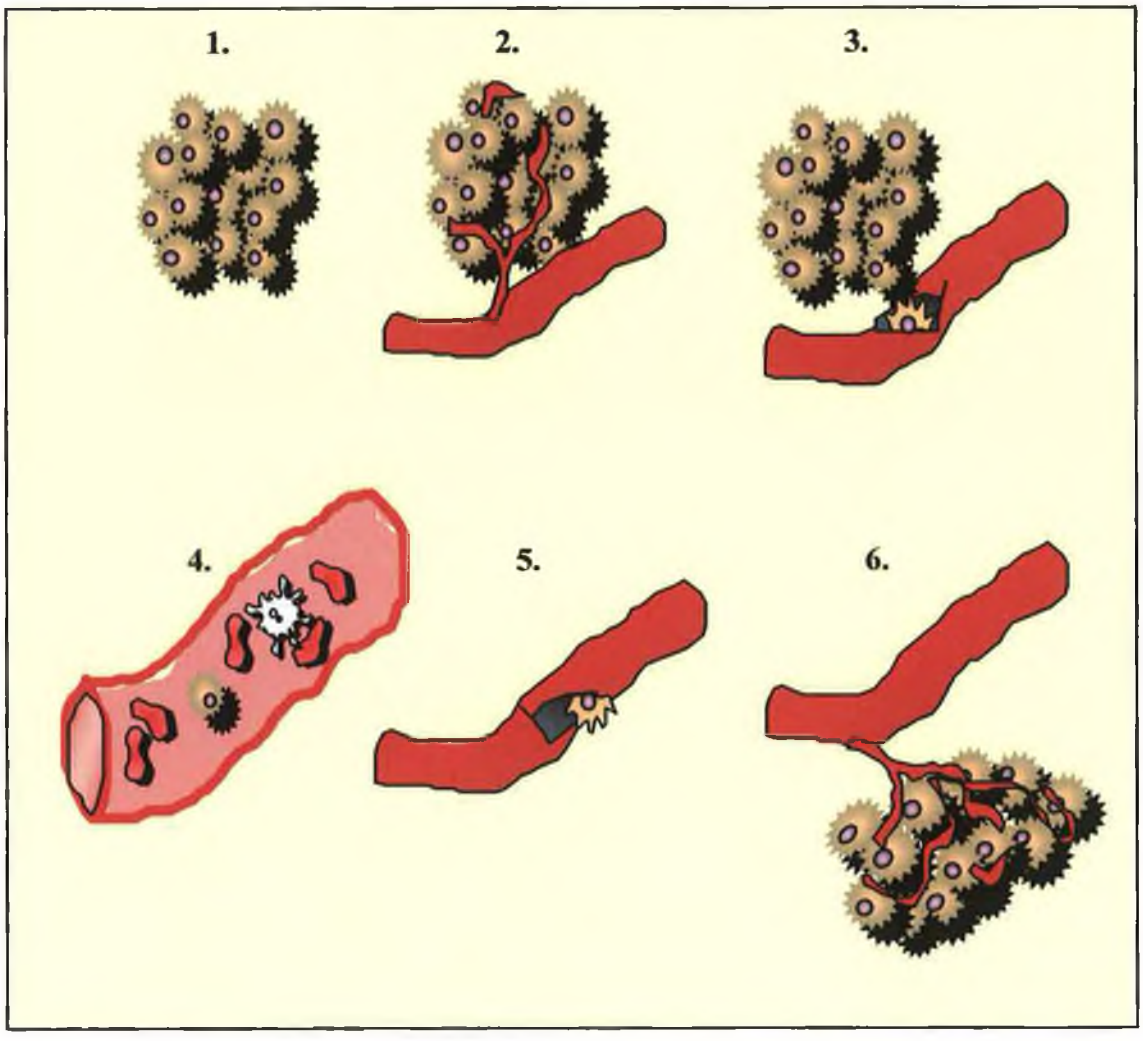

Fig. 1.1: The multistep process of metastasis.

1. A subset of cells within the primary tumour acquire the metastatic phenotype.
2. Vascularisation of the tumour occurs.
3. Invasion of the blood vessel.
4. Circulating tumour cells evade immune surveillance and mechanical destruction.
5. Arrest and extravasation of the tumour cell in downstream organs.
6. Multiplication and vascularisation of the secondary deposit at the distant site.

(Fidler and Ellis, 1994)

1.4 Angiogenesis

Blood vessels are an essential requirement for any living tissue, providing a constant supply of oxygen and nutrients and removing the harmful waste products of cellular metabolism. Angiogenesis is the process of generating new capillaries from a pre-existing blood supply (Folkman, 1982). In the developed adult, angiogenesis is the sole method for the formation of new blood vessels (Tischer *et al.*, 1991). The process is fundamental to healing, reproduction and embryonic development. The proliferation rate of endothelial cells is very low compared to many other cells in the body. More than 10^{12} endothelial cells line the interior of blood vessels covering an area of 1000 m^2 in a 70 Kg adult (Jaffe, 1987). The turnover of these cells can exceed 1000 days. Under normal physiological circumstances there is little need for new vessel formation, however the vasculature retains the ability to form new vessels if necessary, for example following injury (Edgington, 1995).

Because angiogenesis is crucial to a number of physiological processes it is tightly regulated. Angiogenesis is required within the female reproductive system for the development of the corpus luteum, the endometrium during the menstrual cycle and the placenta throughout gestation (Reynolds *et al.*, 1992; Wilting and Christ, 1996). Angiogenesis is also an essential component of wound healing, delivering nutrients and immune cells to the wound site as well as removing wound debris (Arbiser, 1996). In both of these circumstances angiogenesis is switched on for a brief period and is then completely inhibited.

Pathological angiogenesis occurs when the body loses its control over angiogenesis, resulting in either excessive or insufficient blood vessel growth. For instance, conditions such as venous ulcers, strokes and heart attacks may result from an absence of angiogenesis that is normally required for natural healing (Szabo and

Vincze, 2000; Marti and Risau, 1999). On the other hand excessive angiogenesis is a feature of a number of pathological conditions including rheumatoid arthritis, diabetic retinophathy and psoriasis (Koch, 1998; Folkman and Shing, 1992; Malhotra *et al.*, 1989). However, the most serious consequence of pathological angiogenesis is observed in tumour growth.

1.5 Tumour angiogenesis

Accumulating evidence indicates that progressive tumour growth is dependent on angiogenesis. Tumours can remain *in situ* for long periods of time in an avascular, quiescent state. During this stage, the tumour may contain only a few million cells. Growth of these avascular deposits require a subset of the cells within the tumour to switch to an angiogenic phenotype. This switch to the angiogenic phenotype occurs when the local equilibrium between positive and negative regulators of angiogenesis is tipped in favour of blood vessel growth (Hanahan and Folkman, 1996). New blood vessels provide nutrients to proliferating cancer cells, thereby favouring tumour growth. On the other hand, capillaries provide a route for the delivery of anti-neoplastic drugs, thus allowing the chemotherapeutic treatment of cancer patients. Unfortunately, these new capillaries have incomplete basement membranes and as such are 'leaky', causing increases in interstitial pressure that limits drug diffusion within the tumour and favours tumour cell dissemination in the blood stream (Yuan, 1998).

There is substantial evidence that tumour growth is angiogenesis dependent (Folkman, 1990). The requirement of angiogenesis for tumour growth was noted over 50 years ago in a mouse sarcoma model. Tumours implanted sub-cutaneously in transplant chambers did not grow until the development of new blood vessels was observed, after which growth was almost exponential (Algire *et al.*, 1945). In another

experiment, a rat sarcoma in the chick chorioallantoic membrane (CAM) did not grow for 72 hours when no blood vessel supply was visible. However, tumour growth rapidly increased within 24 h of the in-growth of capillaries (Ingber *et al.*, 1986). Similarly, homologous tumour implants grown in the anterior chamber of the rabbit eye formed dormant spheroids because angiogenesis doesn't occur at this site. The tumours remained viable for 34 days after which, when they were placed on the iris where vascularistaion could occur, they grew rapidly reaching 16,000 times their original size within 2 weeks and became locally invasive (Gimbrone *et al.*, 1972). These experiments provide direct evidence that the acquisition of a vascular supply from the host is essential for the local establishment of a tumour.

The relationship between tumour angiogenesis and malignancy has also been investigated in an experimental model where diploid mouse fibroblasts were passaged subcutaneously and then separately assessed for their angiogenic potential using the CAM assay (Ziche and Gullino, 1982). Fibroblasts could initiate angiogenesis by passage 5, but malignancy began at passage 15, indicating that angiogenesis preceded the development of the malignant phenotype. Recent evidence shows that angiogenesis suppression within tumours leads to a clinically dormant state where cellular proliferation is balanced by cell death enabling the tumours to reach an equilibrium with deposits limited to 2 mm^3 (Holmgren *et al.*, 1995). There is evidence that angiogenesis occurs at an even earlier stage in tumour growth. In a study by Li *et al.* 40-50 4T1 mammary carcinoma cells were injected into dorsal chambers in mice. Modification of the host vasculature was observed when tumour mass reached approximately 60-80 cells, and functional new blood vessels were seen when the tumour mass reached as little as 100-300 cells (Li *et al.*, 2000).

1.6 Tumour vascularisation

The use of microvessel quantitation or extent of angiogenesis in solid tumours as a prognostic indicator in cancer has been the subject of intense investigation. The extent of angiogenesis within tumour sections can be measured by staining the tumours for endothelial cell specific markers and then counting the number of blood vessels manually or estimating the percentage of endothelial cells using an interactive image analysis system. The endothelial cell specific markers commonly used to date include factor VIII-related antigen, platelet endothelial cell adhesion molecule (PECAM or CD31) and CD34 antigen (Weidner *et al.*, 1991; Horak *et al.*, 1992; Goulding *et al.*, 1995). A number of studies have demonstrated that breast tumours are significantly more vascularised than normal breast tissue or benign tumours (Horak *et al.*, 1992; Goede *et al.*, 1998). Increased vessel density within tumours has been associated with various clinical outcomes. Horak *et al.* (1992) found that the number of vessels in primary breast tumours was associated with nodal metastases, as well as tumour size and grade. Microvessel counts and density grades have also been associated with metastatic disease and increased tumour aggressiveness in cancer of the breast (Weidner *et al.*, 1991; Bosari *et al.*, 1992), non-small-cell lung cancer (Macchiarini *et al.*, 1992), prostate cancer (Fregene *et al.*, 1993) and squamous cell carcinoma of the head and neck (Albo *et al.*, 1994). Recently, a study of 531 breast cancer patients, with a follow-up greater than 6 years, reported microvessel density (MVD) to be a significant prognostic indicator for relapse-free and overall survival using multivariate models (Gasparini *et al.*, 1998). However a number of reports have disagreed with these findings, suggesting that MVD counts may be highly variable and of no prognostic significance (Goulding *et al.*, 1995; Costello *et al.*, 1995). Indeed, results from our laboratory suggest that the measurement of VEGF as an indicator of

angiogenesis is more reliable prognostically than the measurement of microvessel density (Callagy *et al.*, 2000). In order for tumour vascularity to be used clinically as a reliable prognostic factor in cancer, standardisation of quantitation methods needs to occur. Recent developments in technology in the area of automated image analysis systems could provide the standardisation required to address these issues.

1.7 Steps in angiogenesis

In order to support continued tumour growth, the tumour vasculature constantly remodels itself and as such angiogenesis is an ongoing process continuing indefinitely until the tumour is removed or killed, or the host dies. The angiogenic process can be divided into four sequential steps:

1) Release of tumour angiogenic factors (TAFs) and endothelial cell activation
2) Endothelial cell migration
3) Endothelial cell proliferation
4) Microvessel differentiation and lumen formation

A simplified version of the process is shown in figure 1.2. The process of angiogenesis begins with the release of tumour angiogenic factors (TAF) which diffuse into the surrounding tissue. Many of these factors have been identified to date and a list of some of the most common is outlined in table 1.1. The release of these factors is provoked by factors both endogenous and exogenous to the tumour cells including local environmental conditions, such as hypoxia (Battegay, 1995). The site of action for these TAFs can vary considerably, some acting nearly exclusively on endothelial cells such as vascular endothelial growth factor (VEGF) (Jakeman *et al.*, 1992), while others such as basic fibroblast growth factor (bFGF) act on fibroblasts, endothelial and smooth muscle cells (Gospodarowicz, 1990). Some angiogenic factors, such as bFGF, lack

signal peptides necessary for extracellular secretion but are released from extracellular matrix and basement membranes by enzymatic cleavage (Klagsbrun and D'Amore, 1991). Other factors such as transforming growth factor-β1 (TGFβ1) (Frater-Schroder *et al.*, 1987), tumour necrosis factor-α (TNF-α) (Leibovich *et al.*, 1987) and IL-6 (Motro *et al.*, 1990) inhibit the proliferation of endothelial cells *in vitro*. They have been suggested to have an indirect mechanism of action, such as the stimulation of other cells (e.g. macrophages) to release angiogenic factors. Alternatively, they may promote angiogenesis by causing endothelial cell differentiation (e.g. tube formation or matrix production) rather than endothelial proliferation (Klagsbrun and D'Amore, 1991).

Each of these angiogenic factors act with varying degrees of potency with VEGF being the most potent identified to date. The relative importance of each of the angiogenic peptides as mediators of tumour angiogenesis is uncertain. The majority of these factors are produced by tumour cells while others are released by immune cells in the tumour micro-environment following signals from the tumour cells. The first reaction to these tumour derived angiogenic factors is the activation of quiescent endothelial cells in the neighbouring vessels. The morphology of the endothelial cells is altered in response to these factors, increasing their relative amounts of endoplasmic reticulum, Golgi apparatus and mitochondria and the cells form protrusions on the abluminal side (Fox *et al.*, 1996).

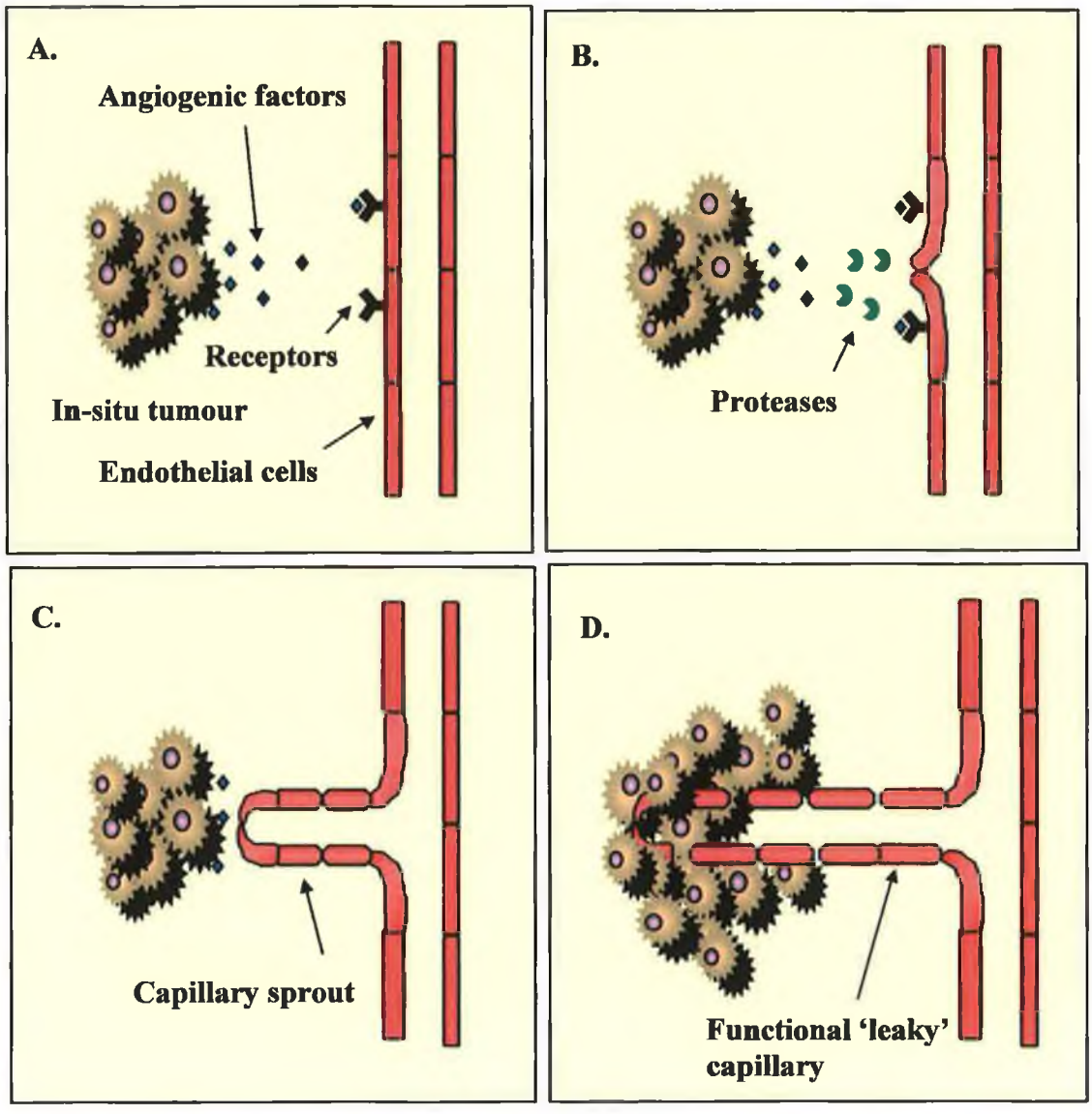

Fig. 1.2 : Sequential steps in angiogenesis.

(A) Tumour or immune cells release tumour angiogenic factors (TAFs) which bind to receptors on endothelial cells. (B) Activated endothelial cells release proteolytic enzymes which degrade the extracellular matrix and migrate along a chemotactic gradient. (C) Endothelial cells proliferate at the base forming a capillary sprout. (D) Capillaries mature and a functional lumen allows the delivery of oxygen and nutrients to support tumour expansion.

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

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

In order for new vessel invasion to occur, degradation of the extracellular matrix must happen. This is facilitated by the production of proteolytic enzymes including plasminogen activators (urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA)) and metalloproteinases (MMPs) secreted by endothelial, immune or tumour cells (Bacharach *et al.*, 1992; Hiraoka *et al.*, 1998). Urokinase plasminogen activator generates the broad spectrum protease plasmin which is capable of degrading most matrix components (Fox *et al.*, 1996). Inhibition of this factor inhibited angiogenesis and tumour growth of B16 melanoma cells in a murine model (Brock *et al.*, 1991). uPA receptors on endothelial cells may be increased by VEGF, and therefore VEGF can aid in the co-ordination of matrix degradation (Mandriota *et al.*, 1995). Matrix degradation and remodelling is carefully controlled by alterations in the proteolytic balance (Pepper and Montesano, 1990).

Degradation of the basement membrane allows the endothelial cells to accumulate in the region where the concentration of angiogenic factors has first reached a threshold level. The endothelial cells from the capillary venules migrate and form sprouts. The capillary sprouts begin to grow by recruiting endothelial cells from the parent vessel. The endothelium initially forms a tube-like solid cord, but eventually intra- and inter-cellular spaces appear to form lumina (Folkman and Haudenschild, 1980). The sprouting vessel is elongated by endothelial cell proliferation some distance from the top of the sprout. The proliferation rate of endothelial cells is some 40 times faster in tumours than in normal tissue where they are normally quiescent (Vartanian and Weidner, 1994).

Neighbouring sprouts eventually fuse together at their tips to form loops or anastomoses (Polverini, 1995). The looped vessels may bud or fuse with other vessels until a complex vascular network develops. These newly formed capillaries have wide

cell junctions, numerous trans-endothelial channels and an irregular basement membrane (Fox *et al.*, 1996). Vascular endothelial growth factor, also known as vascular permeability factor (VPF) causes hyper-permeability of blood vessels within the tumour (Senger *et al.*, 1983). The capillary networks within tumours are therefore 'leaky' due to their structure and the action of VEGF. These events are constant within the growing tumour, with new vessels being constantly recruited, therefore the tumour vasculature is highly unstable (Fox *et al.*, 1996).

1.8 Vascular Endothelial Growth Factor

Vascular endothelial growth factor is the most potent directly acting angiogenic cytokine identified to date. Also known as vascular permeability factor (VPF), it was originally isolated from guinea pigs with hepatocellular carcinomas as a factor regulating permeability (Senger *et al.*, 1983). It was independently discovered as a soluble factor produced by bovine pituitary folliculo stellate cells that stimulated the growth of adrenal endothelial cells (Ferrara and Henzel, 1989). Vascular permeability factor was subsequently found to be an angiogenic endothelial cell mitogen, and structural analysis by protein sequencing and cDNA cloning showed that the two proteins were identical (Connolly *et al.*, 1989).

VEGF is an approximately 46-kDa homodimeric protein with heparin binding activity (Connolly *et al.*, 1989). Molecular cloning has revealed at least 5 different isoforms containing 121, 145, 165, 189, and 206 amino acids respectively. All are encoded on a single gene and are generated by alternative splicing of the mRNA (Tischer *et al.*, 1991; Poltorak *et al.*, 1997). These isoforms differ in their properties due to the presence of different exons. $VEGF_{121}$ fails to bind heparin whereas $VEGF_{189}$ and $VEGF_{206}$ readily bind it (Houck *et al.*, 1992). $VEGF_{165}$ has weak heparin binding

activity. These heparin binding affinities reflect their bioavailability, with $VEGF_{121}$ being secreted freely as a soluble protein, $VEGF_{189}$ and $VEGF_{206}$ retained at the cell surface or extracellular matrix, and $VEGF_{165}$ displaying intermediate behaviour, some being freely secreted and some apparently bound to the cell surface (Houck *et al.*, 1992). $VEGF_{145}$ is the most recently discovered isoform, with heparin binding activity that keeps it bound to the cell surface upon secretion (Poltorak *et al.*, 1997). All isoforms contain glycosylation sites that are essential for efficient secretion but not for their biological activity.

All of the VEGF isoforms contain 8 cysteine residues required for both homo and hetero-dimerisation of the protein. They have a low overall homology with platelet derived growth factor (PDGF), another endothelial growth factor, but the location of the cysteine residues is particularly conserved. Other members of the same family of endothelial growth factors include, placenta growth factor (PlGF), PDGF-A and PDGF-B, VEGF-B, VEGF-C, VEGF-D, and VEGF-E (Maglione *et al.*, 1991; Olofsson *et al.*, 1996; Joukov *et al.*, 1996; Yamada *et al.*, 1997; Ogawa *et al.*, 1998). VEGF-B shares approximately 45% homology with VEGF and is expressed in a variety of human tumours including melanomas, sarcomas, breast, and head and neck tumours (Salven *et al.*, 1998). The remaining members vary in their degree of homology from 20-45%.

1.9 Regulation of VEGF

VEGF expression is regulated by a variety of factors both intrinsic and extrinsic to the tumour cells. PlGF can significantly enhance the activity of low levels of VEGF by its binding to the VEGF type 1 receptor, Flt-1 (Park *et al.*, 1998). PDGF has a more direct effect by enhancing the transcription of the VEGF gene (Finkenzeller *et al.*, 1992). Epidermal growth factor (EGF) and transforming growth factor beta (TGFβ) have also

been shown to have similar effects in human malignant glioma cells, vascular smooth muscle cells and the monocyte like U937 cells, with TGFβ1 inducing a 1.8 fold increase in VEGF expression in the U937s (Goldman *et al.*, 1993; Brogi *et al.*, 1994; Dolecki and Connolly, 1991; Donovan *et al.*, 1997). Other factors that can potentiate VEGF production include fibroblast growth factor 4 (FGF-4) (Deroanne *et al.*, 1997), tumour necrosis factor α (Ryuto *et al.*, 1996), keratinocyte growth factor (KGF) (Frank *et al.*, 1995), IGF-I (Goad *et al.*, 1996), interleukin 1β (IL-1β) (Li *et al.*, 1995) and IL-6 (Cohen *et al.*, 1996). Increased VEGF expression has also been reported following stimulation of hormone receptors. Both oestrogen and progesterone have been shown to considerably increase VEGF mRNA expression (Cullinan-Bove and Koos, 1993). The prostaglandins E_1 and E_2, which stimulate bone formation, a process strongly reliant on angiogenesis, have also been shown to increase VEGF mRNA and protein levels in an oesteoblastic cell line (Harada *et al.*, 1994).

Hypoxia and hypoglycemia also stimulate VEGF expression (Shweiki *et al.*, 1992; Harmey *et al.*, 1998). Hypoxia-induced transcription of VEGF mRNA is mediated in part by the binding of hypoxia-inducible factor 1 (HIF-1) to a binding site in the VEGF promoter (Levy *et al.*, 1996; Liu *et al.*, 1995). This factor can also be induced by the oncogene v-*src* thereby overriding the hypoxia sensing mechanism and ultimately leading to increased VEGF expression (Jiang *et al.*, 1997). Hypoxia also promotes the stabilisation of VEGF mRNA by proteins that bind to sequences located in the 3' untranslated region of the VEGF mRNA (Stein *et al.*, 1995; Claffey *et al.*, 1998).

Inactivation of tumour suppressors is another mechanism whereby VEGF may be constitutively upregulated in tumour cells. The von Hippel Landau (vHL) suppressor gene has been shown to inhibit the production of many hypoxia-regulated proteins including VEGF (Siemeister *et al.*, 1996). This effect was shown to be mediated at both

the transcriptional and post-transcriptional level, by SP1 binding and inhibition of protein kinase C (PKC) activity, respectively (Mukhopadhyay *et al.*, 1997; Iliopoulos *et al.*, 1996). Binding of the transcription factor SP-1 by von Hippel-Lindau protein has been shown to inhibit SP-1 mediated VEGF expression, and inhibition of PKC results in decreased stability of the VEGF mRNA as active PKC mediates the interaction of stabilising proteins with VEGF mRNA (Mukhopadhyay *et al.*, 1997; Levy *et al.*, 1996). P53 tumour suppressor gene can also regulate VEGF expression. Loss of wild-type P53 is associated with increased angiogenesis (Vanmeir *et al.*, 1994) and wild-type P53 was identified as an inhibitor of VEGF (Mukhopadhyay *et al.*, 1995), while mutated P53 potentiated its expression, mediated again by PKC (Kieser *et al.*, 1994)

1.10 VEGF receptors

The biological effects associated with VEGF are mediated by its binding to one of at least two receptors. The first, VEGFR-1, is fms-like tyrosine kinase (flt-1) and was shown to be a high-affinity receptor (K_D ca. 20pM) for VEGF (DeVries *et al.*, 1992). The second one, VEGFR-2, fetal liver kinase 1 (flk-1) also known as kinase insert-domain receptor (KDR), has a lower binding affinity for VEGF with a K_D of 75mM (Terman *et al.*, 1992). Both receptors are type III receptor tyrosine kinases containing seven immunoglobulin-like domains in their extracellular region, a transmembrane domain and two kinase domains separated by an interkinase linker (Terman *et al.*, 1992). Both receptors are predominantly expressed on endothelial cells (Peters *et al.*, 1993; Quinn *et al.*, 1993), although some additional types of cells express one or both of the receptors. VEGFR-1 has been shown to be expressed on trophoblast cells (Charnockjones *et al.*, 1994), monocytes (Barleon *et al.*, 1996) and renal mesangial cells (Takahashi *et al.*, 1995), while VEGFR-2 is also expressed on hematopoietic stem cells,

megakaryocytes, and retinal progenitor cells (Katoh *et al.*, 1995; Yang and Cepko, 1996). They are reported to have different signal transduction properties resulting in different patterns of responses (Waltenberger *et al.*, 1994; Seetharam *et al.*, 1995).

Both receptors are glycosylated, but in cells only expressing VEGFR-1, stimulation with VEGF induces no significant receptor autophosphorylation and no mitogenic response (Waltenberger *et al.*, 1994). In contrast, VEGFR-2 can autophosphorylate upon stimulation with VEGF and is capable of mediating a mitogenic response. In an experiment by Seetharam *et al.*, NIH3T3 cells expressing recombinant VEGFR-2 phosphorylated phospholipase Cγ (PLCγ), GTP-ase activating protein (GAP) and mitogen activated protein kinase (MAPK) following treatment with VEGF, and a mitotic response was observed (Seetharam *et al.*, 1995). When VEGFR-1 was transfected into the same cells MAP kinase was not activated. It is therefore possible that VEGFR-1 does not induce cellular proliferation because it fails to activate MAP kinase.

Recently neuropilin-1, a receptor for the collapsin/semaphorin family that mediate neuronal cell guidance was identified as an isoform specific receptor for $VEGF_{165}$ (Soker *et al.*, 1998). It was previously recognised that these receptors existed and were not related to the VEGFR-1 or VEGFR-2 receptors, which bind both isoforms $VEGF_{121}$ and $VEGF_{165}$ (Gitay-Goren *et al.*, 1996). These receptors are expressed in unusually high amounts by many types of prostate and breast cancer cells, including the MDA-MB-231 cell line (Soker *et al.*, 1996; Soker *et al.*, 1998). These neuropilin receptors, so called because receptors expressed on the MDA-MB-231 cells were found to be encoded by neuropilin-1, have short intracellular domains and are therefore unlikely to function as independent receptors. When cells expressing neuropilin-1, but no other VEGF receptor, were stimulated with VEGF no mitogenic activity or VEGF

mediated chemotaxis in endothelial cells was observed (Soker *et al.*, 1998). However, it has been shown to be crucial to blood vessel development as mouse embryos lacking a functional neuropilin-1 gene fail to develop a functional cardiovascular system and die (Kitsukawa *et al.*, 1997). Recent studies have shown that neuropilin-1 acts as a $VEGF_{165}$ co-receptor for VEGFR-2 potentiating its binding to the receptor and increasing the subsequent migratory response of the cell (Soker *et al.*, 1998). The identification of the neuropilin-1 receptor may explain why the $VEGF_{121}$ isoform, which does not bind to this receptor, does not appear to be as strong a mitogen as $VEGF_{165}$. Indeed more interestingly perhaps, is why there are substantial amounts of the receptor on tumour cells which appear to not express substantial amounts, if any, of the other VEGF receptors. Perhaps they may act as receptors for autocrine regulation of tumour cells by VEGF, or as mediators of cross-talk between the tumour and endothelial cells, similar to that observed between inflammatory and endothelial cells (Krishnaswamy *et al.*, 1999).

VEGF signalling in tumours requires the presence of VEGF receptors in the vasculature adjacent to the tumour. For colon carcinoma it was shown that only the endothelial cells of nearby stromal blood vessels expressed the mRNA for both VEGF receptors. Endothelial cells located some distance from the tumour did not stain for either receptor (Brown *et al.*, 1993a). Similarly in renal cell carcinoma, endothelial cells of small tumour vessels stain strongly for the mRNA of the two receptors (Brown *et al.*, 1993b). These data suggest that receptor expression is observed in vessels penetrating the tumour or adjacent to it, and is absent from endothelial cells of normal blood vessels. This implies that the tumour may secrete factors that are capable of upregulating the expression of the VEGF receptors on endothelial cells, making them more susceptible to VEGF activation. One factor capable of inducing VEGF receptor expression is bFGF, which has been shown to up-regulate the mRNA for both receptors

in human vascular endothelial cells (Barleon *et al.*, 1994). Another factor capable of up-regulating VEGF receptor expression is heparin (Gitay-Goran *et al.*, 1992; Terman *et al.*, 1994). In addition to inducing receptor expression, low concentrations (0.1 - 1 μg/ml) of heparin can enhance the binding of VEGF to both receptors in bovine aortic endothelial cells (Gitay-Goren *et al.*, 1992). Other micro-environmental factors such as hypoxia can induce VEGF receptor expression. This was shown in a cerebral slice culture system, where hypoxia increased VEGFR-2 expression (Kremer *et al.*, 1997). In the same system addition of VEGF increased VEGFR-2 expression, an effect that was lost by the addition of anti-VEGF antibodies. Recently VEGF has been shown to upregulate the expression of KDR in human endothelial cells (Shen *et al.*, 1998), indicating that VEGF can regulate its own receptor expression. The importance of the VEGF signalling system for its biological effects make it an attractive target for novel therapeutic strategies. Indeed the VEGF signal may be regulated by the production of a soluble VEGFR-1 binding VEGF with high affinity (Kendell and Thomas, 1993). Transfection of this soluble VEGFR-1 into human fibrosarcoma cells inhibited tumour growth, metastasis and mortality when implanted into nude mice (Goldman *et al.*, 1998). On the basis of the body of knowledge available regarding the VEGF receptor system, many inhibitory compounds have been developed as a mechanism of regulating tumour angiogenesis, such as PTK inhibitors developed by Novartis.

1.11 Role of VEGF in tumour growth

VEGF is a potent mitogen for vascular endothelial cells, but it is devoid of mitogenic activity for other cell types (Ferrara and Henzel, 1989; Ferrara *et al.*, 1992). It also promotes the migration of endothelial cells, their invasion into collagen gels and their

formation into tube-like structures. It has also been widely reported that VEGF specifically induces the formation and growth of new blood vessels in the chick chorioallantoic membrane (CAM), the mesentery of the small bowel and corneal implants (Oh *et al.*, 1997; Norrby, 1996; Kenyon *et al.*, 1996). Studies have shown that when VEGF signalling is inhibited, tumour angiogenesis and, consequently, tumour growth are impaired (Kim *et al.*, 1993; Millauer *et al.*, 1994). The angiogenesis-inducing activity of VEGF is accompanied by an increase in vascular permeability (Keck *et al.*, 1989). This function of VEGF may be implicated in the mechanism whereby tumour cells enter and leave the circulation to facilitate metastases.

At the cellular level, VEGF can increase the mRNA levels of plasminogen activators and plasminogen activator inhibitor-1 (PAI-1) in endothelial cells (Olofossen *et al.*, 1996). It has also been shown to induce interstitial collagenase expression (Wang and Keiser, 1998). The induction of plasminogen activators and collagenases promotes a pro-degradative environment that is necessary for the migration of endothelial cells, whereas PAI-1 can serve to regulate and balance the process. Therefore it is obvious that VEGF can regulate multiple steps involved in the development of metastasis, and as such is more than just an endothelial cell specific mitogen.

The essential role of VEGF in vessel formation can be seen as early as the development of the embryo. In mice embryos containing a homozygous VEGF deficiency (VEGF -/-) there was massive impairment of vascular and cardiovascular development resulting in the death of the embryos mid-gestation (Carmeliet *et al.*, 1996). Evidence for its role in the regulation of tumour growth is strong. Embryonic murine stem cells with homozygous deficiencies for VEGF display dramatically reduced ability to form tumours in nude mice compared to normal stem cells, and the tumours that do form have significantly reduced blood vessel density (Ferrara *et al.*,

1996). In addition administration of monoclonal antibodies directed against VEGF inhibited the growth of a variety of experimental tumours in mice including human rhabdomyosarcoma, glioblastoma and fibrosarcoma (Kim *et al.*, 1993; Asano *et al.*, 1995; Borgstrom *et al.*, 1996). VEGF is therefore a crucial factor in both the spread and development of tumours, and a potential target for tumour therapies.

1.12 Role of cell death in tissue maintenance

Cancer may be described as a net accumulation of mutated cells, which is generally attributed to excessive cellular proliferation. However, the overall size of cell populations is governed, not only by cellular proliferation, but by the homeostatic balance between cell production and cell death. Factors which interfere with the fine physiological tuning process regulating cell numbers could contribute to the development of a neoplastic phenotype either by promoting cell proliferation and/or inhibiting cell death.

Cell death can occur by one of two processes. Necrosis is a result of acute, non-physiological injury (such as that following oxygen deprivation, a physical blow or at the site of toxic action). This sort of cell death is characterised by swelling of the cell, its subsequent rupture and the release of the intercellular contents into the surrounding milieu, thus sparking an inflammatory response. Under normal circumstances inflammation limits infection and aids in the clearance of cellular debris. However, in extreme cases, the activities and secretions of white blood cells may result in damage to normal healthy tissue in the vicinity of the original injury.

The second form of cell death was discovered by Kerr *et al.* over 20 years ago and is termed 'apoptosis' (derived from the Greek word meaning 'falling off') (Kerr *et al.*, 1974). This form of cell death, also termed 'cellular suicide', is a purposeful, self-

destruction mechanism in which the cell plays an active role. Cells undergoing the 'programmed' form of cell death rapidly shrink, losing normal intercellular contact and subsequently exhibit chromatin condensation, nuclear fragmentation, cytoplasmic blebbing and cellular fragmentation into small apoptotic bodies (Wyllie, 1992). Unlike necrosis, the cytosolic contents are not released into the intercellular milieu and therefore no inflammation is triggered. Apoptotic cells are removed rapidly (within hours) by scavenging cells in the vicinity such as macrophages, thereby preventing either direct damage to neighbouring tissue and avoiding the possible adverse side effects of an inflammatory response.

Apoptotic cell death is an integral part of tissue modelling in vertebrate development (Duke *et al.,* 1996). In general unwanted, injured, or virus-infected cells are eliminated by apoptosis. When this process is disturbed, severe diseases can result. Perhaps the most critical discovery was that of the importance of apoptosis in the pathogenesis of many forms of cancers and their likelihood to form resistance to many conventional apoptosis-inducing forms of therapy.

1.13 Regulation of apoptosis

Apoptosis is regulated by a number of genes that influence cellular susceptibility to enter the process. The p53 gene is the most common gene associated with apoptosis. Mutations in the p53 gene and its regulators are very common, occurring in 50-70% of human cancers (Levine, 1997). p53 protein acts by inducing cell cycle arrest and apoptosis in response to DNA damage. In many tumours, genetic damage fails to induce apoptosis because the cells have an inactivated *p53* gene. Loss of functional *p53* correlates well with tumour aggressiveness in a variety of tumours. In addition, people carrying defects in either 1 or 2 of the genes coding for p53 are clinically diagnosed

with Li-Fraumeni syndrome and develop cancer at a much higher rates and incidence than the normal population (Hetts, 1998)

The p53 protein induces apoptosis by acting as a transcription factor, regulating many other apoptosis mediating genes. One of these mechanisms is through p53 suppression of *Bcl-2* expression. Bcl-2 is an anti-apoptotic protein whose over-expression has been reported in many forms of malignancy (Reed, 1994). The function of this survival protein is thought to be in the protection of cells whose loss would be detrimental to the body. Many normal cells, such as melanocytes, express relatively high levels of Bcl-2 and are subsequently more likely to give rise to aggressive tumours when they become cancerous (Solary *et al.*, 1996). While Bcl-2 acts by suppressing apoptosis, other members of this family of proteins serve to induce apoptosis, working in conjunction with the anti-apoptotic members to regulate the process. Bcl-2 family members are capable of forming dimers, often including homodimer and heterodimer pairs. When Bcl-2 is heterodimered with a pro-apoptotic member of the family, Bax, its anti-apoptotic action is lost and apoptosis is induced. Interestingly, p53 has been shown to upregulate the expression of Bax in lung cancer cells and this is another possible mechanism whereby p53 induces apoptosis in cells (Pearson *et al.*, 2000). In a study by Yin *et al.* it was reported that mice lacking the *Bax* gene developed fast growing tumours with 50% fewer apoptotic cells compared to similar tumours in non knock-out mice.

Other apoptosis-regulating genes include *abl*, *c-myc* and *ras*. Hence, it appears that even within cancer cells there are many different regulators of apoptosis. This in itself creates a further problem when designing treatments aimed at inducing apoptosis by disrupting the homeostatic balances regulating cancer growth.

1.14 Tumour dormancy

In many forms of cancer, secondary tumours may remain clinically asymptomatic and undetectable for months or years after primary tumour resection, only to reappear at a later date. The lag time between treatment and recurrence varies widely, and led to the speculation that the tumours undergo a dormant or 'sleepy' state with growth restraint for prolonged periods (Wheelock *et al.*, 1981). Little is known about the cellular or molecular mechanisms controlling the fate of these tumour cells during dormancy. It was initially viewed as a state in which tumour cells remained in G_0 and was characterised clinically as a state of remission. The long disease free survival associated with recurrence may be explained by one of two mechanisms. The first is founded on uninterrupted tumour growth, where tumour growth starts at the time of seeding and continues until clinical recurrence is documented. In this scenario considerable interpatient variability is suggested and late recurrence denotes slower growing tumours. However, when the interval between excision and reappearance of the tumour as secondary foci exceeds periods such as 5 years, as it often does, it becomes increasingly difficult to believe that cellular proliferation in the residual tumour fragments has been continuous and uninterrupted.

Clinical studies of tumour resection in melanoma and breast cancer patients dispel this theory and actually show that tumour cells can undergo a period of quiescence or dormancy followed by rapid growth during relapse (Crowley & Seigler, 1992; Demicheli *et al.*, 1994). This hypothesis claims that tumour deposits can remain 'dormant' for prolonged periods and then resume their growth at a later date presumably following some critical event. Recent evidence and the discovery of the homeostatic balance governing cell growth lends credibility to this hypothesis. In such case, a population of cells may be considered dormant if the net rate of increase in tumour cell

numbers is equalled by the rate of cell loss. Recently Holmgren *et al.* (1995) linked the concept of balanced proliferation and cell death with angiogenesis suppression into a unified hypothesis of tumour dormancy in three experimental models. In addition, in the case of dormant tumours the vast majority of cells may lack the angiogenic phenotype. Therefore, the reawakening of dormant cells may be explained by events leading to deregulated apoptosis, localised increases in survival or growth factors or induced neovascularisation.

1.15 Cancer treatment

Over the past couple of decades significant progress has been made in the treatment of most forms of cancer. These treatments have led to improvements in the survival of patients and consequent reductions in mortality rates. In the case of breast cancer, as with most other cancers, the choice of systemic treatment depends on the tumour characteristics and stage of the disease. Additional factors such as the patients age, state of health, menopausal status and oestrogen receptor (ER) status also influence the modality of treatment used in breast cancer. At present the main treatment options include surgery, chemotherapy, radiation therapy and endocrine therapy. Traditionally, endocrine therapy has been reserved for post-menopausal women, whereas combination chemotherapy is more commonly used in premenopausal women. Surgical removal of the tumour is the most common treatment for primary disease and is usually followed by adjuvant endocrine, radiation or chemotherapy.

A variety of chemotherapeutic drugs have been shown to induce extensive apoptosis in rapidly proliferating normal cells, lymphoid tissue and tumours. Thus, enhanced apoptosis is responsible for many of the adverse effects associated with chemotherapy such as alopecia, low blood count and infertility. However, induced

apoptosis also occurs within the tumours and this is the ultimate aim of the chemotherapy or radiation therapy. Understanding that anti-cancer regimes mediate their therapeutic effects by triggering apoptosis explains some of the possible mechanisms responsible for failed therapy. Apoptosis is a tightly regulated process that is capable of being inhibited or activated. The failure of chemotherapy is due to the fact that in tumour cells the death program is often deregulated as described previously and therefore only a subset of tumour cells are chemosensitive (Kerr, 1994). Thus the development of resistance to chemotherapy is a major clinical problem. Patients presenting with recurrent disease usually have tumours more resistant to chemotherapy than the primary tumour due to natural selection. In order to obtain successful antitumour therapy in all forms of cancer the self destruction mechanisms within tumours have to be activated in the tumour itself or in any remaining cells following radical tumour resection.

1.16 Anti-angiogenic therapy

As angiogenesis is essential for progressive tumour growth it constitutes a very promising therapeutic target. Successfully inhibiting tumour angiogenesis may represent a potential strategy to circumvent the problem of acquired therapeutic resistance to more 'direct' anti-cancer agents caused by the genetic instability of tumour cells (Kerbel, 1991), as endothelial cells are less heterogenous and are therefore more stable targets. Angiogenesis is regulated at a number of checkpoints and can be inhibited by decreasing the levels or effects of angiogenesis factors, increasing levels of angiogenesis inhibitors, inhibiting the action of proteases or by inhibiting endothelial cell proliferation, migration or tube formation. There is considerable evidence suggesting that targeting VEGF and/or its receptors can serve as an effective way of inhibiting

tumour associated angiogenesis (Ferrara *et al.*, 1996; Kim *et al.*, 1993). Injection of tumour bearing mice with neutralising antibodies to VEGF inhibited tumour growth and resulted in the reversion of growing malignancies to dormant micro-colonies (Ferrara *et al.*, 1996; Borgstrom *et al.*, 1996). Other tumour derived angiogenesis factors (TAFs) capable of stimulating angiogenesis *in vivo*, such as bFGF, have also been successfully targeted by using neutralising antibodies (Hori *et al.*, 1991). Many other factors have shown promise as angiogenic inhibitors. Thrombospondin inhibits endothelial cell migration *in vitro*, and therefore can act as an angiogenesis inhibitor (Good *et al.*, 1990). Linomide has been shown to be both cytostatic and inhibitory to endothelial chemotaxis, suggesting that its anti-angiogenic properties may account for its anti-tumour effects observed in mice and rats (Vukanovic *et al.*, 1995). Antibodies to $\alpha_v\beta_3$-integrin inhibited angiogenesis in the CAM assay by inducing apoptosis in endothelial cells (Brooks *et al.*, 1994).

To date more than 200 compounds have been reported to have anti-angiogenic properties (Holmgren and Bicknell, 1997). Two of the most recently identified of these are angiostatin and endostatin. Produced by the cleavage of plasminogen and collagen XVIII respectively, both are endogenous inhibitors of tumour-related angiogenesis selectively inhibiting endothelial cell growth (O'Reilly *et al.*, 1994; O'Reilly *et al.*, 1997). They have been shown to act by increasing apoptosis within endothelial cells, and angiogenesis suppression has been associated with tumour dormancy. Anti-angiogenesis strategies are numerous, reflecting the complexity of the process and the number of levels at which intervention is possible. The list of agents is rapidly expanding and includes agents whose mechanism of action is as yet unidentified. As yet, success with this approach has been limited and confined to the experimental and clinical trial setting. However, it is very likely that anti-angiogenics will form an

integral part of cancer treatment either alone or in combination with more conventional treatments.

1.14 Surgical removal of tumours

Surgery still remains the predominant method of limiting the progression of solid tumour growth. However, at the time of diagnosis, up to 50 % of cancer patients already have metastatic deposits (Fidler and Ellis, 1994). While the surgical procedure may prove beneficial in the removal of macroscopic disease, there is evidence to suggest that the surgery may induce the rapid growth of microscopic deposits (Arai *et al.*, 1992; Kodoma *et al.*, 1992).

Surgical removal of the tumour may be associated with immunosuppression, tumour cell spill or alterations in the physiological balances that govern tumour growth and angiogenesis. Many studies detail suppressive effects of surgery or trauma on host immune function. One such study outlined the effects of surgical operations on the immune system in 35 patients with benign disease. Circulating lymphocyte sub-populations fell significantly following surgery and the magnitude and duration of the reduction was related to the degree of surgical trauma (Lennard *et al.*, 1985). Arai *et al.* (1992) reported modulation of macrophage function following manipulation of primary melanoma in mice. Following the removal of the primary tumour, the growth of pulmonary metastases were enhanced significantly and this was related to a reduction in macrophage cytotoxicity and increases in prostaglandin E2 secretion by macrophages (Arai *et al.*, 1992). It is recognised that patients with advanced malignancy already suffer from depressed immune function as a consequence of their disease (Angevin *et al.*, 1993). Surgical trauma may therefore further deplete an already depressed immune system resulting in ideal conditions for tumour growth (Lennard *et al.*, 1985).

Tumour manipulation *per se* during surgery may increase tumour cell dissemination into the blood stream resulting in the seeding of tumour cells in distant organs and the establishment of secondary deposits directly. In a study of 61 patients undergoing a variety of oncological operations, tumour cells were detected in the blood shed during surgery in 93% of patients, whereas 20% had circulating tumour cells at the end of the surgical procedures (Hansen *et al.*, 1995). Multiple local, systemic, host and tumour-related factors determine the fate of these circulating tumour cells and whether they will ultimately seed and present clinically as metastatic lesions.

The concept of minimally invasive surgery with its associated reduction in inflammatory responses stimulated by minimal trauma appeared to be a promising development. However, more recently, work has suggested that metastasis to wound sites following laparoscopic procedures may present real problems in minimally invasive cancer surgery (Bouvy *et al.*, 1996), whereas other reports suggest no benefit (Lacy *et al.*, 1998). In the first years of laparoscopic surgery, incidences of portal-site metastases as high as 21% were quoted for resection of colorectal cancer, diagnostic laparoscopy for digestive cancer or laparoscopic removal of gall bladders with occult cancer (Wexner and Cohen, 1995). These rates were probably due to poor surgical technique during early experience because more recent studies on larger patient bodies have documented incidences of port-site metastases as low as 2% (Ballantyne, 1995; Lord *et al.*, 1996). It has been suggested that insufflating gas into the peritoneal cavity augments the growth of cancer cells at portal sites. This has been attributed to gas turbulence increasing the transport of cells to the portal site and leakage of gas around the trocar causing a 'chimney effect' and accumulation of cancer cells (Kazemier *et al.*, 1995). However a growing body of evidence suggests that the minimally invasive laparoscopic procedures are associated with significant reductions in post operative

tumour growth (Allendorf *et al.*, 1998; DaCosta *et al.*, 1998). In a study by Allendorf *et al.* (1998), murine mammary carcinomas were more easily established and grew to 1.5 times the size of tumours in open cecectomy groups compared to controls receiving anaesthesia only. This was confirmed in another study by DaCosta *et al.* (1998) in which B16 melanomas grew significantly larger following open laparotomy compared to laparoscopy. These effects were suggested to be mediated at least in part by immune function, however other reports show that open surgery leads to translocation of abdominal bacterial endotoxin (Watson *et al.*, 1995), which has been associated with increased tumour growth previously (Kawai *et al.*, 1993).

In the light of restraints in the development of other therapies, it is likely that surgery will remain the primary treatment modality for most cancers and therefore research developments into its immunological and biological implications for the host may lead to better survival statistics in oncology patients.

1.15 Endotoxin

Septic shock is a clinical condition commonly occurring in post-operative patients in response to severe overwhelming infections with gram-negative organisms such as *E.coli*, *P.aeruginosa*, *Klebsiella* or *Proteus*. Characteristic clinical manifestations of the condition include fever and/or hypothermia, hypotension, cachexia and multiple organ failure, with an associated mortality of greater than 50% (Brun-Buisson, 2000). The poor prognosis associated with septic shock has been attributed to the role of endotoxin, or lipopolysaccharide (LPS).

Endotoxin, or lipopolysaccharide, is a cell wall constituent of most gram-negative, and some gram-positive bacteria that is capable of stimulating a wide variety of cell types including macrophages, polymorphonuclear leukocytes and endothelial

cells (Ulevitch and Tobias, 1994). The stimulation of these cells initiates a cascade of events involving the release of a number of cytokines including TNF-α, IL-1 and IL-6 that mediate a plethora of effects including changes to the micro-vasculature (Rietschel et al., 1994; Ulevitch and Tobias, 1994; Raetz, 1991). The overall result is an uncontrolled amplification of the inflammatory response contributing to gram negative septic shock, characterised by increased vascular permeability (Brandtzaeg *et al.*, 1995) and ultimately leading to organ failure, adult respiratory distress syndrome (ARDS) and death.

Endotoxin from Enterobacteria consist of three structurally similar regions: the O antigen (a highly variable region), a core consisting primarily of heptose and 2-keto-3-deoxyoctane (kdo) (a relatively conserved region) and the lipid A moiety (Raetz *et al.*, 1991). The majority of biological activities associated with LPS have been attributed to the lipid A region. Recent studies have shown it to be the endotoxically active centre, as the deleterious effects observed with LPS are equally elicited by polysaccharide-deprived free lipid A (Galanos *et al.*, 1984). The general structure of lipopolysaccharide is outlined in Fig. 1.3. The initiation of septic shock may be attributed to the binding of lipid A to its receptor (CD14) on the surface of a variety of host cells leading to the production and release of excess immunomodulatory proteins / cytokines. The CD14 receptor is a cell-

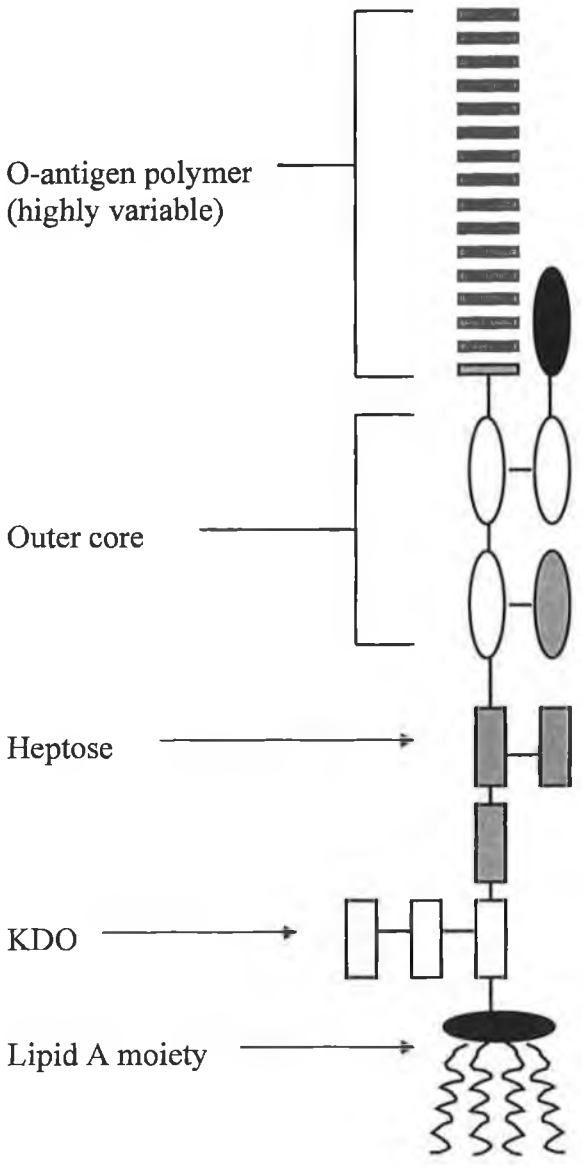

Fig. 1.3 : Schematic representation of the structure of endotoxin / lipopolysaccharide (LPS). [Reproduced from Raetz *et al.*, 1991]

surface, 55-kDa phosphatidylinositol-linked protein on macrophages, monocytes and neutrophils (Schumann *et al.*, 1990; Wright *et al.*, 1990). The receptor recognises and binds complexes formed between LPS and circulating LPS binding protein (LBP) and septins. The LBP forms a complex with LPS and enhances the binding to CD14 manyfold. One molecule of LBP is capable of forming multiple LPS-CD14 complexes, therefore it acts as a lipotransferase (Tobias *et al.*, 1989), meaning even small amounts of LPS are capable of stimulating monocytes / macrophages.

Following binding to CD14, LPS activates a number of protein tyrosine kinases (PTKs) such as p56, lyn, p58 and mitogen-activated protein kinase A (MAPK A) and leads to the mobilisation of the ubiquitous transcription factor NF-κB (Weinstein *et al.*, 1992). This leads to the triggering of a wide range of parallel signalling pathways resulting in the production of a wide variety of cytokines, each capable of binding to different receptors on cell surfaces and stimulating the production of further chemicals and/or cytokines (Sweet and Hume, 1996).

1.16 Role of endotoxin in tumour growth / angiogenesis

Chronic inflammation has been implicated in the pathogenesis of many forms of cancer including gastric cancer following atrophic gastritis (Correa, 1992) and squamous carcinoma in draining sinuses of chronic osteomyelitis (Schiller, 1988). Indeed, an epidemiological study on 2,982 bladder cancer patients in the US reported that a previous history of urinary tract infection was a significant causal factor in their disease (Kantor *et al.*, 1984). More recent studies on rats have taken these obsevations a step further by relating the development of neoplasia with bacterial infections. Infection with heat killed *E.coli* significantly enhanced the growth of N-methyl-N-nitrosourea induced tumours in rats (Yamamoto *et al.*, 1992). This increased growth was characterised by 6-

40 fold increases in the number of tumour nodules and marked capillary proliferation in the tumour stroma. In another study by Davis *et al.*, long term infection with *E.coli* was associated with hyperplastic alterations, dysplasia and early lesions consistent with neoplasia (Davis *et al.*, 1984). Similar results have been reported by other authors, and the enhanced tumorigenesis associated with urinary tract infection has been shown to be due to lipopolysaccharide / endotoxin (Kawai *et al.*, 1993; Johansson *et al.*, 1987). Higher tumour burden in these studies was associated with increased angiogenesis in the tumour stroma, suggesting a possible angiogenic mechanism of action for LPS.

Recent reports have shown that endotoxin is angiogenic, inducing neovascularisation in the rabbit corneal implant model (Li *et al.*, 1991; BenEzra *et al.*, 1993) as well as in the rat mesentery (Mattsby-Baltzer *et al.*, 1994). The latter report concluded that endotoxin mediated neovascularisation could be a component of inflammation and wound healing , as the doses used were realistic in the clinical setting (2ng/ml - 20μg/ml). Endotoxin may mediate its angiogenic activity through the induction of macrophages to secrete cytokines such as TNF-α, IL-1, IL-6 and bFGF, all of which have previously been shown to promote angiogenesis (Austgulen & Nissen-Meyer, 1988; Motro *et al.*, 1990; Fajardo *et al.*, 1992). However, endotoxin can also stimulate human pulmonary artery endothelial cells to produce PDGF mRNA and PDGF-like protein (Albeda *et al.*, 1989). LPS has also been shown to upregulate the expression of VEGF in pulp cells, through a sCD14 mechanism (Matsushita *et al.*, 1999). Therefore, LPS is capable of stimulating the production of multiple cytokines and angiogenic factors that may work alone or in parallel to mediate endotoxin induced angiogenesis.

1.17 Anti-endotoxin strategies

With the recognition that there was an unacceptably high mortality rate associated with sepsis, many anti-endotoxin strategies have been developed to block endotoxins activation of cellular responses. These approaches include neutralising endotoxin before it interacts with the cell receptors, competition for the receptors, inhibition of LPS induced signal transduction and reduction of the inflammatory mediators produced following stimulation. With the realisation of a possible role for endotoxin in both tumour growth and angiogenesis, it is feasible that these strategies could be utilised in the inhibition of endotoxin induced effects on tumour growth.

One molecule that has been given a lot of attention is bactericidal permeability increasing protein (BPI) which is released from polymorphonuclear leukocytes with unique antibacterial activity to specifically kill and permeabilise gram-negative bacteria (Marra *et al.*, 1992). Its specificity results from avid binding of the protein to the LPS constituent of the bacterial wall and it also shares striking homology (45%) to the LPS-binding protein LBP (Abrahamson *et al.*, 1997). This homology suggests that BPI is part of the mechanism whereby the human host regulates its response to bacterial endotoxin. BPI blocks LPS by binding with a high affinity and preventing the LBP-LPS interaction which is required before binding to the CD14 receptor. BPI has been shown to inhibit endotoxin induced TNF-α secretion both *in-vivo* and *in-vitro* in human macrophages (Marra *et al.*, 1992) and to significantly reduce the LD50 in mice (Marra *et al.*, 1994). LPS binding is a property of the N-terminal fragment of BPI and LBP, while the C-terminal region of LBP is required for its interaction with CD14 (Abrahamson *et al.*, 1997). The N-terminal fragment of BPI has been isolated and characterised (Elsbach, 1994) and is up to 30 times more potent than holo-BPI, possessing essentially all of BPI's endotoxin-neutralising activity. The mechanism whereby BPI interferes with LPS mediated effects is outlined in Fig. 1.4.

Another method of neutralising endotoxin is by targeting its active Lipid-A moiety. Recently a new lipid A antagonist termed E5531 has been synthesised that is non-toxic, derived from the lipopolysaccharide of *Rhodobacter capsulatus* (Christ *et al.*, 1995). This compound proved therapeutic in mice, reducing the lethality to *E.coli* under antibiotic treatment. Another lipid A binding murine monoclonal antibody, E5, had been shown to have some promise in humans reducing morbidity and mortality in sepsis patients (Greenman *et al.*, 1991; Greenberg *et al.*, 1992). However, other reports found both HA-1A and E5, two monoclonal antibodies directed against the lipid A moiety, to have unsatisfactory endotoxin-neutralising activity *in vivo* (Baumgartner *et al.*, 1991; Warren *et al.*, 1993; Young *et al.*, 1989). Indeed the latest comparative study comparing E5, HA-1A and BPI reported that neither monoclonal antibody was as effective as BPI at binding or neutralising the effects of LPS *in vitro* or *in vivo* (Marra *et al.*, 1994), and since then E5 has been removed from clinical trials.

As membrane CD14 is required for the myeloid cell responses to LPS it is feasible that a soluble form of the receptor may inhibit LPS activation of cells and therefore thus may be an effective therapeutic target for endotoxin shock. A recombinant form of the soluble receptor, rsCD14, was produced using a Baculovirus expression system and was effective in inhibiting TNF-α release by human mononuclear cells (Haziot *et al.*, 1994). It was also found that mice treated with this rsCD14 and LPS had 100% survival compared to mice receiving saline and LPS (100 μg), in which there was a 60% mortality rate. At present the clinical use of anti-endotoxin agents is still being assessed. Whether any of these agents may prove effective in reducing endotoxin induced angiogenesis and subsequent tumour growth is as yet undetermined.

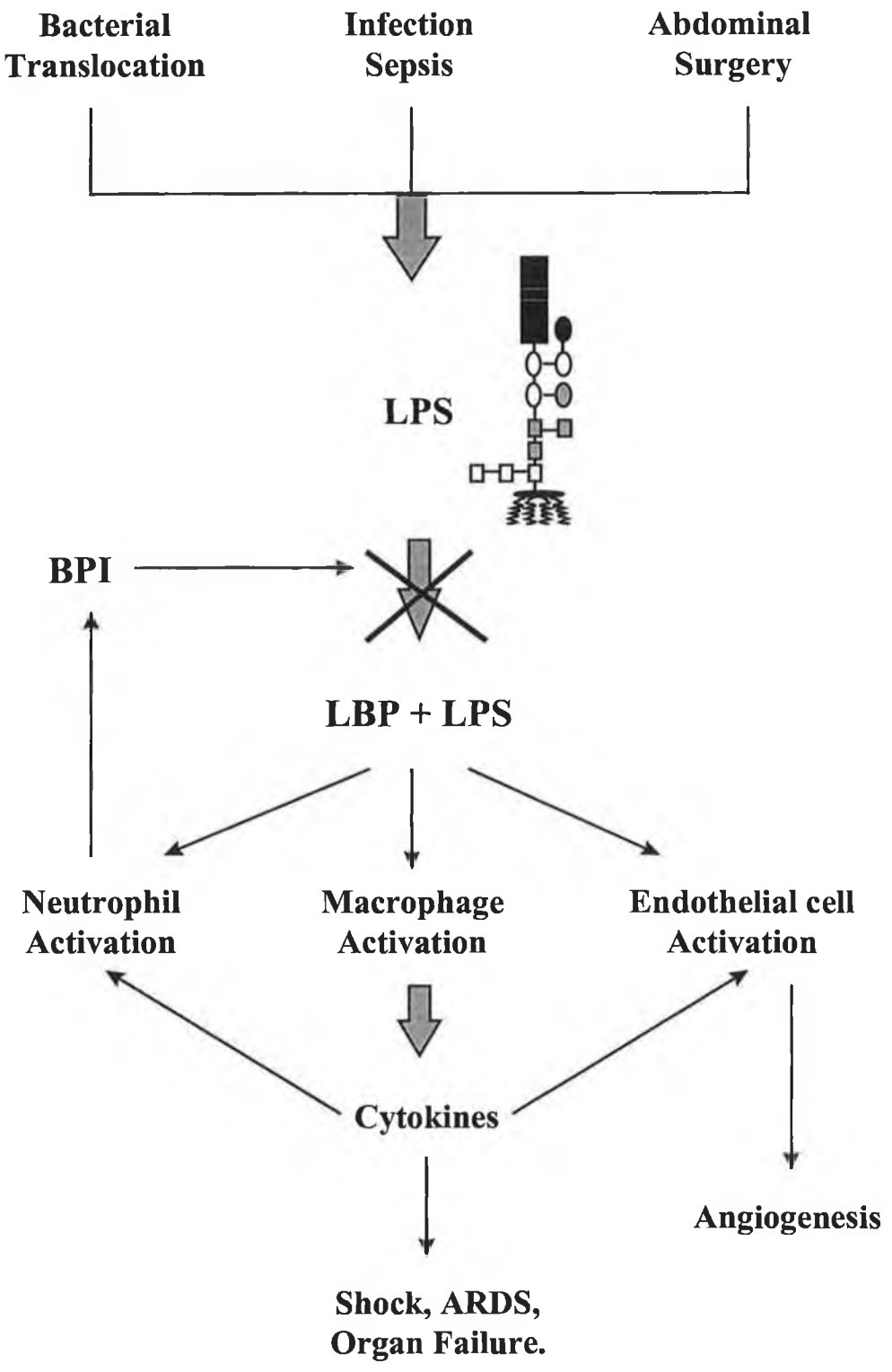

Fig. 1.4 : Mechanism whereby BPI and LBP regulate LPS activity.

1.18 Aim

The surgical removal of a primary tumour is often associated with a subsequent growth of previously dormant metastases. The growth of these tumour deposits is regulated by the balances governing proliferation and apoptosis within the cellular population, as well as those regulating angiogenesis within the metastases. Endotoxin or lipopolysaccharide, a cell wall constituent of gram-negative bacteria, is ubiquitously present in air and may be introduced during surgery. Having previously been shown to induce angiogenesis, endotoxin is a potential angiogenic and metastatic candidate in cancer. Vascular endothelial growth factor is the most potent pro-angiogenic factor identified to date and is regulated by a number of factors. The aims of this thesis were as follows:

- Determination of the effect of surgery on metastatic tumour growth by examining the balances governing their growth and the levels of post-operative serum VEGF and plasma endotoxin in a murine model of experimental metastasis.
- Determination of the effect of endotoxin on metastatic tumour growth and angiogenesis in a murine model, and the examination of the potential therapeutic use of anti-endotoxins to reduce endotoxin- and surgically-induced metastatic tumour growth.
- Elucidating the role of endotoxin in the regulation of VEGF and examination of endotoxin's angiogenic potential in vivo.
- Examination of the role of VEGF and endotoxin as survival factors for tumour cells.

Chapter 2

Materials and Methods

2.1 Preparation and handling of reagents

General lab chemicals were of analytical grade and were purchased from Sigma Chemical Company (St. Louis, Mo, USA) and BDH Chemicals Ltd. (Poole, UK) unless otherwise stated. All chemicals and reagents were stored according to the manufacturers instructions. Solid chemicals were weighed using a Sartorius analytical AC1215 electronic balance (Göttingen, Germany) and prepared in sterile distilled water (SDW) with a conductivity of greater than 18 mΩ. The pH of solutions was measured using a radiometer PHM82 pH meter (Copenhagen, Denmark). Following preparation, solutions were autoclaved within a few hours. Liquid transfer for volumes less than and including 1 ml were carried out using a designated set of Gilson pipettes and for volumes greater than 1 ml an electronic pipette aid was used (Drummond, Broomall, PA, USA) with plastic pipettes (Sarsedt, Wicklow, Ireland).

2.2 Cell Culture

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

2.2.1 Breast Cancer Cell lines

Two breast cancer cell lines were used for the duration of this project. Murine mammary metastatic 4T1 cells were a gift from Mr. E. Coveney (Waterford Regional Hospital, Ireland) and Fred Miller (Duke Univ. Ms, US). Human mammary metastatic MDA-MB-231 (ECACC92020424) cells, isolated from a pleural effusion, were purchased from the European Cell and Culture Collection (ECACC). 4T1 cells were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin sulfate in 25 cm^2 or 75 cm^2 vented 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 Leibovich L-15 medium supplemented with 10% (v/v) heat-inactivated FCS with antibiotics at 37°C. L15 is a CO_2 independent medium and therefore sealed flasks were used. FCS was heat inactivated by incubating at 56°C for 15 min and stored in 50 ml aliquots at -20°C. For experiments requiring cells to be plated in 12, 24 or 96-well plates MDA-MB-231 cells were cultured in Dulbelco's Modified Eagle's Medium (DMEM) with no loss of viability or obvious morphological changes. RPMI, L15, DMEM and FCS were obtained from Gibco-BRL (Paisley, Scotland). Tissue culture flasks were from Sarsedt (Wicklow, Ireland).

2.2.2 Cell Subculture

Cells were examined daily using an inverted phase contrast microscope (Nikon TMS, Tokyo, Japan) and sub-culturing was performed when the cells reached 80-90% confluency. Both cell lines in this study grew in an anchorage dependent manner forming a monolayer

of cells. Cells were detached by trypsinastion for subculturing. Growth medium was decanted and cells were washed with 5 mls of 0.01M phosphate buffered saline (13.8 mM NaCl, 2.7 mM KCl, pH 7.4) (PBS) to remove residual FCS. 1 ml (25 cm^2 flasks) or 2.5 mls (75 cm^2 flasks) 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. Flasks were incubated at 37°C for approximately 5 min when the cells had clearly detached from the flask surface. 10 mls of complete medium (medium containing 10% FCS) was added and cells were transferred to a sterile tube and pelleted by centrifugation (MSE Instruments Mistral 2000, London, UK) at 300 X g for 5 min. Following centrifugation, the supernatant was discarded and the cell pellet resuspended in 5 - 10 ml of complete medium and this suspension was used to seed fresh flasks at a ratio between 1:6 and 1:12. 5 ml or 10 ml of complete medium was added to the cells in 25cm^2 and 75cm^2 flasks respectively. Cells were then maintained as described (2.2.1).

2.2.3 Preparation of frozen cell culture stocks

Cell stocks were stored frozen under liquid nitrogen in a cryofreezer (L'air Liquide RCB40A. Champigny, France). The stocks were prepared from cells growing in log phase at less than 80% confluency. When frozen stocks were being prepared cells were washed with 5 ml PBS and trypsinised as above (2.2.2). Trypsinised cells were layered carefully on a cushion of 2 - 3 ml FCS and pelleted by centrifugation at 300 X g for 5 min. FCS was decanted and the cell pellet was resuspended in 2 ml of FCS. An equal volume of 10% (v/v) dimethyl sulfoxide (DMSO) in FCS was added dropwise to the cell suspension mixing between each addition. 1.5 ml aliquots were added to cryovials and placed in a styrofoam

rack at - 80°C for 24 h. Following this, the vials were transferred to the liquid nitrogen tank for long term storage.

2.2.4 Revival of stored cells

Cryovials were removed from liquid nitrogen and placed at 37°C to thaw rapidly. Cells were transferred to a centrifuge tube and 10 mls of complete medium was added. The cells were pelleted by centrifugation at 300 X g for 5 min. The supernatant was decanted and the pellet of cells resuspended in 7 ml of complete medium and transferred to a 25cm^2 flask. Cells were moved to the incubator and stored at 37°C for 24 h. The medium was replaced the following day to remove any dead cells, and the flasks maintained and passaged as previously described (2.2.2).

2.2.5 Human Umbilical Vein Endothelial Cells

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from fresh human umbilical cords by an experienced technician and used in experiments over a maximum of 4 passages. In brief, fresh cords were thoroughly cleaned using 70% alcohol in a laminar air flow cabinet. A sterile catheter was inserted and the cord was flushed with PBS buffer containing penicillin (250μg/ml), streptomycin sulfate (250μg/ml) and fungizone (0.625μg/ml). Thereafter the cord was filled with 20 ml of 0.1% collagenase I until distended, tied off and incubated at 37°C for 20 min. Following this the cord was massaged lightly to free cells from underlying collagen / smooth muscle layer. This was continued by pinching and stripping down the length of the cord. The contents of the cord were then emptied into a Falcon tube and 20 ml of M199 medium containing 20% FCS was added to

inactivate the collagenase. The suspension was centrifuged at 300 x g for 10 min and resuspended in 5 ml of M199 containing 20% FCS. The cells were transferred to a culture flask coated with gelatin and 150 ml of endothelial cell growth factor (ECGF) was added. Cells were incubated at 37°C in a 5% CO_2 atmosphere and subcultured as described previously, however a 0.1% trypsin solution was used and all plastics were coated in gelatin prior to seeding the HUVECs.

2.2.6 Cell Counting

Suspensions of cells were counted using a Neubauer haemocytometer slide. Viability of cells was examined using a trypan blue dye exclusion assay. 20 µl of the cell suspension was added to 180 µl of trypan blue dye. The mixture was allowed to stand for 1 min and then approximatey 10 µl added to the counting chamber of the slide (enough to fill the compartment fully). Viable cells had an intact cellular membrane and appeared white while dead cells appeared blue due to a damaged cell membrane. The four corner squares of the grid, each $1mm^2$ in size, were counted and the number of viable cells calculated using the following equation:

$$\text{cells / ml} = \frac{No.CellsCounted \times 10,000 \times 10(dilutionfactor)}{4}$$

2.2.7 Mycoplasma testing of cells

When first receiving cell cultures all stocks were examined for mycoplasma contamination. Thereafter all cell lines were routinely examined for mycoplasma every 6 months. A commericial PCR ELISA (Boehringer Mannheim, Mennheim, Germany) was used for

mycoplasma testing according to the manufacturers instructions. However the ELISA part of the test was omitted. In brief, 1 ml of medium from the flask of cells to be tested was taken. Cellular debris was removed by centrifugation at 300 X g for 5 min. The supernatant was removed and mycoplasma were pelleted by centrifugation at 13,000 X g for 10 min. The supernatant was decanted and the pellet was resuspended in 10 µl of sterile distilled water (SDW). A positive control (supplied with the kit) and a negative control (SDW) was also included with each batch of samples. 10 µl of lysis reagent was added and the mixture was incubated for 1 h at 37°C. 30 µl of neutralisation buffer was added per tube. Thereafter 10 µl of this sample was mixed with 25 µl of PCR ready-to-go solution and 10 µl of SDW in a PCR thermo-tube. The tubes were transferred to a PCR thermal cycler machine (PTC 2000 Peltier Thermal Cycler, MJ Research, MA, USA) and the required product was amplified using the following program:

5 min at 95°C, 40 cycles of 95°C for 30 sec, 62°C for 30 sec, 72°C for 1 min and a final extension of 10 min at 72°C. The resulting PCR product was visualised on a 1.2% agarose gel with ethidium bromide staining following electrophoresis (2.3.6).

2.2.8 Isolation of monocytes

30 ml of blood was removed from human volunteers into heparinised tubes (Sarsedt, Wicklow, Ireland). A density gradient was prepared by layering 10 ml Histopaque 1077 carefully over 10 ml Histopaque 1199 (Sigma, St. Louis, Mo, USA). The 30 ml of blood was layered carefully onto the gradient. Cells were separated out by density centrifugation at 400 X g for 30 min. Different layers of cells were clearly visible following centrifugation as seen in figure 2.1. The top plasma layer was removed and filtered through a 0.2 µm

syringe filter (Gelman, Ann Arbor, MI, USA). The layer containing lymphocytes and monocytes was then collected and washed with approximately 30 ml DMEM by centrifugation at 300 X g for 10 min. Following this the cell pellet was washed twice with 30 ml PBS, collecting with centrifugation between washes. After the final wash the cell pellet was resuspended in 2 ml of DMEM and counted (2.2.5). The percentage of monocytes in the cell suspension was determined using Flow Cytometry staining for CD14/CD45 (2.2.10). The cells were finally resuspended at a concentration equivalent to 1 X 10^6 monocytes / ml in DMEM containing 20% (v/v) autologous plasma. Cells were plated into tissue culture plates and monocytes were allowed to adhere by incubating at 37°C for 3 h. Non-adherent lymphocytes were removed by washing twice with sterile PBS. Fresh DMEM containing 20% (v/v) autologous plasma was added to the monocytes.

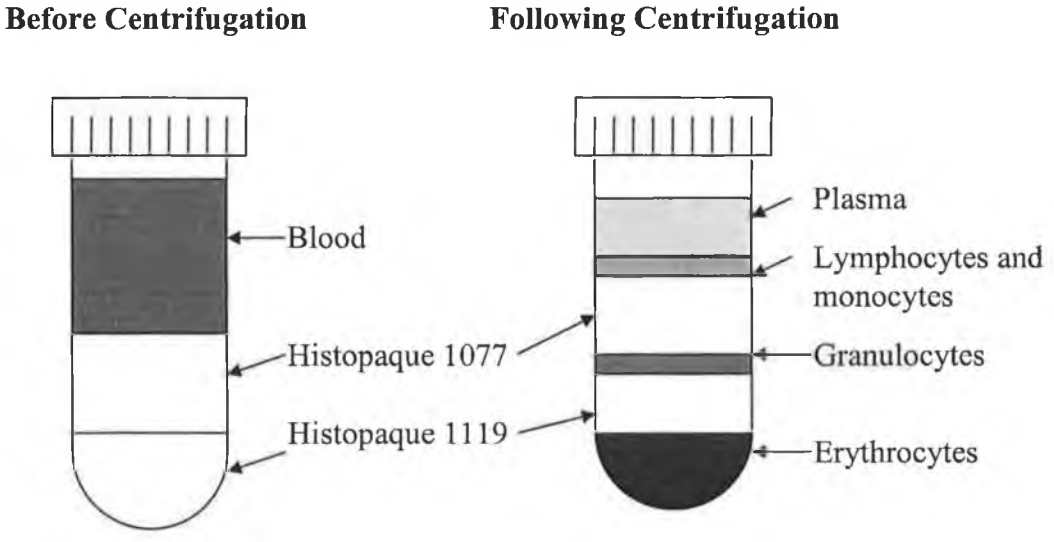

Fig. 2.1: Cell layers formed before and after density centrifugation of whole blood through a Histopaque gradient.

2.2.9 Preparation of monocyte derived macrophages

Monocytes isolated according to the procedure outlined in 2.2.7 were matured for 3 or 5 days in 12-well or 96-well plates respectively in DMEM containing 20% autologous plasma. During this maturation period cells were seen to enlarge. Following this, the cells were washed with sterile PBS and activated for 24 h with 100 ng/ml recombinant interferon γ (rhIFNγ 10^3 units/ml) (Gibco-BRL, Paisley, Scotland) in DMEM with 10% (v/v) FCS. Following activation monocytes appeared to attain spindle like appearance and macrophage like morphology. This method was developed previously in the laboratory (Harmey *et al.*, 1998)

2.2.10 CD14/CD45 Flow cytometry

The percentage of monocytes present in cell samples was determined using flow cytometry counting cells expressing the cell surface receptors CD14 and CD45. CD45 is present on the majority of leukocytes whereas CD14 is a specific marker for monocytes. A 100 µl aliquot of cell suspension (1 x 10^5 cells) was incubated with 10 µl of CD14/CD45 labeled monoclonal antibodies (Becton Dickenson, San Jose, CA, US). The sample was carefully mixed, protected from light exposure with foil and incubated for 30 min at 4°C. Cells were washed with 2 ml of cold PBS and centrifuged at 300 x g for 5 min. Supernatant was carefully removed and cells resuspended in FACS fluid and analysed (FACScan, Becton Dickenson, San Jose, CA, US). The percentage of CD14 positive leukocytes was calculated. A sample dot plot for CD14/CD45 flow cytometric analysis is shown in Fig. 2.2.

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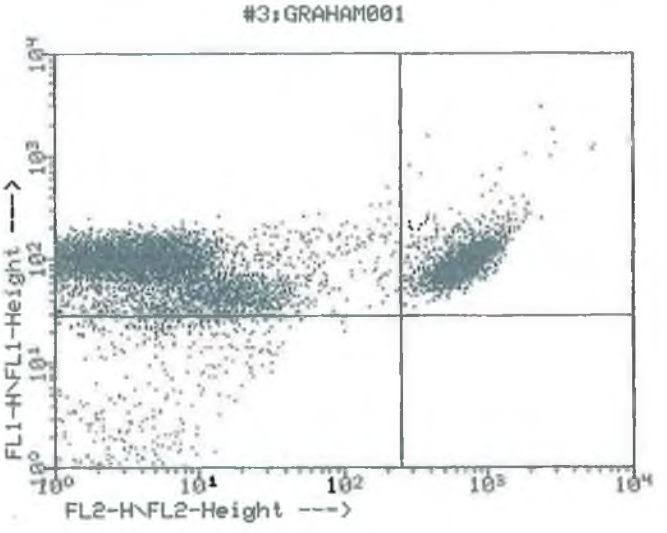

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Quad	Events	% Gated	% Total	Xmean	Ymean
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2 UR	2492	24.92	24.92	701.95	100.84
3 LL	623	6.23	6.23	8.06	12.67
4 LR	0	0.00	0.00	--	--

Fig. 2.2 : Sample dot plot for CD14/CD45 flow cytometric analysis.

2.2.11 Preparation of cells

Cells required for experiments were obtained by trypsinisation and counted. When measuring VEGF protein production, 200 µl of cells were seeded into 96-well plates at a density of 50,000 cells/ml (tumour cells) or 25,000 cells/ml (HUVEC). For proliferation experiments cells were seeded at a concentration of 5,000 cells/ml in 96-well plates. For reverse transcription polymerase chain reaction (RT-PCR) 1 ml of cells at 200,000 cells/well were seeded into 12-well plates. For apoptosis assays, cells were seeded at a concentration of 50,000 cells/chamber. Cells were allowed to recover after trypsinisation for approximately 16 h in RPMI or DMEM with 10% (v/v) FCS. Treatment with lipopolysaccharide or cytokines was carried out by preparing the reagents in the appropriate medium with 10% (v/v) FCS. In the proliferation experiments the lipopolysaccharide was prepared with and without 10% (v/v) FCS for comparison. In the case of VEGF protein analysis medium was removed following the incubation period and stored at -80°C. 100 µl of sterile PBS was added per well and the plates were stored at -80°C for total cell protein determination as described in section 2.4.2.

2.2.12 Proliferation Assays

Cells at a concentration of 5 x 10^3 / well were seeded into 96-well plates in RPMI (4T1) or DMEM (MDA-MB-231 and HUVEC) with 10% (v/v) FCS and incubated for 18 - 24 h. Lipopolysaccharide (Sigma, St. Louis, MO, US) or recombinant bactericidal permeability increasing protein rBPI (Xoma Co., Berkley, CA,US) was added at various concentrations in 100 µl of complete medium and incubated for 48 h. Cell proliferation was assessed using one of two proliferation assays. The first was a Cell Titre 96 Non-Radioactive Cell

Proliferation Assay (Promega, Madison, WI, US) based on cellular metabolism and was used according to manufacturers instructions. In brief, cells were incubated with 15 µl of tetrazolium dye for 4 h and living cells metabolise the tetrazolium dye to a coloured formazan product. 100 µl of solubilisation solution was added to the wells and incubated overnight at 37°C to solubilise the formazan product. Absorbance was measured at 570 nm in a plate reader. The colour and absorbance reading were directly proportional to the number of cells present in the wells. The proliferation of control cells (medium only) was expressed as 100% and proliferation in treated cells expressed as % of control.

The second assay was a BrDU 96 well proliferation assay. The assay was performed according to manufacturers instructions (Boehringer Mannheim, UK). 10 µl of labeling solution was added to each well for 6 h at 37°C. Cells were washed with wash medium containing 10% bovine serum. Cells were then fixed with 200 µl of fixative solution for 30 min at -20°C. Cells were washed again and incubated with 100 µl of nuclease solution for 30 min at 37°C. Thereafter cells were washed again and 100 µl of the anti-BrDU antibody was added for 30 min. Cells were washed and finally 100 µl of peroxidase substrate solution was added for 15-30 min (until strong colour change was apparent). Absorbance was measured at 405 nm with reference wavelength at 490 nm. In a similar way to the first assay, the proliferation of controls was set to 100% and treated cells expressed as % of controls.

2.2.13 Apoptosis assay in culture chamber slides

4T1, MDA-MB-231 or HUVEC cells were trypsinised as outlined in section 2.2.2 and 2.2.5. Cells were counted and a solution of 50,000 cells / ml was prepared in DMEM

containing 10% FCS. 1 ml of this solution was added to each chamber of Falcon glass culture slides (Becton Dickinson, NJ., US). Plastic culture slides were used for 4T1 cells, as these cells did not adhere properly to glass slides. Following 24 h, different treatments were added to the cells prepared in DMEM containing 10% for HUVECs. In the case of the tumour cells the FCS was reduced to 1% to induce a higher rate of apoptosis within the cells and treatments were prepared in this. Cells were incubated for 24 h at 37°C. The medium was then removed and the slides were fixed in 100% acetone. Following this slides were stained using the TUNEL procedure as outlined in section 2.8.2.

2.3 RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

2.3.1 Isolation of total RNA from cultured cell monolayers

As RNA is readily degraded by ubiquitous RNase enzymes a number of precautions were taken when isolating RNA. Gloves, filter-tips and a clean lab coat were used at all times. RNA was isolated from cell cultures growing in 12-well plates. Cells were washed with sterile PBS. 800 µl of TRIzol ® LS (Gibco-BRL, Paisley, Scotland) was added to each well for 5 min at room temperature and transferred to a clean eppendorf tube. 300 µl of chloroform was added per tube. Tubes were vigorously shaken for approximately 20 seconds and allowed to stand at room temperature for 10 min. Phases were separated by centrifugation at 12,000 X g for 15 min (Eppendorf Centrifuge 5417, Hamburg, Germany). Approximately 300 µl of the top aqueous phase was removed to a new eppendorf 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 X g for 10 min. The supernatant was

aspirated and the pellet washed in 1 ml of 70% (v/v) ethanol. RNA was pelleted by centrifugation at 7,500 X g for 5 min. Ethanol was carefully aspirated and the pellet air dried over 2-4 min. Pellets were resuspended in 20 μl of Diethylpyro-carbonate (DEPC) treated water (water treated with 0.01% DEPC overnight and then autoclaved to deactivate DEPC) and immediately placed on ice. The concentration and purity of the RNA was assessed spectrophotometrically as outlined in 2.3.2.

2.3.2 Quantification of RNA

RNA was assessed by measuring the optical densiy at 260 nm, the λmax for nucleic acids, using a Genequant spectrophotometer (Pharmacia, Cambridge, UK). A 40 μg/ml sample of RNA yields an absorbance value of 1 at 260 nm. The purity of RNA was calculated by measuring the ratio of the sample absorbance at 260 nm relative to the absorbance at 280 nm, the λmax for proteins. Pure RNA yields a A260/A280 ratio of 1.6 - 1.8. Lower ratios were an indication of the presence of protein in the samples. Absorbance was measured in a clean quartz cuvette following a 1:500 dilution in SDW.

2.3.3 Reverse Transcription-Polymerase Chain Reaction

RT-PCR was used to examine mRNA levels of VEGF, TGF-β, and bFGF in cells. β-actin, a constitutively expressed gene, was also amplified in each experiment to act as an internal control for cDNA starting quantities and loading differences. The ratio of the target mRNA to β-actin allows semi-quantitative comparison between samples. The conditions used for amplification of VEGF and β-actin have previously been shown by our laboratory to allow semi-quantitative analysis (Harmey *et al.,* 1998).

2.3.4 Reverse Transcription

1 μg of total RNA was used for the reverse transcription reaction. DNase I treatment was carried out to remove any contaminating DNA. 1 μl containing 1 U of DNase I (Gibco-BRL, Paisley, Scotland) was added to 1 μg of total RNA in 10 μl of 1 X DNase buffer in DEPC water and the mixture incubated at room temperature for 15 min. The DNase I was inactivated by adding 1 μl of 25 mM EDTA and heating to 70°C for 10 min. 1μl of random primers (Promega, Madison, WI, US) (500 μg/ml) was added and the samples incubated at 70°C for 10 min and then immediately placed on ice. An RT mastermix was prepared and added to the samples containing the following components: 0.5 μl DEPC water, 0.5 μl RNasin (Promega, Madison, WI, US), 1 μl 5 mM dNTP's (1:1:1:1 dATP, dCTP, cGTP, dTTP) (Promega, Madison, WI, US) (19 U), 4 μl 5X RT buffer (Gibco-BRL, Paisley, Scotland), 2 μl 0.1 M DTT, 1 μl Superscript II□ Reverse Transcriptase (200 U) (Gibco-BRL, Paisley, Scotland). cDNA synthesis was performed for 16 h at 37°C and the cDNA amplified by PCR thereafter or stored at -20°C.

2.3.5 Polymerase Chain Reaction

The PCR 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) (VEGF: Genosys, Cambridge, UK; Bcl-2 and Bax: R&D systems, Kent, UK), 0.5 μl Taq DNA polymerase (2.5U) (Promega, Madison, WI, US) and DEPC water to a final volume of 48.5 μl. Samples were placed in the PCR thermal cycler

(PTC 2000 Peltier Thermal Cycler, MJ Research, MA, US) and amplification was carried out using the appropriate cycle.

The cycle used for the amplification of human VEGF was 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 1.5 min using the following primers:

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 to amplify β-actin was 32 cycles of 94°C for 45 sec, 60°C for 45 sec, and 72°C for 1.5 min using a commercial actin primer mix that amplifies both human and mouse β-actin (Strategene, La Jolla, CA, US) resulting in a 661 bp fragment.

PCR products were visualised on a 1.5% agarose gel as outlined in section 2.3.6.

2.3.6 Agarose gel electrophoresis of DNA

Products were visualised on agarose gels prepared by boiling an appropriate amount of agarose in 50 - 100 ml of 1 X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.0) in a 650 watt microwave. 1.5% (w/v) gels were the most commonly used. The solution was allowed to cool to approximately 60°C (warm to the hand) and then poured into a level gel mold of Easi-E-Cast Gel System (Hybaid, Middlesex, UK). Bubbles were carefully removed and combs placed into the gel and the gel allowed to set. Once set the gel was transferred to the electrophoresis tank filled with 1 X TAE. Samples were prepared by adding a suitable volume of 6 X loading dye (30% (v/v) glycerol, 0.01% (w/v) bromophenol blue) to give a final cocentration of 1 X. Approximately 20 μl of samples or DNA marker was added to each well and the gel run at a constant voltage of 80 V (E-C

Apparatus Corporation EC105, FL, US) until the bromophenol front approached the edge of the gel. Subsequent bands were visualised using a transilluminator (Syngene, Cambridge, UK).

2.4 Protein electrophoresis

2.4.1 Protein isolation from cell cultures

Following treatment, cell monolayers were washed twice with PBS. In the case of samples for analysis of the apoptosis regulating proteins the supernatant was decanted first and centrifuged at 300 x g for 10 min to collect floating (apoptotic) cells. Cells were lysed in 1 ml of lysis buffer prepared as 1 x TNE (5 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM EDTA) containing 0.5% (w/v) SDS, 0.5% (v/v) Triton X-100, 0.005% (w/v) deoxycholic acid and 1 mM phenylmethylsufonyl fluoride (PMSF). Flasks or tubes containing the lysis buffer were kept on ice for 30 min. The lysate was passed through a 20 gauge insulin syringe 10 - 15 times to shear DNA and then boiled for 10 min. Debris was pelleted by centrifugation at 15,000 x g for 10 min. 500 µl of the cleared lysate was added to 3.5 ml methanol and stored at -80°C overnight to precipitate protein. Protein was recovered by ultra centrifugation at 50,000 x g for 30 min at 4°C and resuspended in 2 x Llaemmli buffer (0.03 M Tris-HCl, pH 7.4, 5% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol) at a concentration of 5 µg/µl.

2.4.2 Protein determination

For the determination of total protein in samples the bichinoic acid (BCA) protein assay (Pierce, Rockford, IL, US) was used. The principle behind the assay is that in the presence

of protein Cu^{++} is oxidised to Cu^{+} which reacts with the BCA reagent under alkaline conditions to yield a coloured product. The commercially available kit is provided as two reagents; A, an alkaline bicarbonate solution and B, a copper sulphate solution, which were mixed in a 50:1 ratio prior to use. 200 µl of this working reagent was added to 10 µl of protein standard or sample to be tested in a 96 well plate and incubated at 37°C for 30 min. The absorbance of each well was read at 570 nm (Bio-Tek Microplate Autoreader EL311, VT, US). The protein concentration was determined by extrapolation from a standard curve of known concentrations of BSA between 0 and 1000 µg/ml.

2.4.3 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

A 12% (w/v) gel was routinely used for Western blot analysis. Samples for PAGE were run in the presence of SDS and 2-mercaptoethanol to denature the proteins. Separating gel was prepared by mixing the following solutions.

Separating gel:	12%
acrylamide : bisacrylamide (37.5:1)	9.2 ml
1.875 M Tris-HCl, pH 8.8	4.5 ml
Sterile distilled water (SDW)	8.3 ml
10% (w/v) SDS	176 µl
10% (w/v) ammonium persulphate	120 µl
N,N,N',N'-tetramethyl-ethylenediamine (TEMED)	10 µl

The solutions were mixed and approximately 7 ml were cast in vertical gel plates (Atto Corporation AE-6450 mini-slabs, Tokyo, Japan). A small volume of ethanol was added to

the gel to aid polymerisation by excluding oxygen, removing bubbles and leveling the top of the gel. Once the gel had set the ethanol was poured off and the top of the gel rinsed with distilled water. Following this the stacking gel was prepared and added on top of the resolving gel and 12 well combs inserted.

Stacking gel: 1.7 ml acrylamide:bisacrylamide (37.5:1), 2 ml 0.6 M Tris-HCl, pH 6.8, 6 ml SDW, 100 µl 10% (w/v) SDS, 150 µl 10% (w/v) ammonium persulphate, 10 µl TEMED.

Samples were prepared by mixing an equal volume of sample in 2 X Llaemmli with SDW. Samples were denatured by boiling for 10 min. Protein markers were prepared in a similar way by mixing with 2 X Llaemmli. Samples and markers were added to the wells and electrophoresed at 25 mA / gel in electrode buffer (50 mM Tris base, 384 mM glycine and 0.1% (w/v) SDS) for approximately 1 h 30 min until the dye front approached the bottom of the gel.

2.4.4 Staining with Coomassie brilliant blue

Following electrophoresis gels were stained for protein by immersion in Coomassie brilliant blue (50% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.25% (w/v) Coomassie brilliant blue G250) for 30 min on a shaking table. Gels were destained with 50% (v/v) methanol, 10% (v/v) glacial acetic acid until background cleared and bands were clearly visible.

2.5 Western Blotting

Following electrophoresis gels were overlaid with pre-wetted 0.2 µm nitrocellulose membranes and a layer of Whatman blotting paper. Bubbles were carefully removed and

proteins transferred for 16 h at 80 mA in transfer buffer (0.15 M glycine, 20 mM Tris, 0.1% (w/v) SDS, 20% (v/v) methanol) using a Bio-Rad Trans Blotter (Hercules, CA., USA). During transfer the temperature was maintained at 4°C using a cooling apparatus (Medingen KK5E, Bonn, Germany). Following transfer, membranes were blocked for 1 hour using 5% (w/v) dried milk (Marvel) in TBST (25mM Tris-HCl, pH 7.6, 150mM NaCl, 0.05% (v/v) Tween 20). Thereafter a 1:200 dilution of anti-Bcl-2 (Santa Cruz, CA. USA) was added for 1 hour. Following incubation with the primary antibody the membranes were washed 3 times for 10 min each in TBST. A 1:2000 dilution of anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Dako, Cambridge, UK) was then added to the membrane for 1.5 h with gentle agitation. Following this the membranes were washed six times for 5 min each in TBST. The horseradish peroxidase (HRP) signal was detected using a chemiluminescent substrate (Pierce, Rockford, IL, USA). The working reagent was prepared just prior to use by mixing two solutions in equal volumes and adding to the blot for 5min. Following incubation the solution was poured off and the membrane enclosed in plastic and exposed to X-ray film (Kodak) in a dark room and developed. Exposure times varied between a few seconds to several minutes depending on the protein concentration and age of the antibody used.

2.6 Densitometry

Blots and gels were recorded using a computerised Syngene UV transilluminator and white box in a syngene darkroom with camera. The software utilised was GeneSnap version 2.6. Densitometric analysis was carried out using GeneTools version 2.1 assigning bands as PCR boxed manual areas.

2.7 In-Vivo Experiments

2.7.1 Animals

Male six to eight week old BALB/c mice (Charles River Institute, Margate, Kent, UK) were used throughout the course of the study. The mice were acclimatised for one week before experimentation and caged in groups of five or less in an air conditioned room at ambient temperature of 21-22°C and 50% relative humidity under a 12 h light-dark cycle (lights on at 8am). The animals were housed in a licensed biomedical facility (RCSI Department of Surgery, Beaumont Hospital) and all procedures were carried out under animal licence guidelines of the Ministry of Health, Ireland. Animals had *ad libitum* access to animal chow (W.M. Connolly & Sons Ltd., Kilkenny, Irl.) and water.

2.7.2 Establishment of Pulmonary metastases by tail vein injection

4T1 cells at 80% confluency were removed by trypsinisation as outlined in section 2.2.2. The cells were washed three times in Ca^{++}/Mg^{++} free Phosphate Buffered Saline (PBS), counted (as described in section 2.2.6) and resuspended at a final concentration of 5 X 10^5/ml in PBS. 200 µl of this solution was injected into each mouse via the lateral tail vein following intramuscular administration of 200 µl of 10% (v/v) solution of the anaesthetic and vasodilator Hypnorm™ (Janssen, Buckinghamshire, UK) in the left hind limb. Initially animals were sacrificed at intervals of two days and the lungs removed and processed by histology to examine for visible metastases. The livers and brains of mice were also removed and processed by histology to ensure that no metastases were present in these organs.

2.7.3 Surgical treatment

Two weeks following tumour cell injection mice were randomised into four groups. A control group received the anaesthetic Halothane™ (Rhone-Poulenc Rorer Ltd., Dublin, Irl.) for 30 min with oxygen at a flow rate of 4L/min. A second group underwent laparotomy following the preparation of their abdomen with ethanol. Under anaesthesia a mid-line xiphoid to pubis incision was made with a sharp scissors through the skin, subcutaneous tissues and peritoneum. The peritoneal contents were exposed and agitated for a period of 25 min (replacing the contents at 10 min intervals) before closure (5 min) with a continuous nylon suture (Ethicon, UK) (Figure 2.3a). Animals were returned to their cages thereafter and allowed to recover under a heat lamp.

A third group underwent laparoscopy with sterile carbon dioxide. Under Halothane anaesthesia, after preparation of the abdomen with ethanol, an 18-guage cannula (Venflon, UK) was placed in the epigastrium. A sterile CO_2 pneumoperitoneum was established and maintained for 30 min by passing the CO_2 through a 0.2μm acrodisc PF filter (Gelman sciences, Ann Arbor, MI, USA) at 4-6 mmHg. A second 18-guage cannula was then inserted in the midclavicular line to serve as an outlet port, and the cannula was loosely sealed (Figure 2.3b). This method of trocar insertion was necessary to enable the insertion of the second cannula once the bowel contents were safely separated from the anterior abdominal wall by the pneumoperitoneum. Following this the port-sites were closed by pressure using a sterile metal forceps. Throughout this time the peritoneal cavity was not exposed to any atmospheric air, only to sterile CO_2 used to insufflate the peritoneum.

The final surgical group received laparoscopy with air. The surgical model was identical to that outlined above except for the fact that room air was used to insufflate the peritoneum

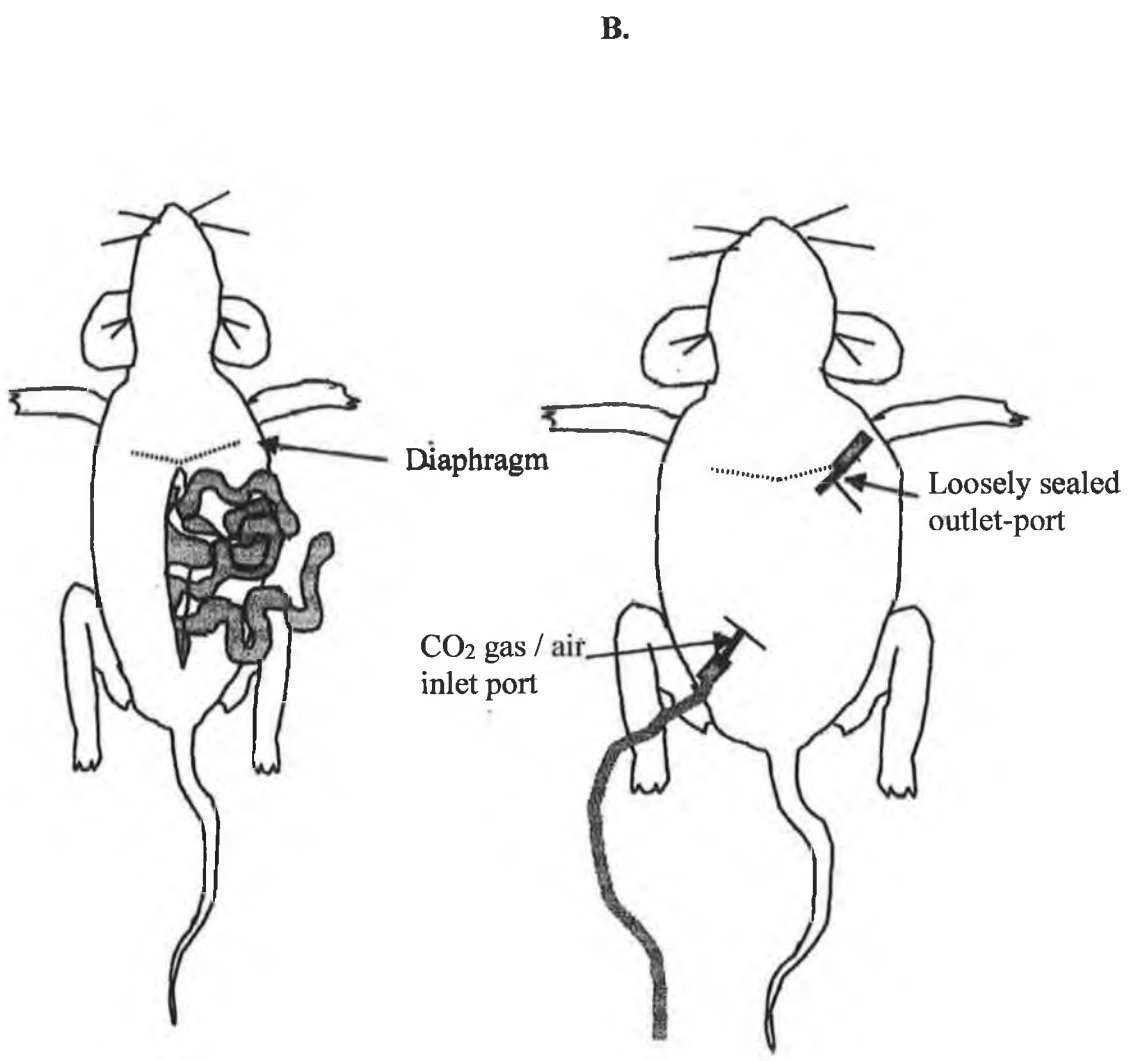

Fig. 2.2 : A. Diagrammatic representation of a mouse undergoing open laparotomy with intestinal agitation. **B**. Diagrammatic representation of a mouse undergoing laparoscopy with sufflation of the peritoneal cavity with either sterile CO_2 gas or room air.

instead of sterile carbon dioxide. In order to deliver the same volume of air a 50 ml syringe was attached to a hypochondrial cannula and the peritoneal cavity insufflated for 30 min with non-sterile room air. Following this time the portal sites were closed as previously described. Following the laparoscopy procedures all animals were returned to their cages and allowed to recover as before under a heat lamp.

2.7.4 Intra-peritoneal injection of mice

Two weeks following tail-vein injection of tumour cells animals were separated into one of two groups. The first group served as a control and received a 200 µl intra-peritoneal injection of sterile saline, while the second group received a 200 µl intra-peritoneal injection of 50 µg/ml (10µg/mouse) commercial LPS derived from *E.coli* (serotype 055:B5 Sigma Corp., Dublin, Irl.) in saline.

2.7.5 Anti-endotoxin intervention

To determine if anti-endotoxin treatment could reduce endotoxin or surgically induced tumour growth the above experiments were repeated in conjuction with a regime of anti-endotoxin treatment. The murine monoclonal antibody E5, recombinant bactericidal permeability increasing protein ($rBPI_{21}$) and thaumatin were a gift from Xoma Corporation, Berkely, CA., US. Animals underwent anaesthesia or laparotomy, saline or endotoxin injection injection as outlined in sections 2.7.3 and 2.7.4 respectively. Animals were then further separated to receive a 200 µl intra-peritoneal injection of 0.4 mg/ml $rBPI_{21}$, E5, thaumatin (a control peptide of 21 KD with no endotoxin neutralising activity) or in the case of controls 200 µl formulation buffer. Animals received further injections of the same

treatment at two hourly intervals for a total of 4 injections and then were allowed to recover overnight. 24 h later the animals received a final injection and were then allowed to recover in their cages.

2.7.6 Blood collection

Mice were anaesthetised with Halothane and their chests cleaned with ethanol. Blood was obtained via closed cardiac puncture by means of a 22-gauge hypodermic needle and a sub-xiphoid approach. Blood was transferred to 1.5 ml capacity serum tubes (Sarstedt, Germany) and allowed to clot for 1-2 h at room temperature. Tubes were centrifuged for 20 min at 1200 X g. Serum was removed, filtered through a 0.22 μm filter and stored in aliquots at -80°C.

For endotoxin analysis, blood was removed as described above 4 h after surgical treatment via closed cardiac puncture. Blood was transferred into pyrogen free Coatex Endo tubes (Chromogenix AB, Mölndal, Sweden). Plasma was separated by centrifugation at 2,200 X g for 15 min and samples were analysed for plasma endotoxin levels immediately using the Limulus Amebocyte Lysate assay (Chromogenix AB, Mölndal, Sweden).

2.7.7 Sacrificing of animals, metastatic burden and tissue processing

Five days after surgical treatment or intra-peritoneal injection the animals were anaesthetised again under Halothane. The animals were weighed and thereafter returned to anaesthetic where blood was removed. Following this the animals were sacrificed by cervical dislocation. The chest cavity was cleaned using ethanol and opened using a sharp scissors. The lungs were carefully excised and weighed immediately. Following this the

lungs were fixed in a 10% formalin solution and processed into paraffin embedded blocks by the histology department. The percentage lung weight : body weight (lung weight/body weight X 100) was used as an indicator of metastatic burden, similar to the method used previously to assess metastatic burden in murine tumour metastases following cytomegalovirus infection (Olson *et al.*, 1980).

2.8 Immunohistochemistry

2.8.1 Preparation of sections from paraffin embedded tissue

Paraffin embedded blocks were cut into 4 micron sections using a Microm HM325 (MSc., Dublin Irl.) microtome. Blocks were cooled on an ice-block and then secured in place in the microtome. The block was brought close to the blade and sections were cut until a full face was taken. A ribbon of approximately 5 to 8 sections was taken at a time and transferred to a clean water bath containing a few drops of ethanol. Sections were taken onto pre-treated glass slides and then wrinkles were removed by dipping the section into a heated water bath (MSc., Dublin, Irl.). The sections were allowed to dry overnight at 37°C and then baked for 3 - 4 hours at 50°C prior to staining.

2.8.2 TUNEL immunohistochemical staining

The number of apoptotic cells within metastases was estimated by labelling apoptotic cells with the TUNEL kit (Boehringer Mannheim, East Sussex, UK). Sections to be stained were re-hydrated by bringing the sections through graded solutions of xylene, 100% ethanol and 70% ethanol, before rinsing in running water. Sections were then treated with proteinase K (20 μg/ml) for 30 min at 37°C. Following this endogenous peroxide activity was blocked by

incubating the slides in a 3% (v/v) solution of hydrogen peroxide. Thereafter ice-cold permeabilisation solution (0.1% (v/v) Triton-X-100, 0.1% (w/v) sodium citrate) was added for 3 min. Following this samples were rinsed in TBS and 50 µl of the working TUNEL reaction mix was added (prepared by mixing two reagents provide in the kit). A negative was included in each batch of samples by adding 50 µl of only one of the working TUNEL mixture components. A cover slip was added to ensure even distribution of the labelling mixture and the slides were incubated at 37°C for 1 hour in a humidified chamber (prepared by placing soaked tissue paper in a sealed plastic tip box below the level at which the slides were kept). Thereafter slides were rinsed with TBS and placed in a TBS bath for 5 min. Following this 50 µl of the converter-POD mixture was added to each section and again a cover slip was added. Slides were returned to the humidified chamber and incubated for a further 1 hour at 37°C. Slides were rinsed again with TBS and placed in a TBS bath for 5 min. Peroxidase activity was visualised by the precipitation of 3, 3' diaminobenzidene (DAB) (Sigma, Dublin, Irl.) for 15 min. Sections were lightly counterstained in haematoxylin, rinsed in running water and then dehydrated by bringing the section through graded alcohol and finally into xylene. Apoptotic cells stained brown against a blue background and were counted using a $1mm^3$ grid counting an average of 500 tumour cells / mm^3 at a magnification x 400. Only cells which stained brown and had the morphological appearance of an apoptotic cell were counted. Necrotic cells were easily distinguished and were not counted. 10 fields were scored and inter-person variation checked by an independent observer. The apoptotic index (AI) was calculated as the mean number of positively stained cells in 10 fields.

2.8.3 CD31 Immunohistochemistry

The extent of angiogenesis within matrigels was estimated by staining gels with anti-CD31 (PECAM) antibody (Santa Cruz, CA., USA). Sections to be stained were re-hydrated by brining the sections through graded solutions of xylene, 100% ethanol and 70% ethanol, before rinsing in running water. Endogenous peroxide activity was blocked by incubating the slides in a 3% (v/v) solution of hydrogen peroxide. Thereafter unspecific binding sites were blocked by the addition of 10% normal goat serum for 30 min. Following this samples were rinsed in TBS and a 1:100 dilution of the goat anti-CD31 primary antibody was applied to the sections for 1 h. A negative was included in each batch of staining by addition of TBS only in place of the primary antibody. Thereafter slides were rinsed with TBS and placed in a TBS bath for 5 min. Following this a 1:100 dilution of rabbit anti-goat biotinylated secondary antibody (Santa Cruz, CA., US) was added for 30 min. Slides were rinsed again with TBS and placed in a TBS bath for 5 min. A 1:100 dilution of strepavidin and biotin (Dako, Dublin, Irl.) in TBS was applied to amplify the signal from the biotinylated antibody. Peroxidase activity was visualised by the precipitation of 3, 3' diaminobenzidene (DAB) (Sigma, Dublin, Irl.) for 15 min. Sections were lightly counterstained in haematoxylin, rinsed in running water and then dehydrated by bringing the section through graded alcohol and finally into xylene. Endothelial cells within matrigels stained brown following CD31 immunostaining and the number of vessels within the matrigels was determined using a $1mm^3$ grid, counting an average of 5 fields.

2.9 VEGF ELISA

VEGF protein levels were determined using ELISA. For human cell culture supernatants a sandwich ELISA that was developed previously in our laboratory was used (Donovan, PhD

thesis, 1999). Plates were prepared by adding 100 µl of the coating antibody (R&D systems, UK, anti-human VEGF neutralising antibody) at a concentration of 0.7 µg/ml in PBS and incubating at room temperature overnight in sealed plates. The following day plates were washed with wash buffer (0.05% (v/v) Tween-20 in PBS, pH 7.4) four times and blocked by incubating with blocking buffer (1% (w/v) BSA and 5% (w/v) sucrose in PSS) for 1 h at room temperature. Following blocking plates were washed again. Samples or standards were prepared in diluent (0.1% (w/v) BSA, 0.05% (v/v) Tween-20 in TBS, pH 7.3) and 100 µl added to the wells for 2 h at room temperature. The plates were mixed gently on a shaking table over this two hour period. Following this the wells were washed 4 times with wash buffer and 100 µl 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 2 h at room temperature with gentle agitation as before. The plate was washed 4 more times and 100 µl of streptavidin HRP (Zymed Laboratories, San Francisco, CA, USA) was added for 30 min at room temperature. Following a further set of 4 washes 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) per well for 40 min at room temperature in the dark. Thereafter the reaction was stopped by adding 50 ml of 0.5 M H_2SO_4. Absorbance was read at 450 nm with a correction wavelength of 570 nm to correct for impurities on the plate. VEGF levels in samples were determined from a standard curve constructed from known standards. A sample standard curve is provided (Appendix 1A).

For murine serum and cell culture samples a mouse-specific commercial ELISA was used in accordance with manufacturers instructions (R&D systems, Abington, UK). In

brief, 50 µl of assay diluent was added to each well, followed by 50 µl of standard or sample prepared in calibrator diluent and the plates incubated for 2 h at room temperature. The contents of the wells were aspirated and the plates washed five times with 400 µl of wash buffer. Following this, 100 µl of prepared conjugate was added per well and incubated at room temperature for further 2 h. Wells were aspirated and washed again as before, and 100 µl of substrate solution was added for 30 min. Thereafter 100 µl of stop solution was added and the absorbance 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. A sample standard curve is provided in the appendix (Appendix 1B).

2.10 Limulus Amebocyte Lysate assay

Plasma endotoxin levels were determined using the limulus amebocyte lysate (LAL) assay according to the manufacturers instructions (Chromogenix AB, Mölndal, Sweden). In brief, samples or standards were diluted 1:10 with LPS free water in sterile falcon plasma tubes in a laminar air flow cabinet. Samples or standards were heated to 75°C in a water bath for 5 min. 50 µl of sample or standard was then added to a 96 well plate, followed by 50 µl of the LAL solution and the plate was incubated for 16 min at 37°C. 100 µl of previously heated (37°C) substrate solution was then added to each well and the plates incubated at 37°C for a further 10 min. Following this 100 µl of 20% acetic acid was added to each well to stop the reaction and the plates were read at 405 nm single wavelength. A standard curve was constructed from known standards and plasma endotoxin levels in samples read from the curve. A sample standard curve is provided in the appendix (Appendix 1C).

2.11 In-Vivo angiogenesis assay

2.11.1 Matrigel preparation and implantation

Matrigel basement membrane matrix (Becton Dickinson, MA, US) is a solubilised basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, containing laminin, collagen IV, heparin sulfate proteoglycans and many growth factors occurring naturally in the EHS tumour. It was prepared for use by placing at 4°C overnight at which it became liquid. In a laminar air flow cabinet different solutions of matrigel were prepared in falcon tubes held on ice. 100 ng/ml VEGF, 10 μg/ml LPS or saline control solutions were prepared in matrigel. 500 μl of each of these solutions was drawn into 1 ml insulin syringes and the syringes laid flat on crushed ice to ensure the matrigel did not set.

Mice were anaesthetised as outlined in section 2.7.3 and placed on their stomachs. Using a clean metal forceps the skin above their left hind limb was lifted and 500 μl of the prepared matrigel solution was injected slowly in one continuous motion. Following injection the gel solidified rapidly and a clear implant was visible to the left side of each mouse. Mice were returned to their cages and allowed to recover.

2.11.2 Extraction and processing of matrigels

5 days after matrigel implantation mice were sacrificed by cervical dislocation. Matrigels were located and an incision was made to the right of the implant with clean sterile metal scissors. The whole matrigel implant along with skin was carefully removed by cutting around the implant initially and then trimming the appropriate perimeter. Skin was pinned to small pieces of cork and the implants and skin were floated (implant down) in 10%

formalin solution overnight, before being processed into paraffin embedded blocks for immunohistochemistry.

2.12 Statistical analysis

Data is graphically represented as mean ± standard error of mean (SEM). Normal probability plots were prepared to determine if the data was normally distributed. For normally distributed data, statistical significance was determined using students t-test or ANOVA for samples comparing more than 2 groups. A Scheffe post-hoc test was used with the ANOVA to determine differences between the groups. Data that was not normally distributed was analysed 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 correlation for normally distributed data, or a Spearman rank for abnormally distributed data. Data was taken to be significant where $p<0.05$. All data was analysed using DataDesk 5.1 for a Macintosh computer (Data Description Inc., Itheca, NY, US).

Chapter 3

Effect of Surgery on Metastatic Tumour Growth

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3.1 Introduction

Rapid growth of previously dormant metastases following the surgical removal of a primary tumour is well documented (Arai *et al*, 1992; Kodama *et al*, 1992). A generalised state of immune-suppression prevails in the postoperative period. While tumour resection may be the only curative measure available to some patients, the procedure itself may compromise host defences and therefore create a permissive environment for the seeding and growth of any circulating tumour cells or micrometastases remaining after the surgery.

Surgical trauma modulates immune cell populations and their function, and may influence metastasis directly by mechanical spillage of tumour cells during resection. Recently, however, there is growing evidence to suggest that the removal of a primary tumour alters the balances governing tumour growth and angiogenesis. In animal tumour models, primary tumours have been shown to inhibit the growth of their own metastases by the secretion of angiogenesis inhibitors, such as angiostatin and endostatin, formed by enzymatic cleavage of plasminogen and collagen XVIII, respectively (O'Reilly *et al*, 1994; O'Reilly *et al*, 1997). Within five days of removal of the primary tumour these angiogenesis inhibitors disappear from the circulation permitting new vessel growth within previously dormant metastases.

A universal factor associated with surgery is the healing wound. Clinical experience has shown that the healing wound provides a favourable environment for metastatic tumour growth (Abramovitch *et al.*, 1998; Bogden *et al.*, 1997, Skipper *et al.*, 1988). It has been reported in rats that when radiolabelled MC28 sarcoma cells reach a healing colonic anastamosis or laparotomy wound within 2 hours of its formation, there is a 1000 fold increase in the probability of the cells forming a viable metastatic deposit

compared to normal tissue (Skipper *et al.*, 1988). Following surgical trauma the cytokine environment within the body is altered to influence the healing process and limit infection (Reid *et al.*, 1996). However, this environment can facilitate tumour recurrence by promoting a pro-angiogenic climate altering the equilibrium which would otherwise maintain micrometastases in a state of dormancy (Holmgren *et al*, 1995). Vascular endothelial growth factor (VEGF) is one possible mediator of tumour recurrence following surgery as it is essential for wound healing but may encourage the growth of micrometastases via its potent angiogenic activity. There are a limited number of clinical studies demonstrating elevated VEGF in the post-operative period in both cancer and non-cancer patients (Harmey, un-published observations).

In addition to removing angiogenic inhibitors, depressing immune function and altering the cytokine balance, surgery is also associated with the introduction of factors that may promote tumour growth. Endotoxin or lipopolysaccharide (LPS) is a cell wall constituent of gram negative bacteria that is present ubiquitously in the atmosphere at concentrations of approximately 1 $\mu g/m^3$ (Rylander *et al*, 1989). Another major source of endotoxin is derived from endogenous gut bacteria and significant translocation of endotoxin into the peritoneal cavity and systemic circulation following open surgery or laparoscopy with air-sufflation has been reported (Watson *et al*, 1995). Endotoxin is angiogenic at clinically relevant doses, inducing angiogenesis in a number of experimental animal models (Li *et al*, 1991, Mattsby-Baltzer *et al*, 1994; BenEzra *et al*, 1993).

The aim of this chapter was to investigate the effect of surgical procedures on metastatic tumour growth in a murine experimental metastasis model where no primary tumour was established. In this model, alterations in tumour growth can not be attributed to the removal of angiogenesis inhibitors, such as angiostatin and endostatin derived

from primary tumours. The effects of open surgery on metastatic growth was compared with a minimally invasive laparoscopic technique performed with either sterile CO_2, containing no endotoxin, or normal room air containing ubiquitous endotoxin. The numbers of proliferating and apoptotic tumour cells were assessed within metastases as these are the ultimate processes governing tumour expansion. Circulating levels of VEGF were measured to determine if an angiogenic mechanism was activated by the surgical procedure. Plasma endotoxin levels were also examined following the different surgical procedures and compared to VEGF to examine the relationship between levels of endotoxin and circulating VEGF.

3.2 Results

3.2.1 4T1 cell line

Figure 3.1 is a representative photograph of cultured 4T1 cells. The 4T1 cell line is a 6-thioguanine-resistant variant of 410.4, a spontaneously arising mammary adenocarcinoma from BALB/c mice. This tumour shares many characteristics with human mammary cancers, closely mimicking their immunogenicity, metastatic properties, and growth characteristics (Pulaski *et al*, 1998). The 4T1 cell line spontaneously metastasises to the lungs early, and the liver and brain later, as indicated by the formation of visible nodules in these organs in mice bearing subcutaneous tumours.

3.2.2 Mycoplasma testing of cell cultures

Mycoplasma is a very common infection contaminating cultured cell lines. All cell lines used in the laboratory were examined for mycoplasma contamination on arrival and every six months thereafter as described (2.2.7). Any cell lines testing positive for mycoplasma were discarded. Culture supernatants were centrifuged to pellet mycoplasma, the resuspended pellet was lysed and mycoplasma DNA amplified by PCR. Figure 3.2 shows a representative 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 template) and a positive control (mycoplasma DNA) were included in each batch of samples to be tested for PCR. No amplified product is visible in the negative control (lane 2) indicating that no contamination of samples occurred. A 660 bp PCR product was amplified from the positive control (lane 3). Smearing of the sample indicates some degradation of the template and is likely to be

due to repeated freeze-thawing of the control DNA. Mycoplasma specific PCR reactions for 4T1 cells are shown in lanes 4 and 5, and MDA-MB-231 cells in lane 6. The absence of any amplified PCR product indicates that these samples are free from mycoplasma contamination. Lane 7 shows a PCR reaction for a cell line obtained from an outside laboratory. It is clear that this cell line was contaminated with mycoplasma and therefore was discarded.

3.2.3 Development of experimental metastasis model

An initial pilot study was carried out to establish the growth kinetics of 4T1 cells in BALB/c mice. 10^5 4T1 cells were injected into the lateral tail vein of BALB/c mice as a single cell suspension in Ca^{2+} and Mg^{2+} free PBS. 7 days later mice were sacrificed (n=3/ timepoint) to examine organ metastases and every second day thereafter. Lungs were removed and weighed and a lung : body weight index recorded as outlined (2.7.7). The liver and brain of any mice containing visible tumour nodules in the lungs was also removed and all organs were examined by histology for the presence of micro-metastases. Fig 3.3 shows the lung : body weight index over time following the tail-vein injection of the 4T1 cells. There is no significant change in lung : body weight index over the first two weeks following tumour injection. At two weeks small nodules were visible in the lungs, and by day 19 these nodules were large enough (minimum 500 tumour cells) to allow histological examination of their mitotic and apoptotic indices. By day 19 there was a significant ($p<0.05$) increase in metastatic burden (0.926 ± 0.03) as indicated by lung : body weight index relative to day 0 (0.592 ± 0.051). Figure 3.4 shows representative H + E stained sections from the lungs (3.4.A), liver (3.4.B) and brain (3.4.C) of mice on day 19. No visible metastases were recorded in the liver or brain of any mice at this time.

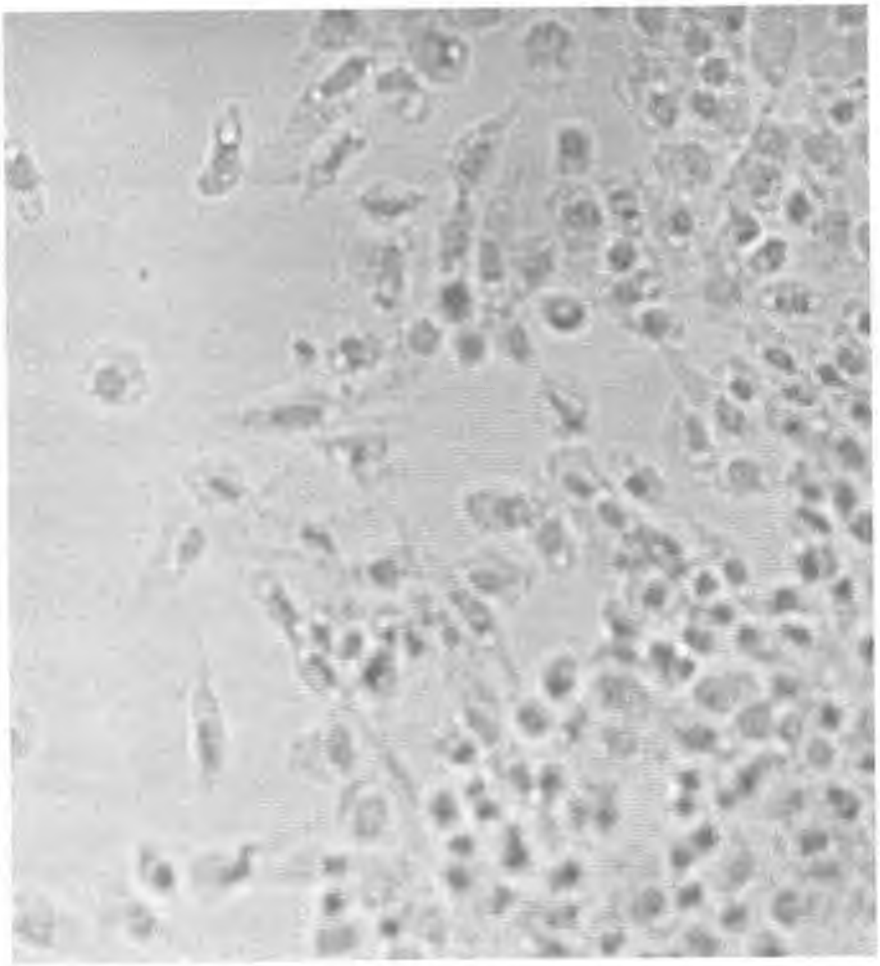


Fig. 3.1: Monolayer culture of 4T1 cells.

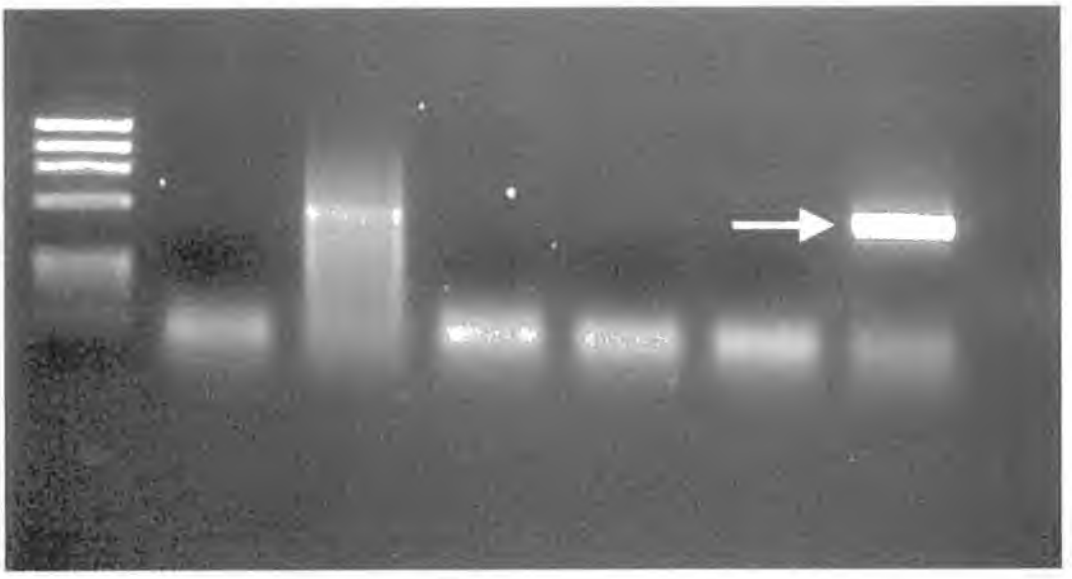


Fig 3.2: Mycoplasma testing of cell lines. Mycoplasma DNA was amplified by PCR. PCR products were resolved on a 1.5% (w/v) agarose gel and photographed under UV light. Lane 1 shows molecular weight markers, ϕX174 digested with *Hae*III. Lanes 2 and 3 show negative and positive controls, respectively. Lanes 4 - 6 contain samples from two 4T1 samples and MDA-MB-231 cells, respectively. Lane 7 shows a mycoplasma positive sample. A mycoplasma specific band of 660 bp is indicated.

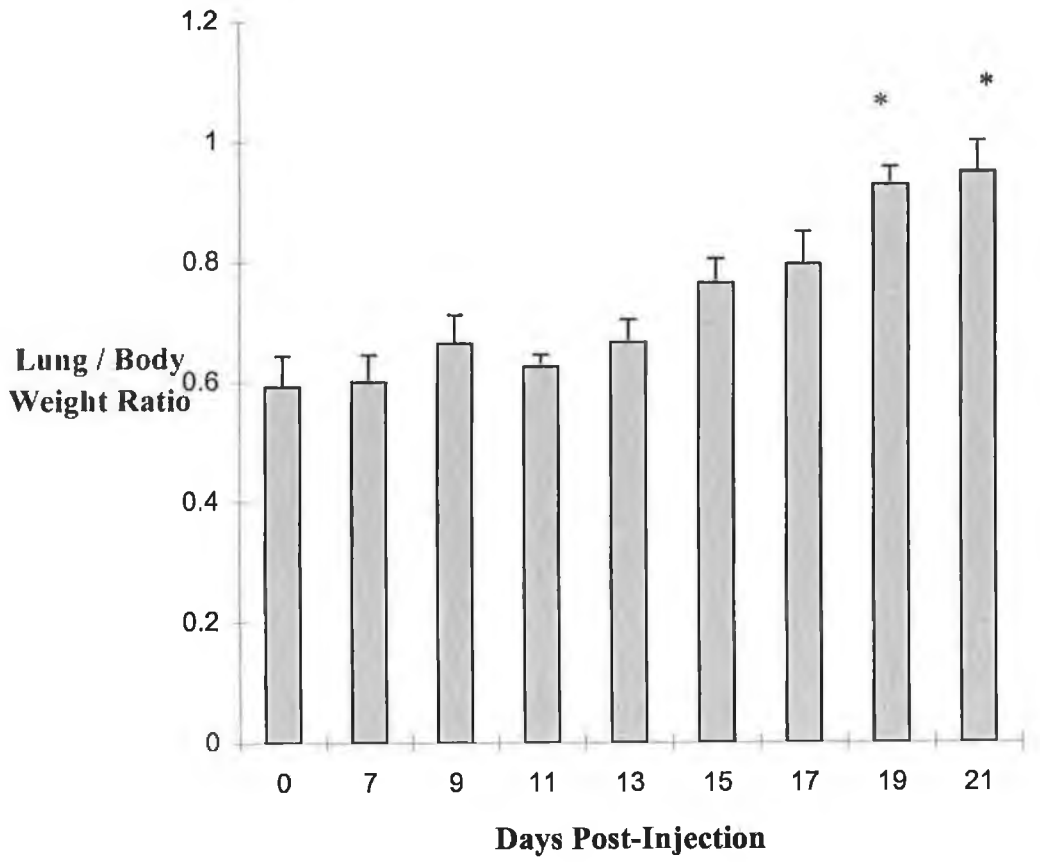


Fig 3.3: Metastatic burden in mice following tail vein injection of 4T1 tumour cells. 10^5 4T1 mammary adenocarcinoma cells were injected and animals sacrificed at the time points shown and lungs were removed and weighed. Tumour burden is expressed as lung weight/body weight x 100 (mean ± S.E.M., n=3). Statistical analysis was by ANOVA, with Scheffe post-hoc correction * ($p<0.05$ vs day 0) .

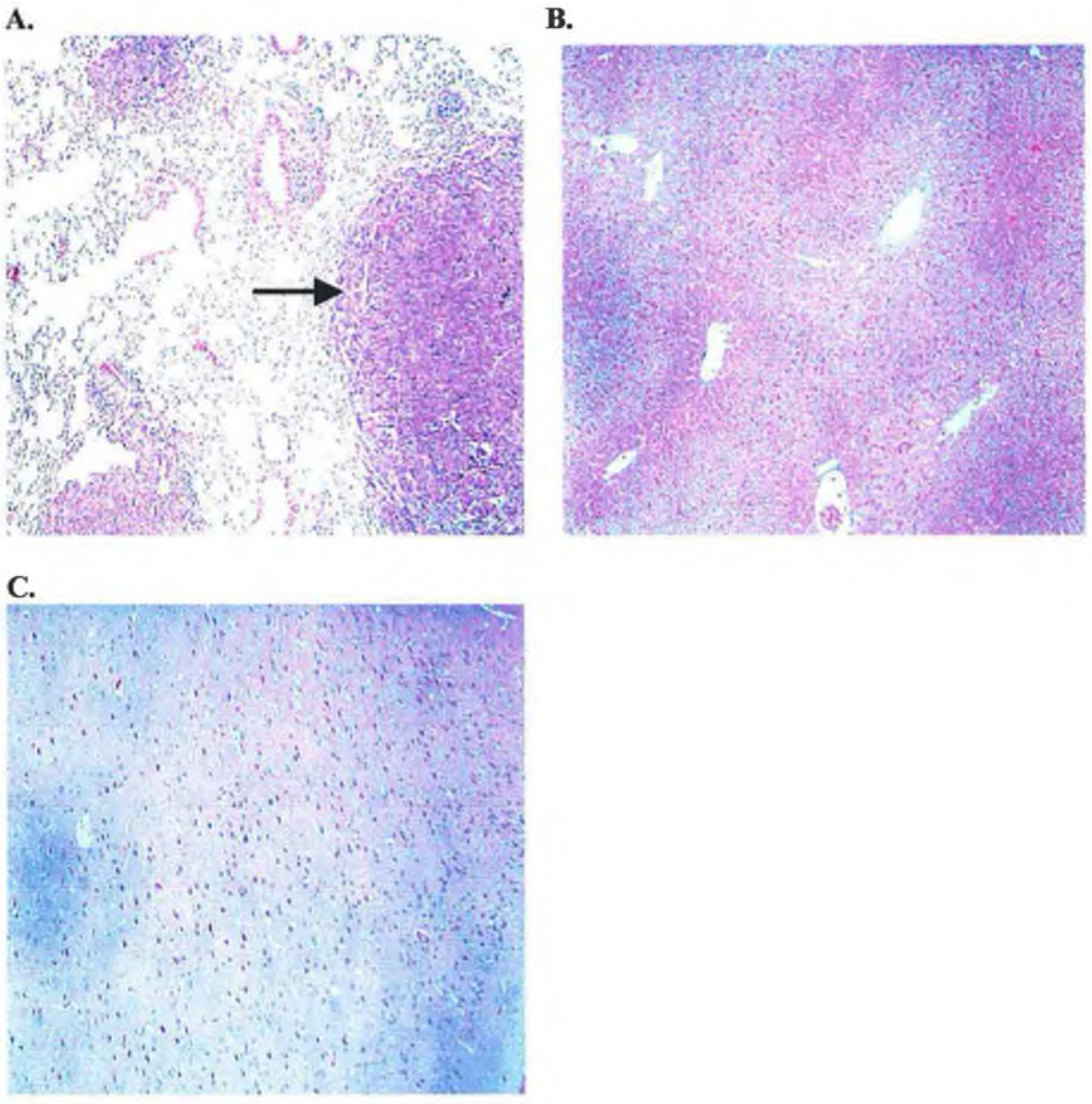


Fig 3.4: Histological sections of lung (A), liver (B) and brain (C) tissue of mice 19 days after tail-vein injection of 10^5 4T1 cells. Sections were stained with haematoxylin and eosin (original magnification X 200). Metastatic deposits containing at least 500 tumour cells are noted by an arrow in the lung section (a).

3.2.4 Effect of surgery on metastatic tumour burden

The effect of different surgical procedures on metastatic tumour growth was examined. 10^5 4T1 cells were injected into the lateral tail vein of mice as a single cell suspension in Ca^{2+} and Mg^{2+} free PBS. Two weeks later the mice underwent a designated surgical procedure (n=8/group). 5 days later animals were sacrificed, lungs removed and weighed and a lung : body weight index recorded as outlined (2.7.7). There was no significant difference between body weights in any of the groups (Fig. 3.5), indicating that the animals were not cachectic (p=n.s.) and no apparent oedema was observed. Animals undergoing either laparotomy (3.37±0.14) or air laparoscopy (2.73±0.11) had significantly ($p<0.001$) higher metastatic burden than anaesthetic controls (1.32±0.12). Animals undergoing CO_2 laparoscopy, where air is excluded had a metastatic burden similar to controls (1.94±0.42, p=n.s.) (Fig. 3.6).

3.2.5 Effect of surgery on mitosis and apoptosis within lung metastases

As it is ultimately the balance between proliferating cells and dying cells within metastases which govern their growth, the mitotic and apoptotic index within the metastases was determined. Similar trends were observed to those seen for metastatic burden. Figure 3.7A and 3.7B are representative sections showing mitotic and apoptotic cells, respectively. Animals undergoing either laparotomy (8.33±0.21) or air laparoscopy (7.99±0.09) had significantly ($p<0.001$) higher mitotic indices than anaesthetic controls (4.39±0.63) or CO_2 laparoscopy (4.71±0.15) groups (Fig. 3.8). Laparotomy (1.31±0.13) or laparoscopy with air (1.33±0.42) resulted in significantly ($p<0.01$) lower apoptotic indices compared with controls (4.46±1.13). In contrast, mitotic (4.39±0.63) and apoptotic (3.17±0.76) indices in animals undergoing laparoscopy with CO_2 were similar to controls (p=n.s.) (Fig. 3.8 and 3.9, respectively).

As net tumour growth is determined by the relative numbers of proliferating and apoptotic tumour cells, the ratio of mitosis : apoptosis, MI/AI was calculated as described in section 2.8. Laparotomy (6.20±0.35) or laparoscopy with air (6.35±1.00) resulted in significantly ($p<0.01$) higher ratios compared with either CO_2 laparoscopy (1.54±0.19) or control (1.02±0.15) groups (Figure 3.10).

3.2.6 Effect of surgery on plasma endotoxin levels

It has previously been reported that surgery results in the translocation of endotoxin, across the gut into the abdominal cavity and systemic circulation (Watson *et al*, 1995). In that study, the levels of endotoxin peaked at 4 hours post-operatively. The levels of plasma endotoxin were therefore measured 4 hours post surgery using the LAL assay, as described in section 2.10. Laparotomy (1.22±0.18 EU/ml) and air laparoscopy (0.50±0.15 EU/ml) groups had significantly higher levels of circulating LPS than controls (0.13±0.19 EU/ml). Animals which underwent laparoscopy with air exclusion (CO_2 laparoscopy) had levels of LPS comparable to controls (0.19±0.14 EU/ml, p=n.s. vs control). (Fig. 3.11)

3.2.7 Effect of surgery on serum VEGF levels

The levels of serum VEGF were measured by ELISA at 5 days after surgery, at the time of sacrifice. Laparotomy (77.92±5.00 pg/ml) and air laparoscopy (61.97±7.14 pg/ml) groups had significantly higher post-operative levels of circulating VEGF compared with either control (14.28±5.44 pg/ml) or CO_2 laparoscopy groups (28.41±13.70 pg/ml) (Fig. 3.12). No significant difference was observed between the control and CO_2 laparoscopy group (p=n.s.). The presence of high levels of this pro-angiogenic factor in the circulation following surgery suggests that an angiogenic mechanism may be

involved in the enhanced metastatic tumour growth observed in the surgical groups exposed to air. The relationship between serum VEGF at the time of sacrifice and the levels of circulating endotoxin in the immediate post-operative period were examined. A significant positive correlation (Pearson Product Moment, r=0.966) was observed between circulating VEGF and LPS levels, when all surgical groups were examined in parallel (Fig. 3.13).

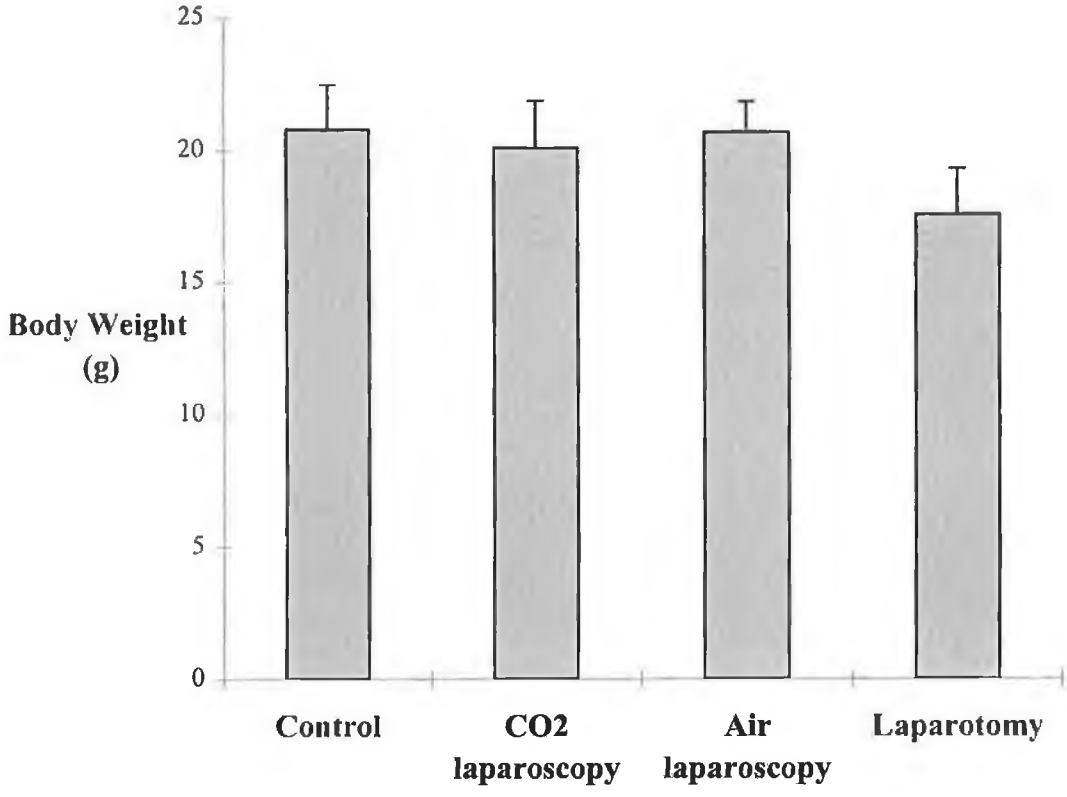


Fig. 3.5: Body weights of mice following surgery. Surgery was performed two weeks after tail-vein injection of 10^5 4T1 mammary adenocarcinoma cells. 5 days later animals were weighed before sacrifice. Statistical analysis was by ANOVA, with Scheffe post-hoc correction (mean ± S.E.M., n=8/group). No significant differences in body weights was observed between any of the groups (p=n.s.).

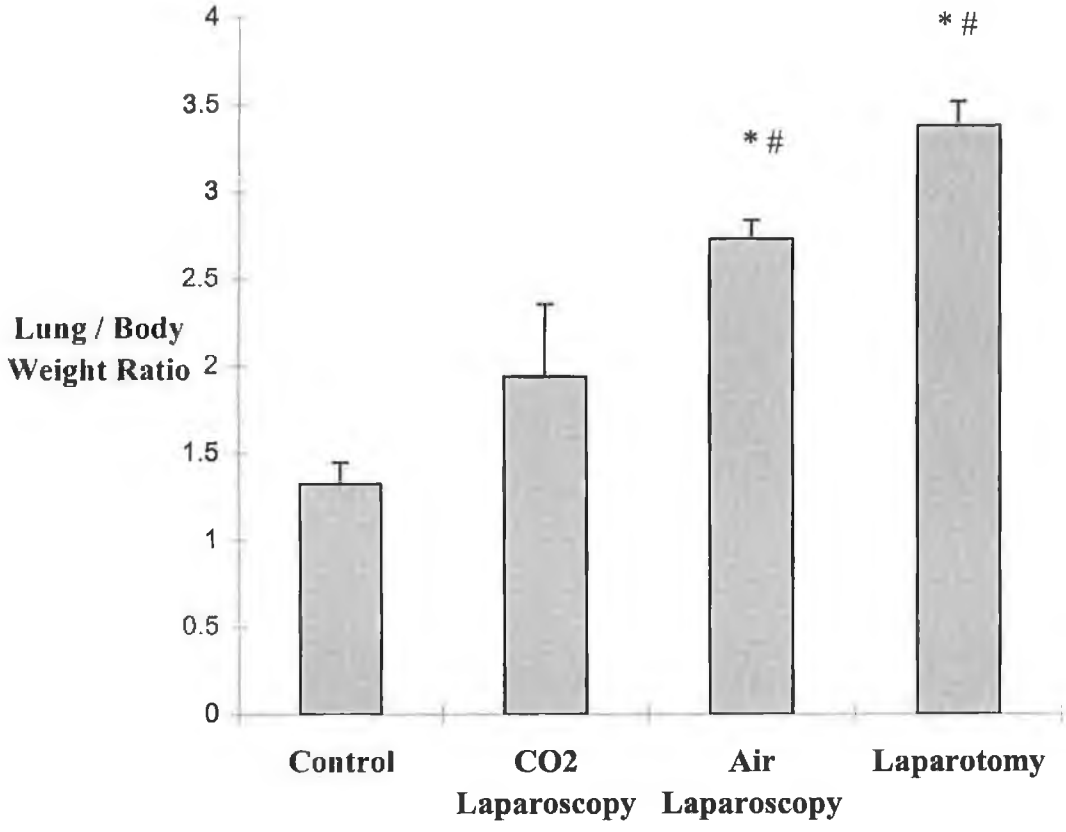
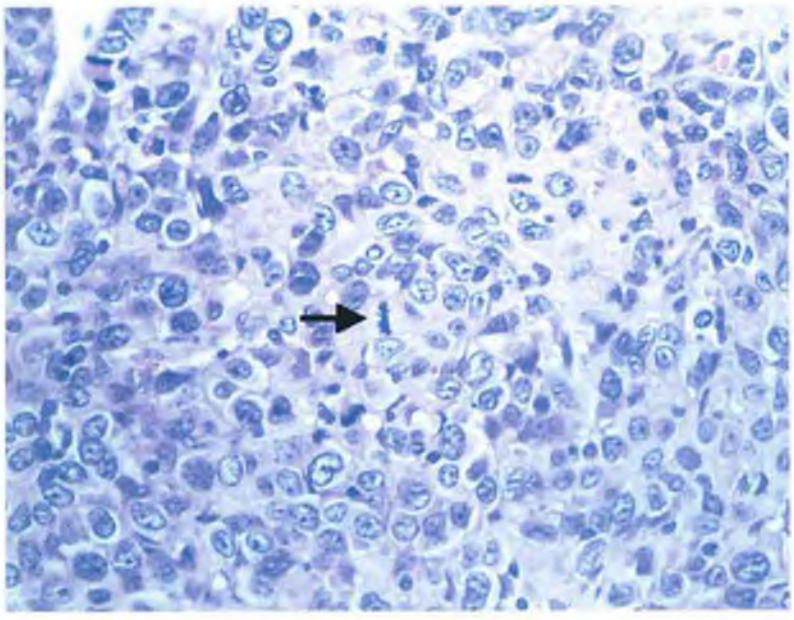


Fig. 3.6: Metastatic burden in mice following surgery. Surgical procedures were performed two weeks after tail-vein injection of 10^5 4T1 mammary adenocarcinoma cells. 5 days later animals were sacrificed and lungs removed and weighed. Tumour burden is expressed as lung weight/body weight x 100 (mean ± S.E.M., n=8/group). Statistical analysis was by ANOVA, with Scheffe post-hoc correction. A significant increase in metastatic growth was observed following laparotomy and air laparoscopy compared to controls * ($p<0.0001$) or CO_2 laparoscopy # ($p<0.001$).

A.



B.

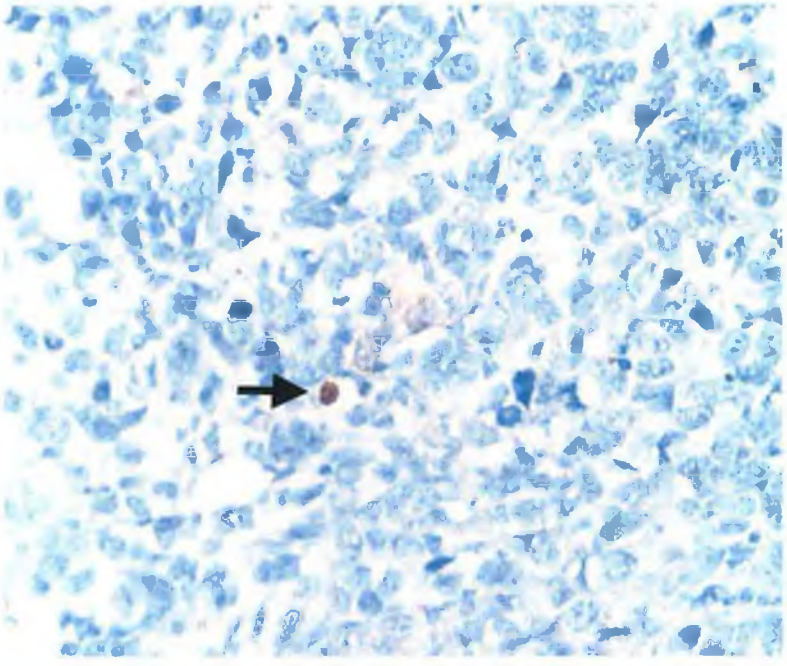


Fig. 3.7: Mitosis and apoptosis within lung metastases following surgery. **A.** Mitotic cells in lung metastases. Arrow indicates cell during metaphase with linear chromosome alignment (original magnification X 400). **B.** Apoptotic cells in lung metastases. Arrow indicates apoptotic cell following TUNEL staining as described in section 2.8.2 (original magnification X 400).

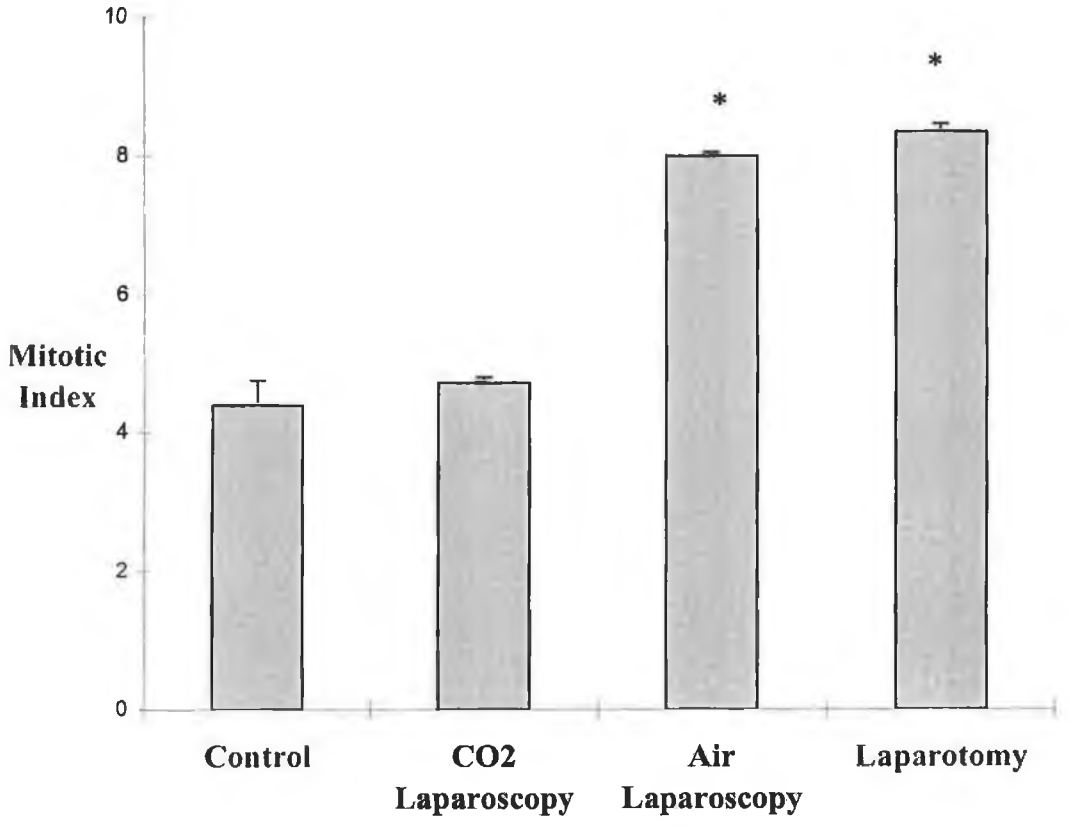


Fig. 3.8: Mitotic index within metastases following surgery. Surgical procedures were performed two weeks after tail-vein injection of 10^5 4T1 cells. 5 days later animals were sacrificed, lungs were removed and processed for histology. Mitotic index is expressed as the average number of mitotic cells counted over 10 fields in a 1 mm^3 grid, counting an average of 500 cells per grid (mean ± S.E.M., n=8/group). Statistical analysis was by ANOVA, with Scheffe post-hoc correction. A significant increase in mitotic indices was observed following laparotomy and air laparoscopy compared to controls or CO_2 laparoscopy *($p<0.001$).

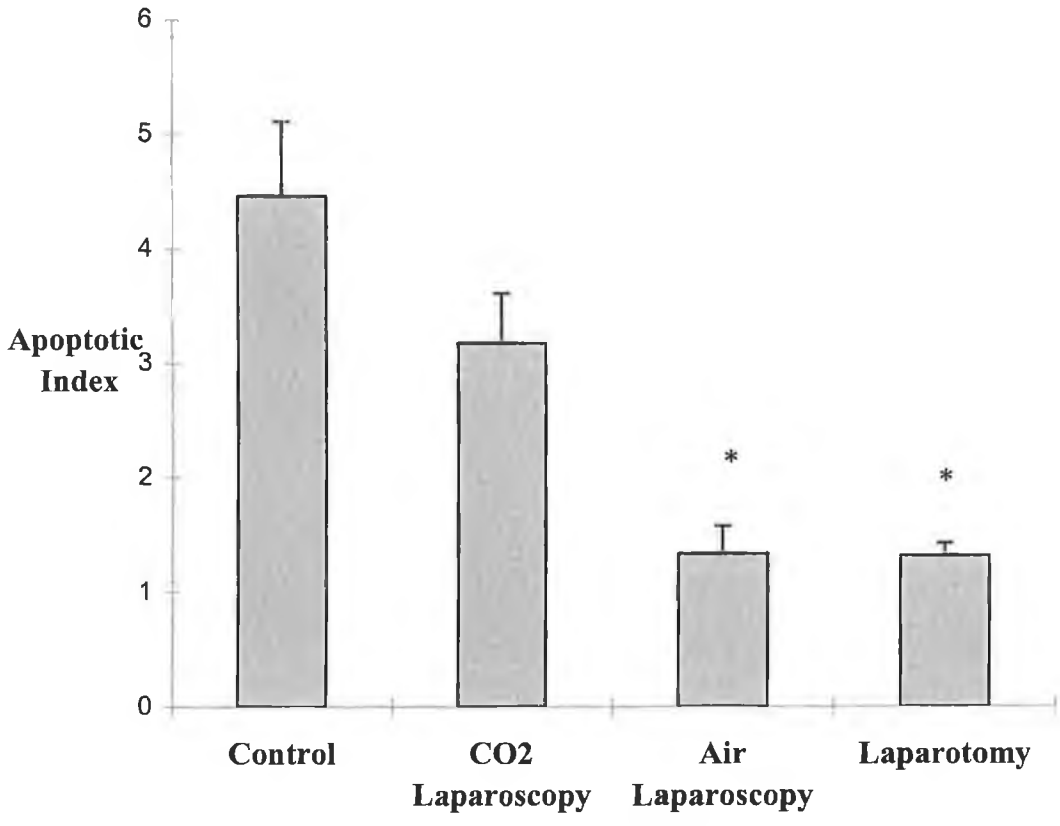


Fig. 3.9: Apoptotic index within metastases following surgical treatment. Apoptotic cells within metastases were identified by TUNEL staining. The apoptotic index is expressed as the average number of positively stained cells counted over 10 fields in a 1 mm^3 grid, counting an average of 500 cells per grid (mean ± S.E.M., n=8/group). Statistical analysis was by ANOVA, with Scheffe post-hoc correction. A significant reduction in apoptosis was observed following laparotomy or air laparoscopy compared to controls or CO_2 laparoscopy *($p<0.01$).

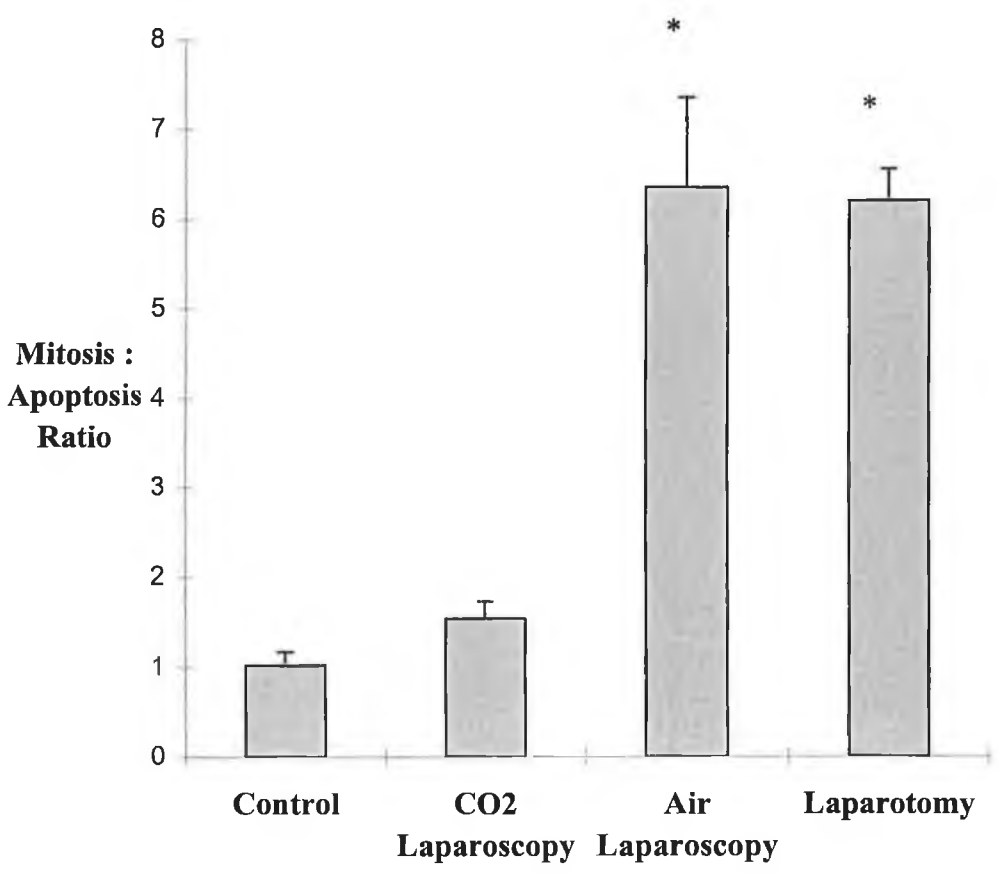


Fig. 3.10: Mitosis : Apoptosis ratios within metastases following surgery. The number of mitotic and apoptotic cells (mean of ten fields) were estimated for each group and the ratio calculated. Data represent the mean ± S.E.M (n=8 / group). A significant increase in mitosis : apoptosis ratios was observed following laparotomy or air laparoscopy * ($p<0.01$) compared to controls or CO_2 laparoscopy. There was no significant difference between the controls and CO_2 laparoscopy groups (p=n.s.)

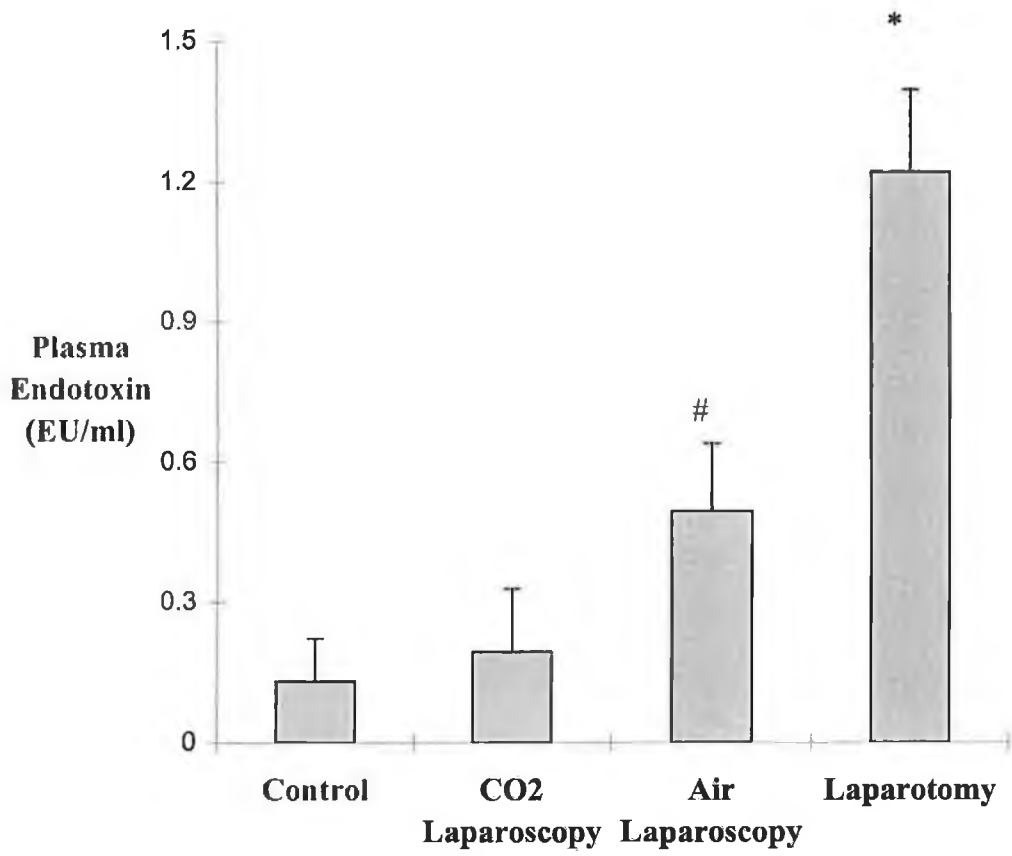


Fig. 3.11: Plasma endotoxin levels following surgery. Blood was collected by cardiac puncture and LPS levels were examined 4 h post-surgery by the LAL. Data represent mean ± S.E.M. (n=8 / group). Laparotomy * ($p<0.001$) and air laparoscopy # ($p<0.03$) both resulted in significantly elevated levels of circulating LPS compared to the controls receiving anaesthesia alone. No significant difference was observed between the control and CO_2 laparoscopy groups (p=n.s.)

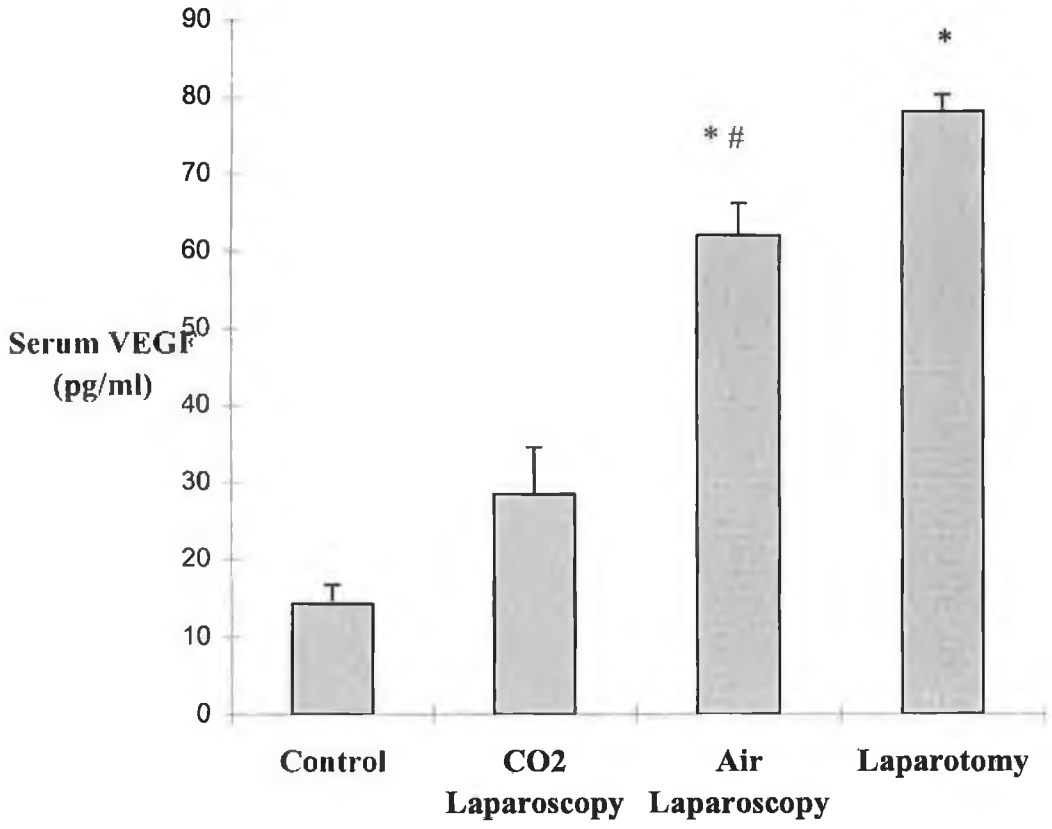


Fig. 3.12: Serum VEGF in mice following surgery. 5 days post-operatively, blood was obtained by cardiac puncture and VEGF was assessed by quantitative ELISA. Data represent mean ± S.E.M. (n=8 / group). A significant increase in circulating levels of VEGF was observed following laparotomy and air laparoscopy compared to controls * ($p<0.001$) or CO2 laparoscopy groups # ($p<0.01$).

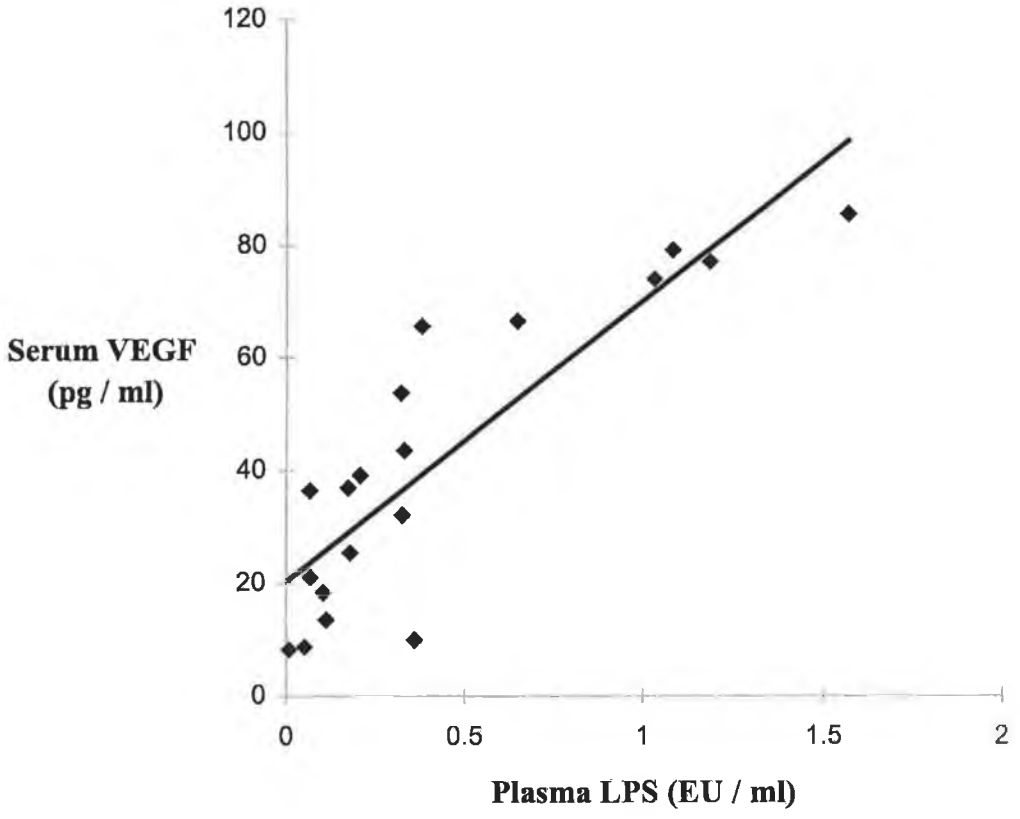


Fig. 3.13: Circulating VEGF in relation to plasma LPS levels (n=20). The relationship between serum VEGF and plasma LPS levels was examined by Pearson Product Moment correlation. A strong positive correlation (r=0.966) was observed between serum VEGF at 5 days and plasma LPS levels 4 h post-surgery.

3.3 Discussion

An experimental model of pulmonary metastasis with no primary tumour was established to examine the effect of surgery on post-operative metastatic tumour growth in mice. Laparotomy, or open surgery, resulted in increased metastatic tumour growth compared to controls receiving anaesthesia only. It has been known for many years that major surgical trauma induces profound immunosuppression (Lennard *et al.*, 1985; Arai *et al.*, 1992). As various forms of anaesthesia have also been implicated in post-operative immuno-suppression (Vose and Moudgil, 1976; Cullen, 1976; Lundy *et al.*, 1978), the control group received halothane anaesthesia for the same period of time to correct for any effects of anaesthesia on tumour growth. These results are in agreement with other recent reports, indicating that open surgical procedures result in significantly higher post-operative tumour growth than minimally invasive techniques (Allendorf *et al.*, 1998; Da Costa *et al.*, 1998). To examine the effect of minimally invasive surgery on metastatic tumour growth, two groups receiving laparoscopy with air or CO_2 insufflation of the peritoneum were included. Mice which received laparoscopy with sterile CO_2, where air is excluded from the peritoneum, had a metastatic burden comparable to controls. However, when the laparoscopy procedure was performed using room air, which contains endotoxin, significant increases in metastastic growth was observed. The level of metastatic growth was, in fact, comparable to that of mice which underwent open laparotomy, implicating an airborne factor in the increased metastatic tumour growth observed in this group.

The removal of a primary tumour (and consequently the anti-angiogenic factors it produces) has been implicated in postoperative tumour metastatic growth (O'Reilly *et al.*, 1994; O'Reilly *et al.*, 1997). In the model used here there was no primary tumour established. Therefore, the increased metastatic tumour growth observed in animals

exposed to air during the surgical procedure cannot be attributed to the removal of anti-angiogenic factors derived from primary tumours. As tumour growth is controlled at the cellular level by the balances between cellular proliferation and apoptotic death within cell populations, the extent of mitosis and apoptosis was examined immunohistochemically within metastases. The increases in metastatic burden coincided with significantly higher tumour cell mitosis and lower apoptosis within lung metastases of both laparotomy and air laparoscopy groups. With both sides of the balance altered in favour of tumour cell expansion, metastases grew rapidly and significant increases in tumour burden were observed after 5 days. Mitotic cells were identified morphologically in haematoxylin and eosin stained tissues. Although this method identified significant differences between groups, reports suggest that staining metastases for antigens associated with cellular proliferation (such as Ki67 or proliferating cell nuclear antigen PCNA) may detect up to 3 times the amount of proliferating cells (Huang *et al.*, 1994). It is therefore possible that the ratio of mitosis : apoptosis within these surgical groups is an under-estimate, and if PCNA or Ki67 staining were performed a much higher ratio may be observed. However, other reports have also suggested that these methods grossly over-exaggerate the proliferating fraction of cell populations (Scott *et al.*, 1991)

It has been suggested that the effect of open surgery on distant tumour growth could be due to the spill over of wound factors, such as sVEGF, into the circulation post-operatively. In this study it is unlikely that this is the case, as similar responses were observed in groups which contained a significant wound (laparotomy) and in the group receiving laparoscopy with air, in which no significant wound was present. These effects indicate that systemic factors were responsible for the alterations in tumour growth. Both laparotomy and air contamination of the peritoneum have previously been shown to result in bacterial translocation across the gut, and the release of endotoxin into the

peritoneal cavity and systemic circulation (Watson *et al.*, 1995). In that study levels of endotoxin peaked 4 hours post-operatively. Plasma endotoxin levels were therefore measured 4 h post surgery in this study. Significantly higher levels of plasma endotoxin were observed in animals which underwent either laparotomy or laparoscopy with air compared to controls. However, when circulating endotoxin levels were examined in the CO_2 laparoscopy group, there was no difference to that of controls. Endotoxin is present ubiquitously in the air at concentrations of approximately 1 μg / m^3 (Rylander *et al.*, 1989). It is also a major component of endogenous gut bacteria. A possible mechanism responsible for the increased metastatic tumour growth observed in this study is through quantities of endotoxin introduced into the peritoneal cavity following laparotomy or laparoscopy with air, which in combination with intestinal agitation, could result in the translocation of endotoxin from gut flora into the systemic circulation. Having entered the circulation, endotoxin could effect the growth of distant metastases. Indeed, it has been directly implicated in cellular proliferation and differentiation, possibly through the phosphorylation and activation of several protein kinases (Weinstein *et al.*, 1992; Shapira *et al.*, 1994).

Vascular endothelial growth factor (VEGF) is the most potent angiogenic factor identified to date (Peters *et al.*, 1993). It's production is regulated by a range of effector molecules and cytokines. It has recently been shown that endotoxin upregulates VEGF expression by human pulp cells through a soluble CD14 mediated mechanism (Matsushita *et al.*, 1999). Angiogenesis is critical for the growth of metastases and its suppression results in tumour dormancy (Holmgren *et al.*, 1995). VEGF expression has been shown to correlate with the level of metastases in experimental melanomas (Claffey *et al.*, 1996). Therefore levels of circulating VEGF were estimated by ELISA. Open surgery or air laparoscopy resulted in significantly elevated levels of circulating

serum VEGF compared with either the CO_2 laparoscopy group or controls. As increased metastatic burden was observed in these groups, it is possible that the increases in VEGF could simply be a consequence of the increased tumour volume. However, separate studies in our laboratory have found increased VEGF expression within tumour nodules from endotoxin treated mice compared to tumour nodules from untreated mice (Harmey, un-published observations). When the levels of plasma endotoxin were compared with circulating VEGF in all groups, a strong positive correlation was observed. This suggests that in addition to promoting tumour growth, the surgical introduction of endotoxin induced the release of the pro-angiogenic cytokine VEGF.

Chapter 4

Endotoxin and Anti-Endotoxin Therapies in Metastatic Tumour Growth

4.1 Introduction

It has been reported that infection, and its associated inflammation, are risk factors in many forms of cancer (Correa, 1992; Schiller, 1988; Kantor, 1984). Increased tumour growth and capillary proliferation into the tumour stroma has been reported in a rat model of bladder cancer following infection with killed *E.coli* (Yamamoto *et al.*, 1992), supporting the theory that bacteria, or components thereof, are angiogenic in the tumour microenvironment. With respect to bladder cancer, other investigators have reported the tumour enhancing effect of urinary tract bacterial infections, and these have been attributed to lipopolysaccharide (LPS) / endotoxin (Kawai *et al.*, 1993; Johansson *et al.*, 1987). These studies also reported increased angiogenesis in the tumour stroma, and endotoxin has been shown to be angiogenic in a number of *in vivo* model systems, inducing angiogenesis in the corneal implant model in rabbits (Li *et al.*, 1991; BenEzra *et al.*, 1993) and also in the rat mesentery (Mattsby-Baltzer *et al.*, 1994).

Bacterial endotoxin has a wide range of biological effects on a number of cells and organs in the host (Morrison and Ryan., 1979; Raetz *et al.*, 1991). There are a number of mechanisms through which it may exert its angiogenic and tumour enhancing effects. Stimulation of macrophages and other cells to release cytokines such as TNF-α , IL-1, IL-6 and bFGF are possible pro-tumour effector molecules (Austgulen & Nissen-Meyer, 1988, Motro *et al.*, 1990; Fajardo *et al.*, 1992). These cytokines have been suggested to be involved in the pathogenesis of psoriasis, which is characterised by increased angiogenesis in the skin and increased proliferation of keratinocytes (Nickoloff *et al.*, 1991; Kupper, 1988). The cytokine network induced by LPS plays a significant role in cellular proliferation in inflammation-induced urothelial hyperplasia (Kawai *et al.*, 1993). Inflammation induces short lived effects on host vasculature, such as increased

permeability and activation of endothelium, as well as chronic effects such as extensive mitosis of the endothelium resulting in the remodeling of capillaries and venules (Thurston *et al.*, 1998). Indeed, recently LPS has been shown to increase VEGF expression by human pulp cells (Matsushita *et al.*, 1999), which could account for these observations. It is likely that inflammation influences metastasis which is facilitated by hyperpermeability and increased proliferation of blood vessels (see section 1.3).

Endogenous gut bacteria are a major source of endotoxin, which can be released following bacterial cell division or upon lysis of the bacterial cell. Significant translocation of endotoxin into the peritoneal cavity and systemic circulation occurs following open surgery or laparoscopy with air-sufflation within as little as 2-4 hours (Watson *et al.*, 1995). Subsequent observations on laparotomy-induced growth of the murine B16 flank tumour suggested that, in addition to post-operative immunosuppression, exposure of the peritoneal cavity to air, and the minute quantities of LPS in this air, are causal factors in this tumour growth (DaCosta *et al.*, 1998).

As there is accumulating evidence to suggest that LPS, either directly or indirectly through the induction of inflammatory cytokines, is a possible regulator of tumour growth and angiogenesis, strategies which neutralise LPS or block the production or biological effects of LPS-induced cytokines, may be useful in cancer therapy. Physiologically, the body has developed regulatory mechanisms to deal with, and control our response to bacterial infection. The polymorphonuclear leukocytes (PMN) play a central role in the host defense against gram negative bacteria. Bactericidal/permeability increasing protein (BPI) is a 55-kDa cationic protein localised in the azurophilic granules of mature neutrophils that specifically kills gram negative bacteria (Weiss *et al.*, 1978). This protein was first purified by Weiss *et al.* in 1978 and has been isolated from both human and rabbit neutrophils. In a similar way to LPS binding protein (LBP), BPI

interacts with LPS to form high affinity complexes. The binding of LPS by BPI prevents binding by LBP, and the subsequent binding to the cellular receptor CD14, therefore neutralising the inflammatory cascade associated with LPS (Marra *et al.*, 1990; Marra *et al.*, 1992).

The LPS binding property of BPI has been localised to its N-terminal fragment (Abrahamson *et al.*, 1997) and a 25-kDa recombinant form of BPI, termed $rBPI_{21}$ has been developed (Ooi *et al.*, 1987). Essentially, $rBPI_{21}$ is the proteolytic N-terminal 193 amino acids of the holoprotein BPI produced from engineered Chinese Hamster Ovary (CHO) cells in culture, with a single amino acid change at position 132 with cysteine being substituted with alanine (communication with Dr. R. Dedrick, Xoma, Ca., US). BPI binds to a region close to lipid A, however minimal binding is observed to monophosphoryl lipid A (Jahr *et al.*, 1995). Results from Jahr and co-workers (1995) indicate that $rBPI_{21}$ has a considerably higher neutralising activity on LPS with long polysaccharide chains compared to LPS which contains only 2-keto-3-deoxyoctane (KDO) sugars (see Fig. 1.3). Differential TNF-α production, in response to different LPS molecules and BPI, led the authors to suggest that $rBPI_{21}$ may have a higher affinity for smooth LPS than for the lipid A moiety and that therefore $rBPI_{21}$ may interact with the polysaccharide part of the LPS molecule. Elsbach *et al.* (1994) compared the inhibitory effect of BPI with $rBPI_{21}$ on bacterial growth and found that BPIs inhibitory effects diminished with increasing polysaccharide chain length (Elsbach *et al.*, 1994). It was suggested that long hydrophilic polysaccharide chains impede BPI binding to the lipid A region which is embedded in the outer membrane of the gram-negative bacterial envelope and that the smaller 25-kDa rBPI fragment penetrated more easily. These observations suggest that LPS and lipid A may use different mechanisms to stimulate monocyte TNF-α production, and implies that CD14 may bind polysaccharide parts of

LPS as well as lipid A. They may also explain why anti-endotoxin therapies, such as the E5 anti-lipid A murine monoclonal antibody, designed to neutralise the lipid A moiety of the LPS molecule, failed to have satisfactory endotoxin-neutralising activity *in vivo* (Baumgartner *et al.*, 1991; Marra *et al.*, 1994).

The aim of this chapter was to investigate the effect of endotoxin exposure on metastatic tumour growth. Metastatic burden, mitosis and apoptosis within the lung metastases were assessed. Circulating levels of serum VEGF were also measured post-LPS injection to examine if LPS induced the release of this pro-angiogenic factor into the circulation. The use of the novel anti-endotoxin agent $rBPI_{21}$ was investigated in comparison to the monoclonal anti-lipid A antibody E5 in blocking LPS induced alterations in tumour growth. A separate group was included which received a control peptide the same size as $rBPI_{21}$, thaumatin, which has no anti-LPS activity. Finally, the use of these anti-endotoxin agents in the post-operative period was investigated, to examine if anti-endotoxin strategies could reduce surgically induced tumour growth and angiogenesis.

4.2 Results

4.2.1 Dose response for endotoxin injection

Endotoxin / lipopolysaccharide (LPS) has been shown to be angiogenic. As angiogenesis is critical for the growth of both primary and metastatic tumours, the effect of LPS on metastatic growth was examined. An initial set of experiments was carried out to establish a clinically relevant dose without associated morbidity or toxicity in mice. Experimental lung metastases were established by tail vein injection of 4T1 mammary adenocarcinoma cells. 14 days after injection animals received an intra-peritoneal injection of LPS (0, 1 ng, 10 ng, 100 ng, 1 μg, 10 μg, 100 μg) in saline. These concentrations reflect the clinical setting where plasma concentrations of 2 ng/ml - 20 μg/ml have been reported and shown to be angiogenic (Mattsby-Baltzer *et al.*, 1994). Animals were sacrificed 5 days later and a lung to body weight ratio calculated as before. 10 μg LPS/animal (2.063 ± 0.287) and 100 μg LPS/animal (2.193 ± 0.285) resulted in significantly ($p<0.02$) increased metastatic burden compared to control animals receiving a saline injection (0.597 ± 0.023) (Figure 4.1). The higher dose of 100 μg, however, was outside the clinically relevant dose previously reported and had an associated mortality rate of 40% by day 4 post-injection. Therefore, a dose of 10 μg/animal was chosen to further investigate the effect of LPS on other parameters involved in tumour growth.

4.2.2 Effect of LPS on metastatic tumour burden, mitosis and apoptosis within lung metastases

There was no significant difference between body weights of animals receiving 10 μg LPS and saline controls (Fig. 4.2.A), indicating that the animals were not cachectic (p=n.s.) and no apparent oedema was observed. As in the dose-response experiment, 10 μg of endotoxin (2.84 ± 0.20) resulted in a significantly ($p<0.001$) higher metastatic

burden compared with the saline controls (1.99±0.10), (Fig. 4.2.B). Photographs of lungs from each group are shown in Figure 4.2.C demonstrating increased macroscopic metastases in mice which received endotoxin injection relative to controls. Mitosis and apoptosis within the lung metastases were assessed by counting mitotic figures and TUNEL stained cells respectively, and calculating the number of cells per grid of 500 cells. The higher metastatic burden was accompanied by a significantly ($p<0.05$) higher mitotic index in the endotoxin injection group (7.68 ± 0.24) compared to the saline injection group (4.74 ± 0.99), (Fig. 4.3.A). In addition, a significantly ($p<0.05$) lower apoptotic index was observed in the endotoxin group (1.27 ± 0.11) compared to the saline group (2.98 ± 0.53) (Fig. 4.3.B). As it is ultimately the balance between mitosis and apoptosis that governs net tumour growth, a ratio of mitosis : apoptosis within the metastases was calculated. The MI/AI ratio in the endotoxin injection group (6.18 ± 0.64) was significantly ($p<0.02$) higher than the ratio observed following saline injection (2.02 ± 0.88), (Fig. 4.3.C). This represented a 3-fold increase in MI/AI ratio in the endotoxin treated animals.

4.2.3 Effect of LPS on circulating levels of serum VEGF

As VEGF is the most potent angiogenic factor identified and LPS has been shown to increase its expression by certain cell types (Matsushita *et al.*, 1999), the levels of serum VEGF were measured by ELISA. The endotoxin injection group (207.85±42.74 pg/ml) was found to have significantly ($p<0.003$) higher levels of circulating VEGF compared with the saline treated group (33.88±7.34 pg/ml) (Fig. 4.4). This represents a six-fold increase in circulating VEGF following LPS injection, demonstrating that LPS has a pro-angiogenic effect, which could contribute to the increased metastatic tumour growth observed in mice following endotoxin injection.

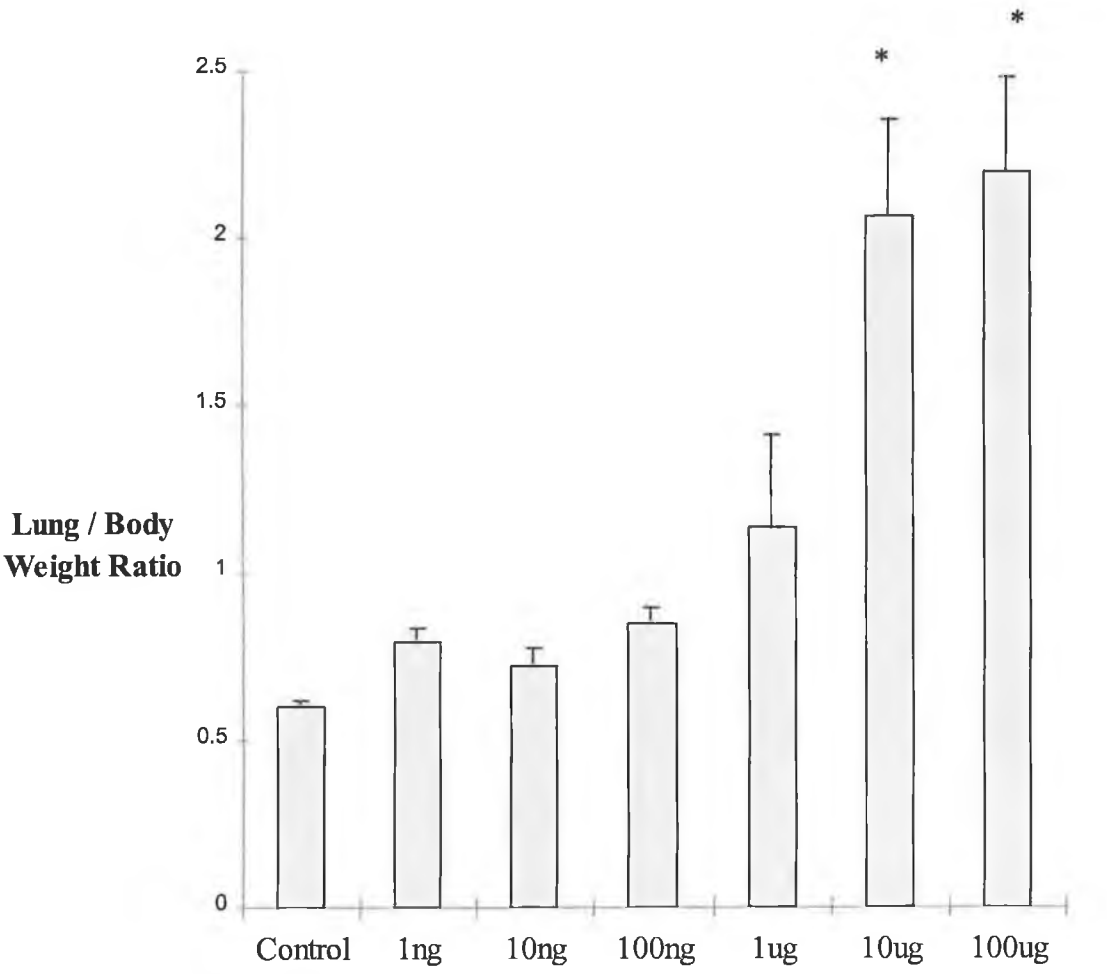


Fig 4.1: Effect of LPS on experimental lung metastases. 14 days after tail vein injection of 10^5 4T1 cells, mice (n=3) received an injection of saline or LPS. 5 days later mice were sacrificed and a lung : body weight index calculated. Data represents mean $\pm$ S.E.M. * $p<0.05$ (ANOVA with Scheffe post-hoc correction).

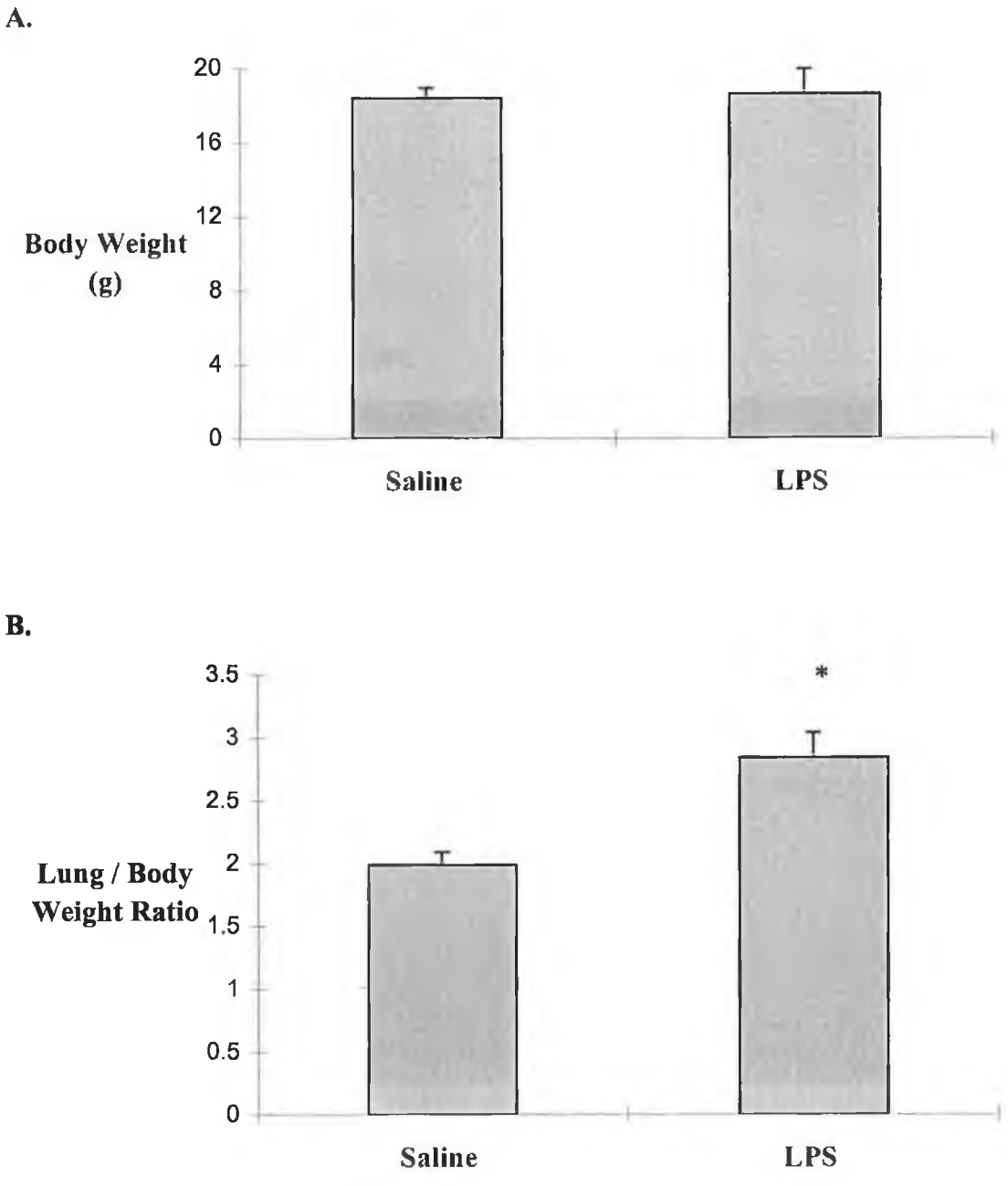
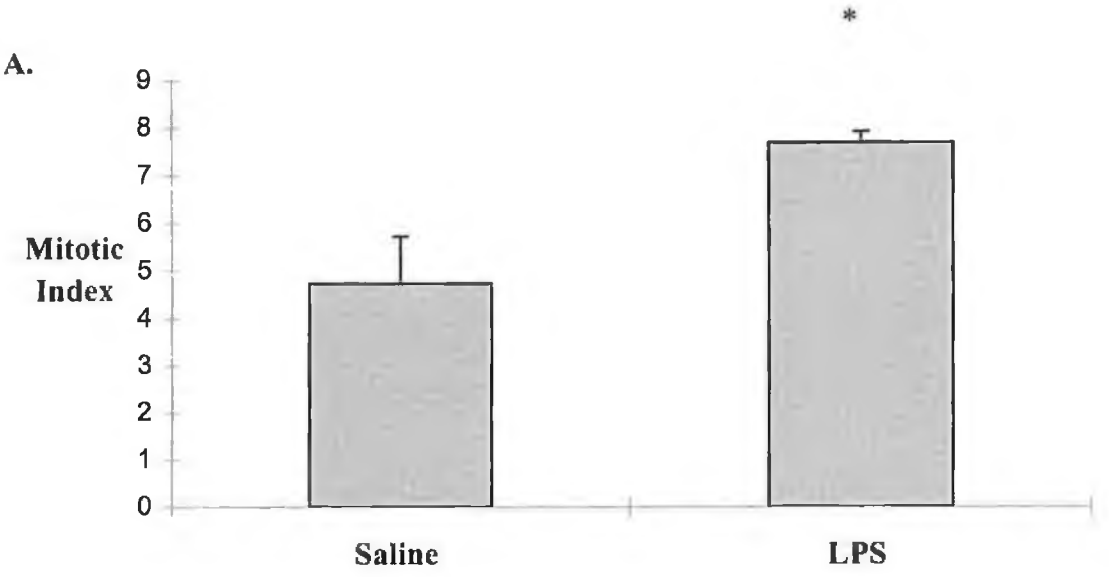


Fig. 4.2: Effect of LPS on metastatic tumour growth. 14 days after tail vein injection of 10^5 4T1 cells, mice (n=8/group) received an i.p injection of saline or 10 mg LPS. 5 days later mice were sacrificed. Data represents mean $\pm$ S.E.M. Statistical analysis was by students t-test. **A.** Body weight p=n.s. **B.** Lung metastatic burden * ($p<0.001$). **C.** Representative lungs.



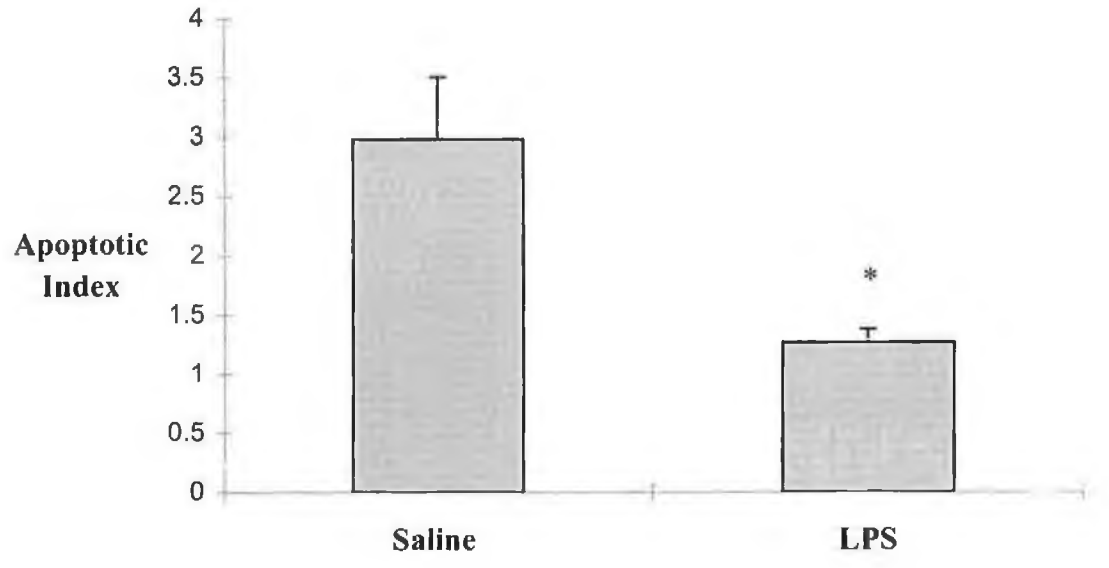


Fig. 4.3: Mitosis and Apoptosis within lung metastases in histological sections. In each case an average of 500 cells/mm^2 grid were scored in 10 fields/section. Mitotic cells were estimated by counting mitotic figures and apoptosis by counting cells stained positive by TUNEL. Data is represented as mean $\pm$ S.E.M (n=8). Statistical analysis was by students t-test. **A.** Mitotic Index within lung metastases, * $p<0.04$. **B.** Apoptotic index within lung metastases, * $p<0.05$.

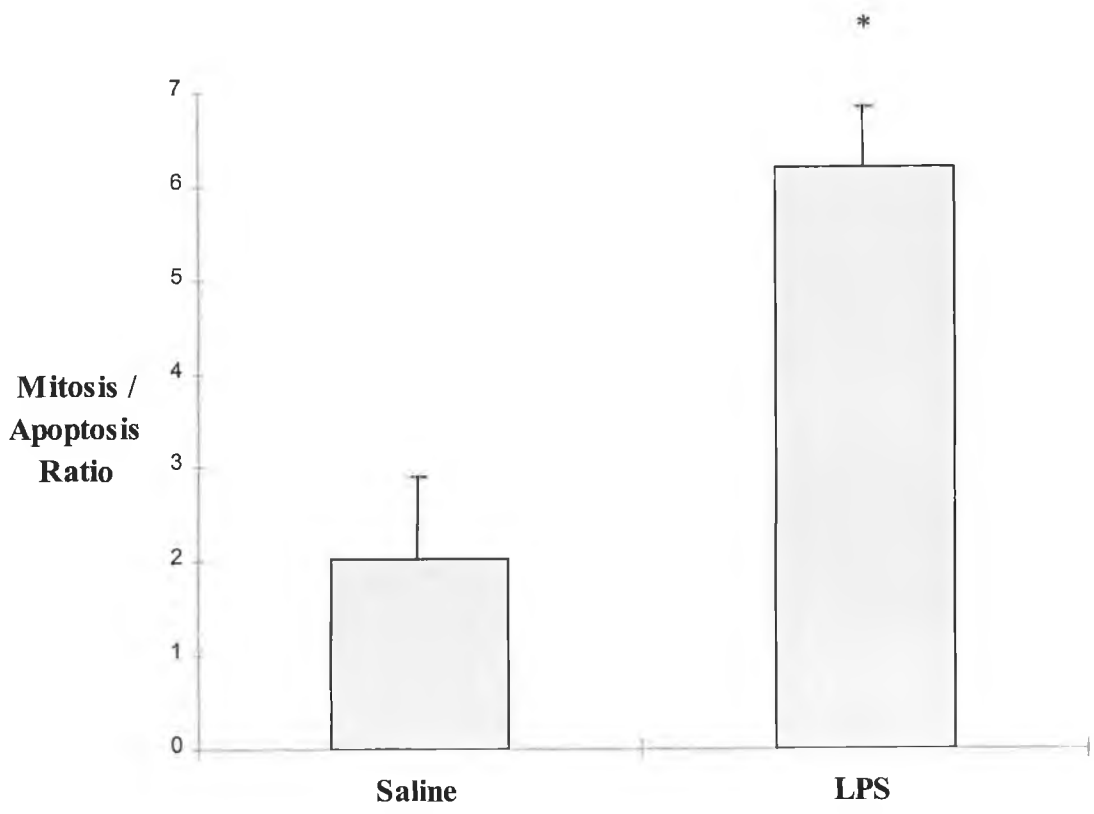


Fig. 4.3: D. Mitosis : Apoptosis ratio within lung metastases, * $p<0.02$.

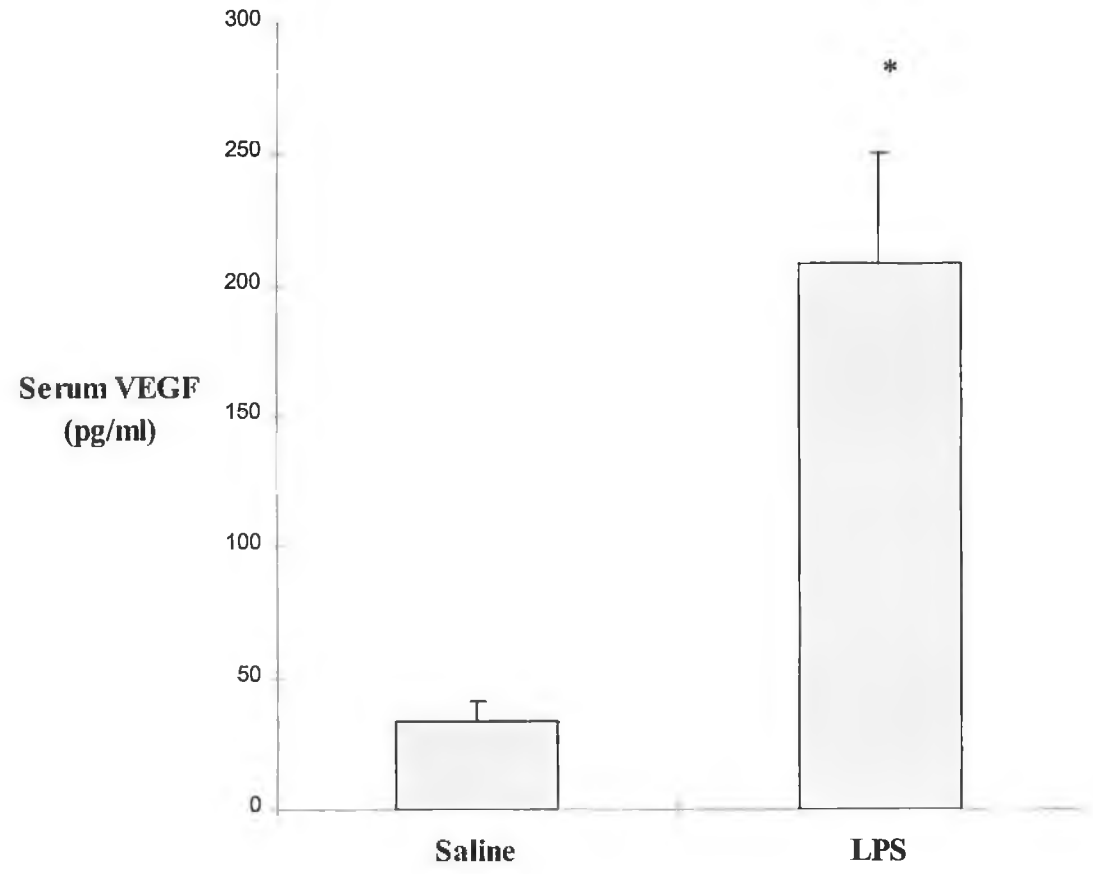


Fig 4.4 : Serum VEGF in tumour bearing mice following endotoxin injection. On the day of sacrifice, blood was obtained by cardiac puncture and VEGF assessed by ELISA. Data represent mean ± s.e.m. (n=8 / group). Statistical analysis was by students t-test, *($p<0.003$).

4.2.4 Effect of anti-endotoxin $rBPI_{21}$ on LPS induced metastatic growth.

Having demonstrated that intra-peritoneal injection of LPS increases metastatic tumour growth in the murine model of breast cancer, the efficacy of anti-endotoxin treatments in attenuating this increased growth was evaluated. A novel recombinant bactericidal permeability-increasing protein (BPI), named $rBPI_{21}$, and a control peptide of the same size, thaumatin, were gifts from Xoma Corporation, CA. US. Mice were injected with 10^5 4T1 cells via the lateral tail vein as previously described. Two weeks later the mice received an intra-peritoneal injection of either sterile saline or 10 μg LPS, in combination with the dose regime of $rBPI_{21}$, thaumatin or formulation buffer outlined in section 2.7.5. Five days after LPS or saline injection animals were sacrificed and a lung / body weight index recorded. LPS injection (1.81 ± 0.12) resulted in a significantly ($p<0.001$) higher metastatic burden compared to saline injected controls (0.83 ± 0.80). However, when $rBPI_{21}$ treatment was injected with LPS (0.804 ± 0.12) the LPS-induced increase in metastatic burden was significantly ($p<0.001$) attenuated (Figure 4.5.A). $rBPI_{21}$ completely blocked LPS-induced metastatic growth, with metastatic burden in these animals comparable to saline controls. The control peptide, thaumatin had no effect on LPS induced metastatic growth (1.869 ± 0.089). Representative lungs taken from each of the six groups clearly show the effect of rBPI21 treatment on LPS-induced lung metastatic growth (Figure 4.5.B).

The altered growth kinetics observed in these experiments relative to previous groups of mice (Section 4.1 and 4.2) is due to the fact that a different batch of 4T1 cells was obtained from another laboratory (Fred Miller, Duke University) following the loss of all cell culture stocks, due to liquid nitrogen failure within our facility. Factors such as the source of cells, their preparation, and the length of time between injections, all contribute

to the inter-batch variations observed between experiments. Although there are differences between metastatic burden, mitotic index (MI) and apoptotic index (AI) between experiments, there is a clear trend reflected in all the groups. Higher MIs are observed in groups with high metastatic burdens, and correspondingly lower AIs are also observed in these groups. Increased metastatic burden was observed in mice receiving LPS relative to controls, demonstrating the validity of the model with the newly obtained cells. A table of the various batches of mice from different experiments is shown in Table 4.1. These data clearly demonstrate the relationship between net tumour growth and the proliferation / apoptotic balance.

4.2.5 Effect of $rBPI_{21}$ on mitosis and apoptosis within metastases.

The mitotic and apoptotic index within the metastases was examined histologically. A similar trend was observed to that seen in the lung/body weight ratios. Endotoxin injection (4.90±0.26 cells/grid) resulted in significantly ($p<0.001$) higher mitotic indices compared to saline controls (1.47±0.29). However, when mice were treated with $rBPI_{21}$ following LPS injection (1.46 ± 0.355) the mitotic index was significantly ($p<0.001$) reduced to the level of saline controls (Fig.4.6 A). rBPI completely blocked LPS induced increases in mitotic index compared to saline controls. Thaumatin treatment (4.70 ± 0.336) had no effect on LPS induced increases in mitotic indices.

When apoptosis was examined, LPS injection (1.58 ± 0.067) resulted in significantly ($p<0.001$) reduced numbers of apoptotic cells within metastases compared to saline injected mice (4.36 ± 0.467). $rBPI_{21}$ treatment (3.79 ± 0.043) significantly prevented LPS mediated inhibition of tumour cell apoptosis ($p<0.003$ vs LPS), while thaumatin treatment (1.13 ± 0.043) had no effect (Fig. 4.6 B). There was almost a complete

inhibition in LPS-induced reduction in apoptosis following treatment with rBPI. The MI/AI ratio was calculated as described in section 2.8. LPS injection (3.436±0.337) resulted in significantly higher ratios compared to saline injected mice (0.365±0.108, $p<0.002$) or mice receiving LPS with $rBPI_{21}$ treatment (0.467±0.059, $p<0.005$) (Figure 4.6 C). Thaumatin had no effect on LPS induced MI:AI indices (4.121±0.681 vs 3.436±0.337, p=n.s). It is clear that $rBPI_{21}$ blocks the stimulation of tumour growth by LPS by inhibiting it's effect on apoptosis and mitosis, the opposing processes governing tumour growth.

4.2.6 Effect of $rBPI_{21}$ on LPS induced serum VEGF.

Intra-peritoneal injection of LPS was previously shown to increase circulating levels of VEGF (4.2.3), therefore serum VEGF levels were examined in mice following LPS injection and $rBPI_{21}$ treatment to establish whether $rBPI_{21}$ could prevent the pro-angiogenic effect of LPS. LPS injection (292.4±65.94 pg/ml) resulted in higher circulating VEGF than saline injected controls (86.07±1.64 pg/ml) as before. Treatment with $rBPI_{21}$ following LPS injection (144.48±79.9 pg/ml) did not significantly reduce LPS-induced circulating VEGF (p=n.s, vs LPS only), although an apparent reduction in the levels of VEGF was observed (Fig. 4.7). Treatment with the control peptide thaumatin (191.3±87.64 pg/ml) did not effect LPS induced VEGF. The effect of $rBPI_{21}$ may not have reached statistical significance because of the small numbers of bloods analysed in the study (n=4) and considerable variation in the levels of VEGF production between mice. Nevertheless the data suggests a trend toward rBPI blocking VEGF induction.

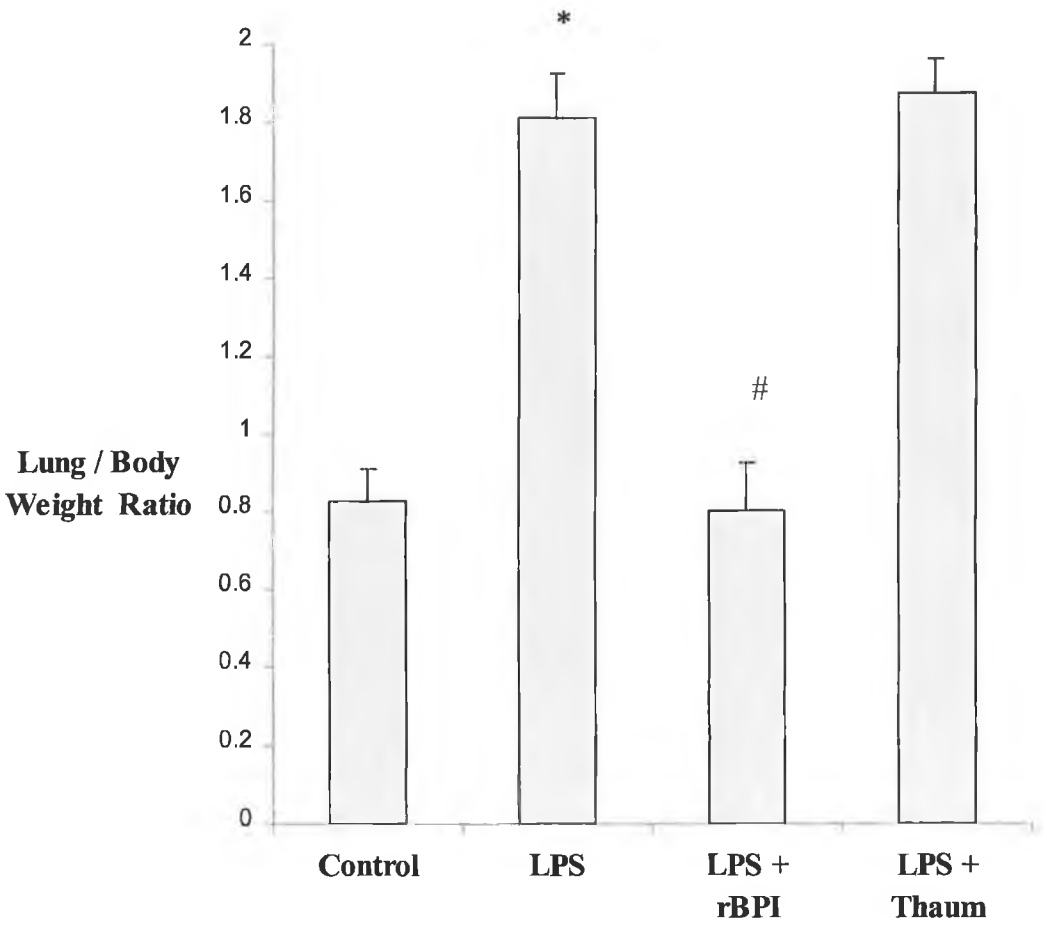
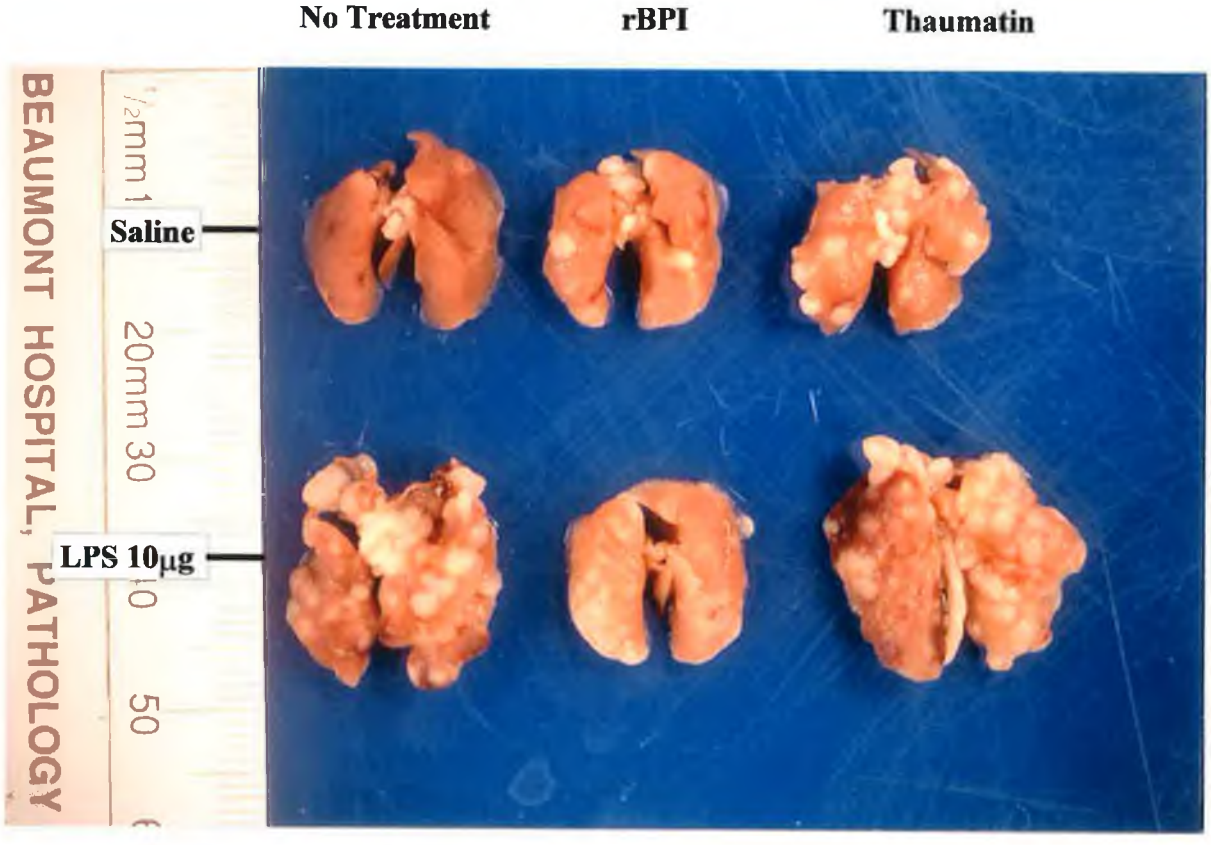


Fig. 4.5 Effect of $rBPI_{21}$ on LPS induced metastatic growth. Mice received 1 x 10^5 4T1 tumour cells by tail vein injection, followed 14 days later by either saline or endotoxin (10 μg/animal) i.p., with or without $rBPI_{21}$ or thaumatin as indicated. Mice were sacrificed 5 days after intra-peritoneal injection and their lungs excised. **A:** Tumour burden is expressed as lung weight/body weight x 100. Data is expressed as mean ± S.E.M. (n=4). Statistical analysis was by ANOVA with Scheffe post-hoc correction * ($p<0.001$ vs control), # ($p<0.001$ vs LPS). **B:** Representative lungs.

B.


No Treatment
rBPI
Thaumatin
Saline
LPS 10μg
BEAUMONT HOSPITAL, PATHOLOGY
20mm 30
50

Experimental Group	Metastatic Burden	Mitotic Index (MI)	Apoptotic Index (AI)	MI / AI
Section 3.2				
Control	1.32±0.12	4.39±0.63	4.46±1.13	1.02±0.15
CO_2 Laparoscopy	1.94±0.42	4.71±0.63	3.17±0.76	1.54±0.19
Air Laparoscopy	2.73±0.12	7.99±0.09	1.33±0.42	6.35±1.00
Laparotomy	3.37±0.14	8.33±0.21	1.31±0.13	6.20±0.35
Section 4.2				
Saline Injection	1.99±0.10	4.74±0.99	2.98±0.53	2.02±0.64
LPS Injection	2.84±0.20	7.68±0.24	1.27±0.11	6.18±0.64
Section 4.2				
Control	0.83±0.80	1.47±0.29	4.36±0.047	0.365±0.108
LPS	1.81±0.12	4.90±0.26	1.58±0.067	3.436±0.337
LPS + rBPI21	0.804±0.12	1.46±0.355	3.79±0.043	0.467±0.059
LPS + thaumatin	1.869±0.089	4.70±0.336	1.13±0.043	4.121±0.681

Table 4.1 Comparison of metastatic burden, mitotic indices, apoptotic indices and MI/AI index between experimental groups.

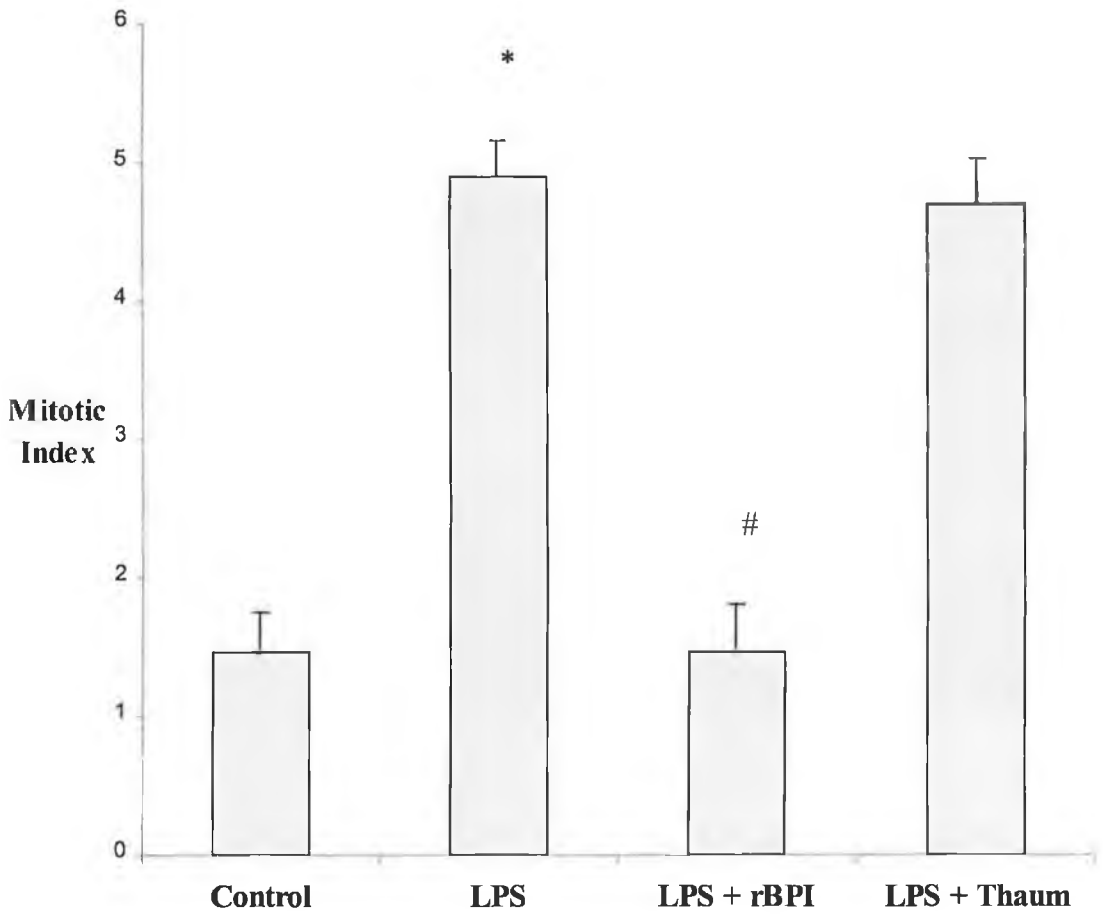
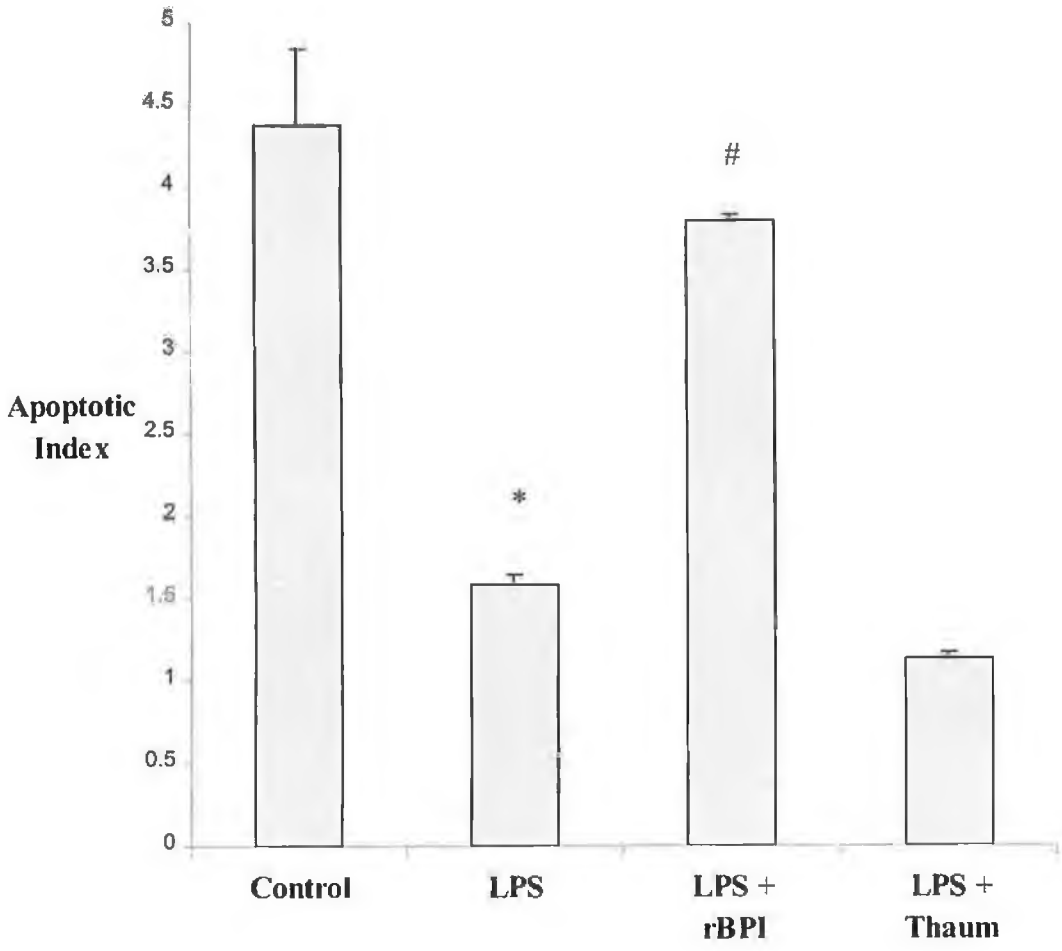
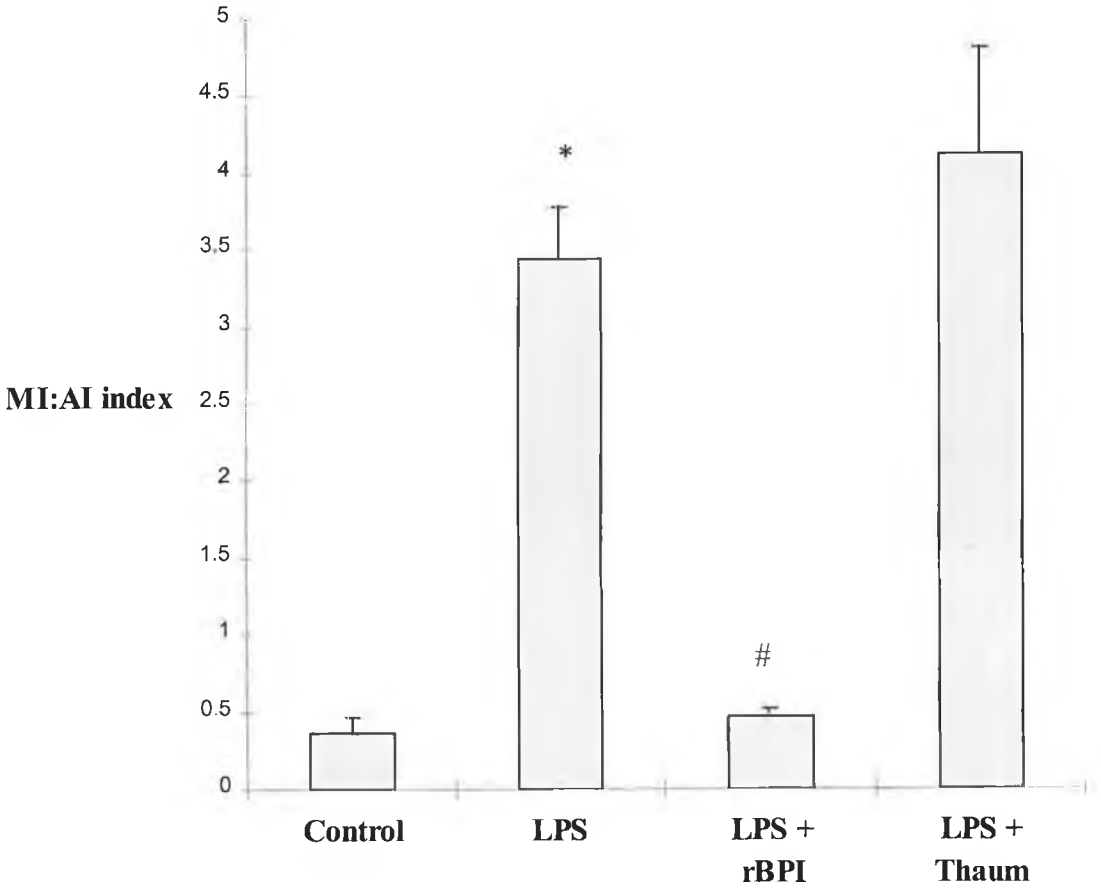


Fig. 4.6 Effect of $rBPI_{21}$ on mitosis and apoptosis within lung metastases following LPS injection. Data is expressed as mean $\pm$ S.E.M. (n=4). Statistical analysis is by ANOVA with Scheffe post-hoc correction **A:** Mitotic index within metastases *($p<0.001$ vs controls) #($p<0.001$ vs LPS injection). **B:** Apoptotic index within metastases *($p<0.001$ vs controls) #($p<0.003$ vs LPS injection). **C.** Mitosis : Apoptosis ratios within metastases *($p<0.002$ vs controls) #($p<0.005$ vs LPS injection).

B.


Apoptotic Index
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
#
Control
LPS
LPS + rBPI
LPS + Thaum

C.


MI:AI index
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
#
Control
LPS
LPS + rBPI
LPS + Thaum

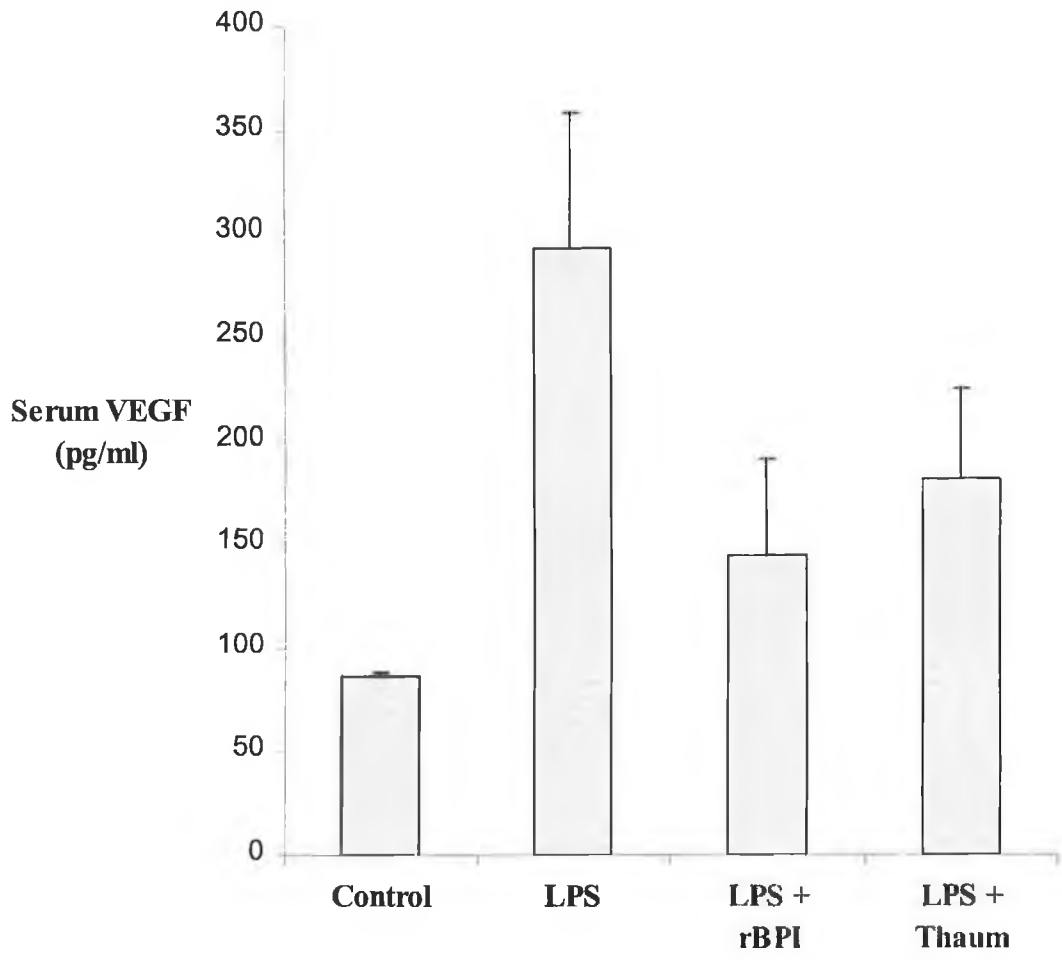


Fig 4.7: Serum VEGF in mice following endotoxin injection and $rBPI_{21}$ treatment. 5 days after injection blood was obtained by cardiac puncture and VEGF assessed by ELISA. Data represent mean ± S.E.M. (n=4 / group). Statistical analysis is by ANOVA with Scheffe post-hoc correction (p=n.s. between all groups).

4.2.7 Effect of $rBPI_{21}$ or E5 on surgically-induced metastatic burden.

Section 3.2.6 showed elevated plasma LPS in mice following surgery with a tight correlation between serum VEGF and LPS (section 3.2.7). Having demonstrated that LPS increases metastatic burden in mice and the anti-endotoxin agent $rBPI_{21}$ blocks this effect, the effect of $rBPI_{21}$ on surgically-enhanced metastatic tumour growth was investigated. $rBPI_{21}$ and the control peptide thaumatin, were used as before. The efficiency of $rBPI_{21}$, which binds to a region on / or close to the lipid A moiety and the anti-Lipid A murine monoclonal antibody, E5, obtained from Xoma Corporation (CA. US) were compared. Mice were injected with 1 x 10^5 cells via the lateral tail vein as before. Two weeks later the mice received a mid-line laparotomy with intestinal agitation or anaesthesia alone for a period of 30 min (described in section 2.7.3), in combination with the treatment schedule of $rBPI_{21}$, thaumatin , E5 or formulation buffer as described (section 2.7.5). 5 days later the animals were sacrificed, their lungs removed and a lung / body weight index recorded (Fig. 4.8). Surgery (1.135 ± 0.124) resulted in a significantly ($p<0.001$) higher metastatic burden compared to saline injected controls (0.674 ± 0.026). $rBPI_{21}$ treatment significantly reduced surgically enhanced metastatic tumour growth (0.637 ± 0.018 vs 1.135 ± 0.124, $p<0.002$). The metastatic burden in rBPI treated animals was comparable to control animals which did not receive a surgical insult. Treatment with the anti-Lipid A antibody E5 had no effect on surgically induced metastatic growth (0.994 ± 0.127 vs 1.135 ± 0.124, p=n.s.) Similarly, the control peptide, thaumatin (1.13 ± 0.152) had no protective effect.

These results implicate LPS as a causative factor in surgically enhanced tumour growth, as the anti-LPS agent rBPI is capable of preventing this enhanced growth. The results also indicate that rBPI is far more effective at blocking LPS and surgically induced

metastatic growth than the monoclonal antibody E5. E5 is no longer in production by Xoma Corp., and it was for this reason that there was only a limited supply available to use in this one study.

4.2.8 Effect of $rBPI_{21}$ and E5 on mitosis and apoptosis within surgically-induced metastases.

The mitotic and apoptotic indices within lung metastases was calculated to determine the numbers of proliferating and apoptotic cells within metastases. Open surgery (5.29 ± 0.226) resulted in significantly ($p<0.001$) higher mitotic indices compared to anaesthesia controls (1.44 ± 0.131). However, when mice were treated with $rBPI_{21}$ following surgery (1.61 ± 0.205) the mitotic index was significantly ($p<0.001$) reduced to that observed in lung metastases of anaesthetic controls (Fig.4.9 A). Treatment with either the monoclonal antibody E5 (4.57 ± 0.327) or thaumatin (5.20 ± 0.20) had no effect on surgically-induced increases in mitotic indices.

When apoptosis was examined, surgery (1.12 ± 0.128) significantly ($p<0.001$) reduced the number of apoptotic cells within metastases compared to mice undergoing anaesthesia alone (4.37 ± 0.288). $rBPI_{21}$ treatment (4.44 ± 0.396) prevented the reduction in apoptosis observed in the lung metastases of animals following surgery ($p<0.001$ vs Surgery) with levels comparable to controls, while E5 (1.28 ± 0.061) or thaumatin treatment (1.28 ± 0.182) had no effect (Fig. 4.9 B). The ratio of mitotic : apoptotic cells (MI/AI) was calculated as described in section 2.8. Open surgery (5.048 ± 0.717) resulted in significantly ($p<0.001$) higher ratios compared to anaesthetic controls (0.341 ± 0.049). $rBPI_{21}$ treatment (0.364 ± 0.042) prevented this increase with a similar MI/AI to control mice ($p<0.001$ vs Surgery) (Figure 4.9 C). Neither E5 (3.637 ± 0.369), nor thaumatin

(4.482±0.735) had any effect on the increased MI/AI observed in mice which received laparotomy. In each case, as before, the MI/AI reflects the metastatic burden, with a high MI/AI in those groups with a high metastatic burden.

4.2.9 Effect of anti-endotoxin $rBPI_{21}$ and E5 on surgically induced serum VEGF.

Chapter 3 showed that laparotomy increased circulating levels of VEGF and this increase was associated with increased circulating LPS in the plasma. The levels of VEGF following surgery and $rBPI_{21}$, E5 or thaumatin treatment were examined. Open surgery (213.56±39.2 pg/ml) resulted in significantly ($p<0.01$) higher circulating VEGF than in animals receiving anaesthesia alone (21.14±6.69 pg/ml). Treatment with $rBPI_{21}$ following surgery (39.69±10.18 pg/ml) significantly ($p<0.02$) reduced circulating levels of VEGF, compared to animals undergoing surgery (Fig. 4.10). However, this level was still twice that observed in the control groups, so $rBPI_{21}$ does not appear to completely block surgically induced VEGF. Thaumatin (184.87±23.22 pg/ml) or E5 (180.80±13.56) failed to reduce circulating VEGF levels following surgery. This indicates that blocking the lipid-A moiety alone is not sufficient to block the angiogenic response to LPS in tumour bearing mice, whereas rBPI which can also bind regions outside of the lipid A moiety can.

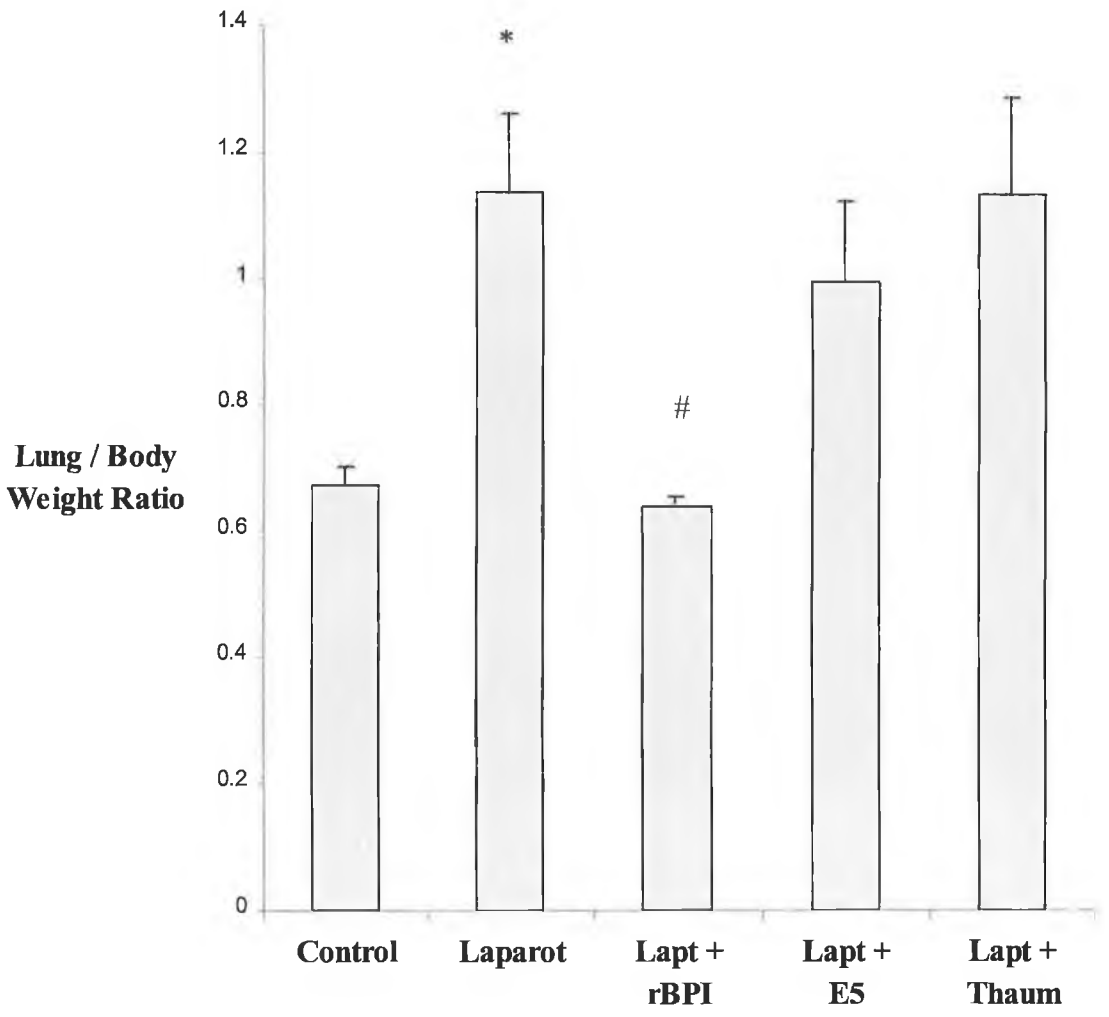


Fig. 4.8: Effect of endotoxin blockade on surgically-induced metastatic growth. Mice (n=5/group) received 10^5 4T1 cells by tail vein injection. 14 days later mice underwent a laparotomy or laparotomy combined with the anti-endotoxin agents rBPI, E5 or thaumatin control peptide. Control mice received anaesthetic only. Mice were sacrificed 5 days later and the lung:body weight index recorded. Data is expressed as mean $\pm$ S.E.M. Statistical analysis is by ANOVA with Scheffe post-hoc correction * ($p<0.001$ vs control) # ($p<0.002$ vs Laparotomy).

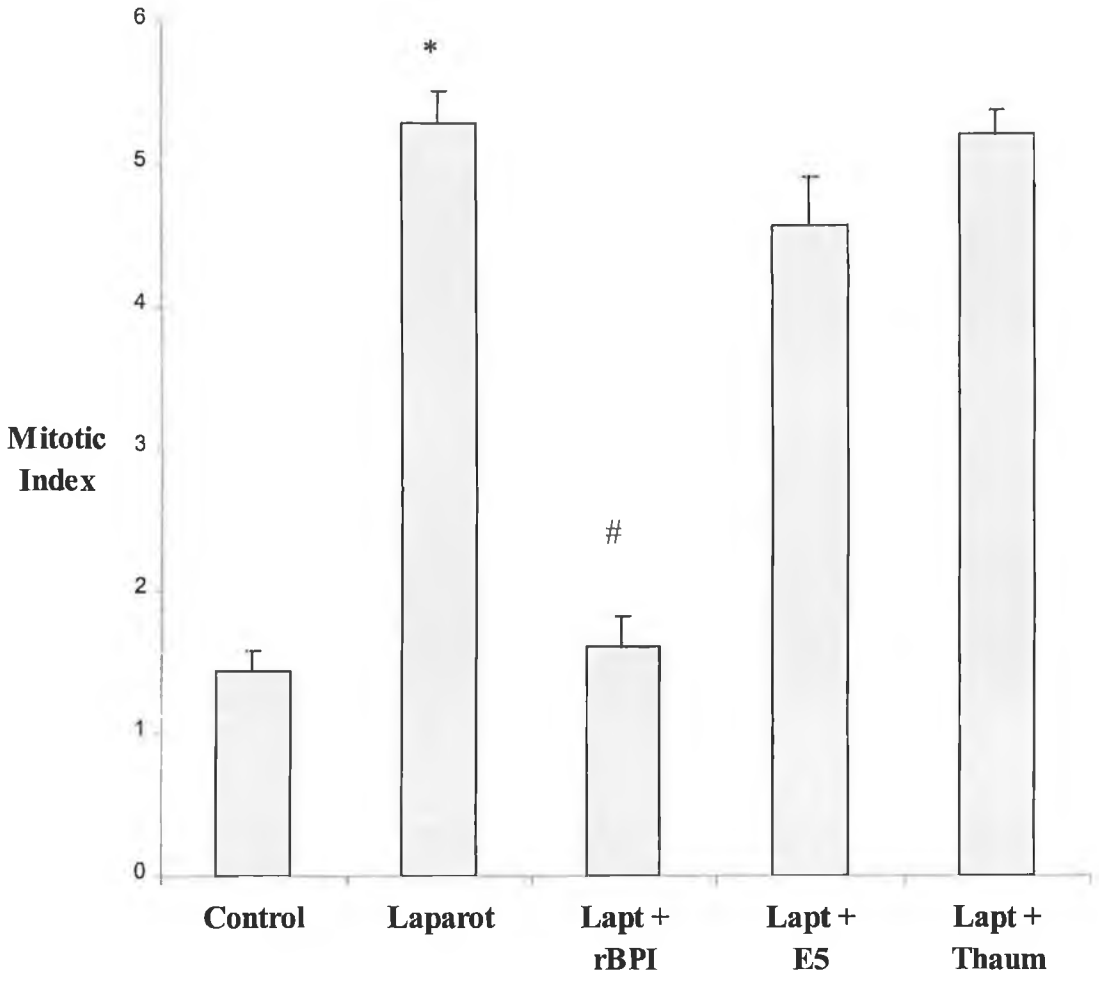
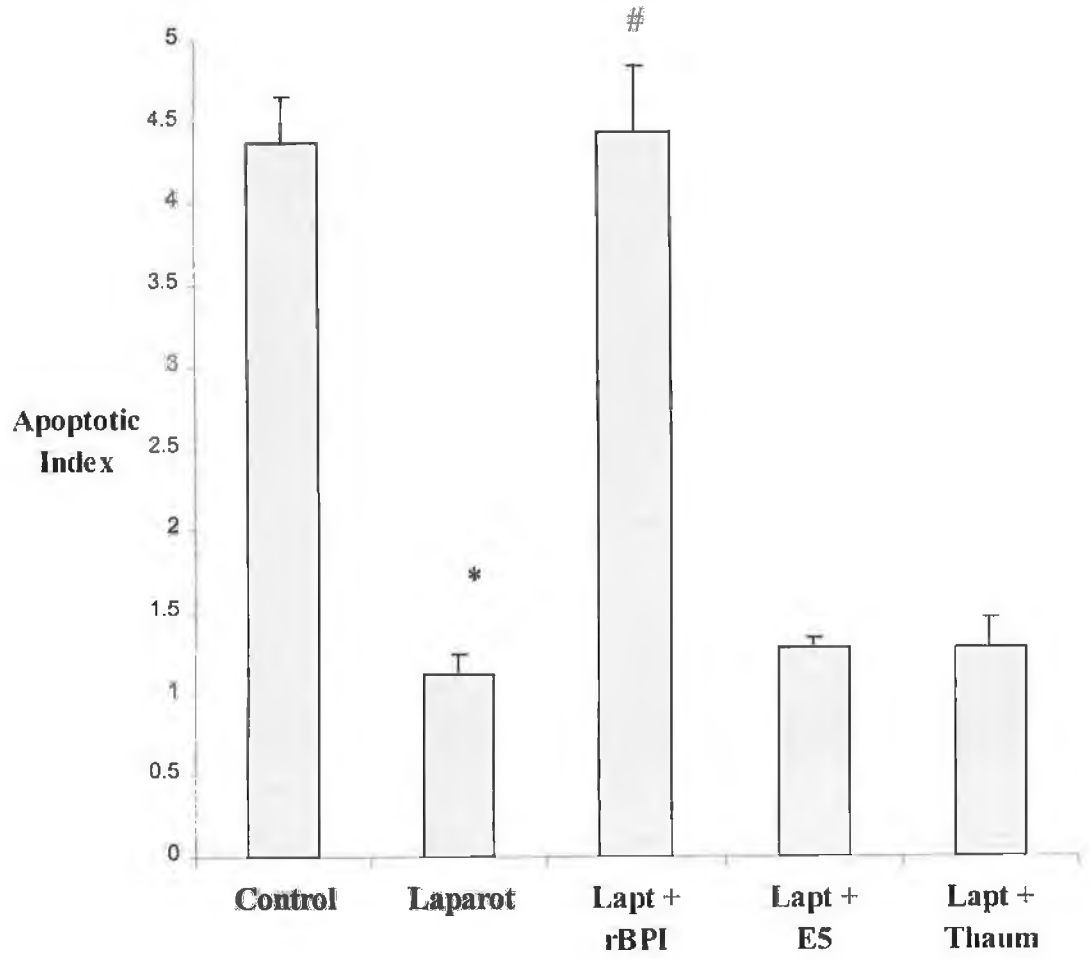
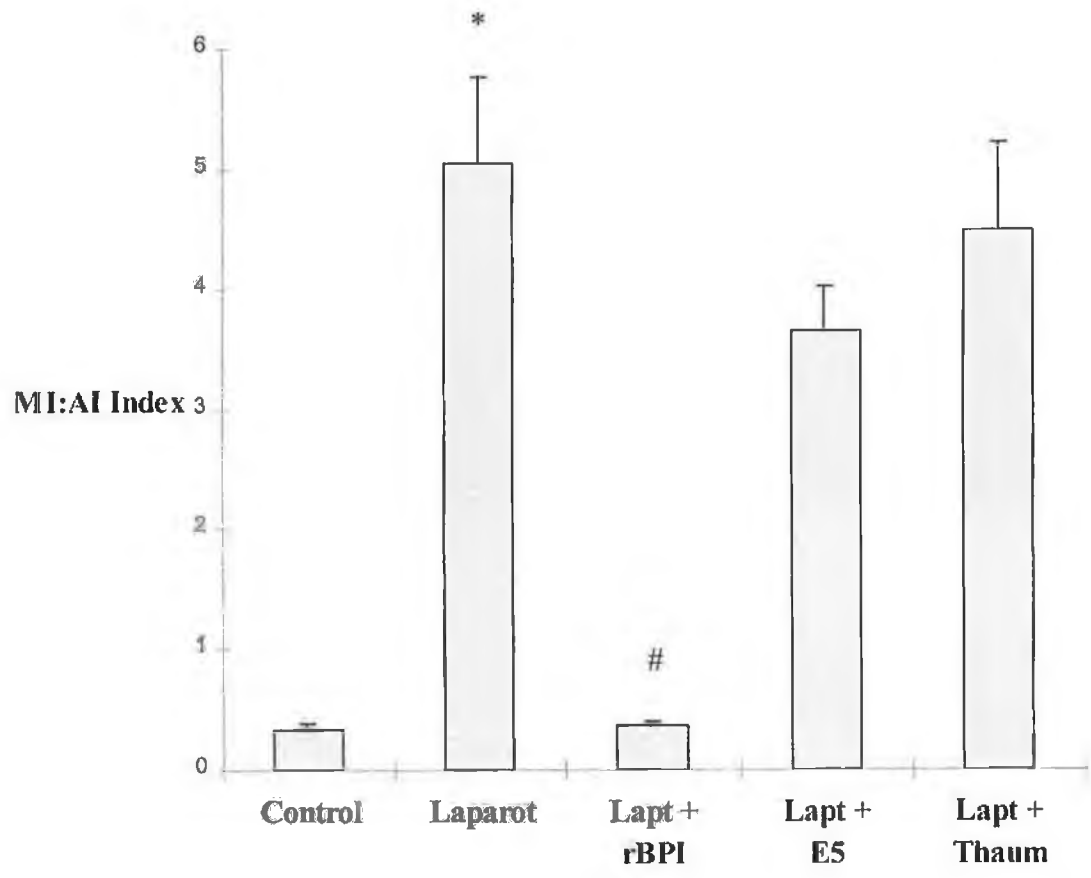


Fig. 4.9. Mice (n=5/group) received 10^5 4T1 cells by tail vein injection. 14 days later mice underwent a laparotomy or laparotomy combined with the anti-endotoxin agents rBPI, E5 or thaumatin control peptide. Control mice received anaesthetic only. Data is expressed as mean $\pm$ S.E.M (n=5/group). Statistical analysis is by ANOVA with Scheffe post-hoc correction. **A:** Mitotic index within metastases * ($p<0.001$ vs control) # ($p<0.001$ vs Laparotomy). **B:** Apoptotic index within metastases * ($p<0.001$ vs control) # ($p<0.001$ vs Laparotomy). **C.** Mitosis : Apoptosis ratios within metastases * ($p<0.001$ vs control) # ($p<0.001$ vs Laparotomy).

B.


Apoptotic Index
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
#
*
Control
Laparot
Lapt + rBPI
Lapt + E5
Lapt + Thaum

C.


MI:AI Index
6
5
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Control
Laparot
Lapt + rBPI
Lapt + E5
Lapt + Thaum

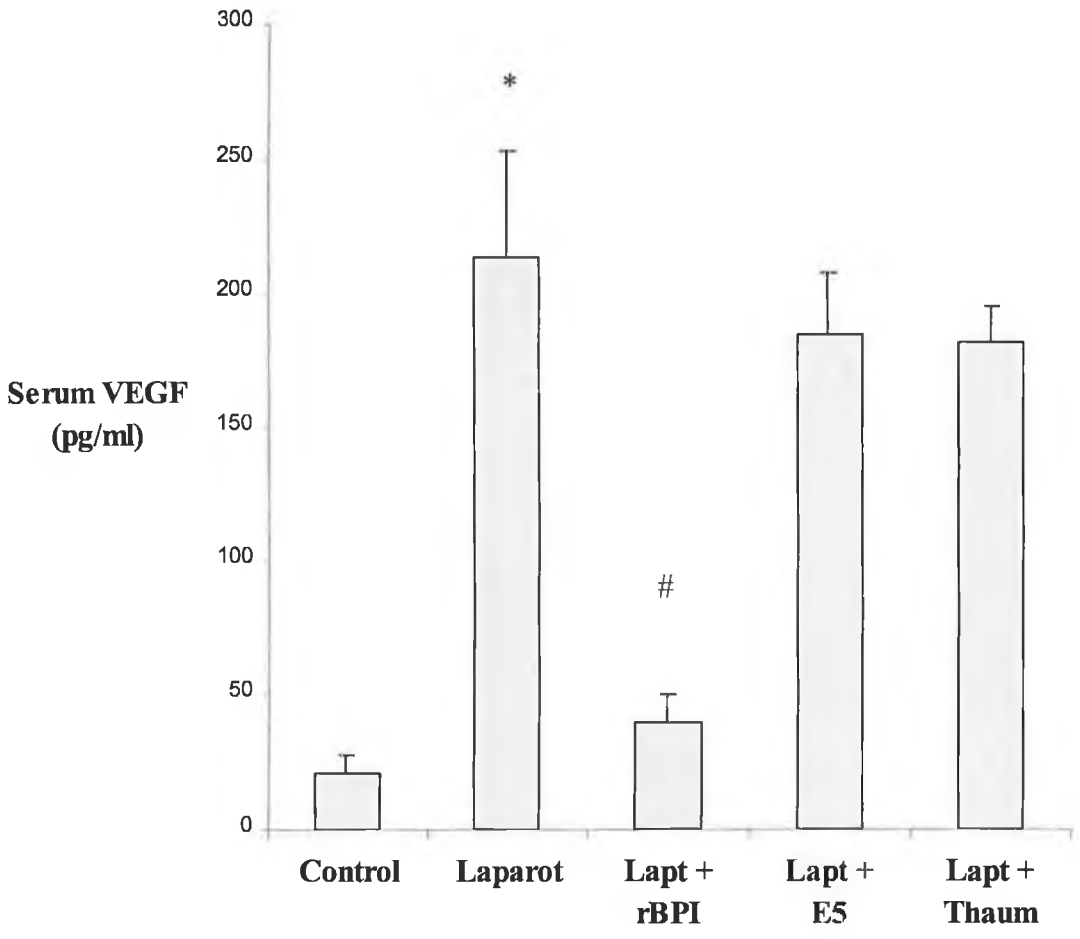


Fig 4.10: Effect of endotoxin blockade on surgically-induced serum VEGF. 5 days post-operatively, blood was obtained by cardiac puncture and VEGF assessed by ELISA. Data represent mean ± S.E.M. (n=5 / group). Statistical analysis is by ANOVA with Scheffe post-hoc correction * ($p<0.01$ vs control) # ($p<0.02$ vs Laparotomy).

4.3 Discussion

The data presented in this chapter show that endotoxin injection into the peritoneal cavity results in enhanced growth of established metastases in the lungs of mice. Following a dose response experiment, a concentration of 10 μg LPS / mouse was shown to result in significantly higher metastatic burden compared to saline controls, reflected by increased lung / body weight indices. This dose was well tolerated in mice, with no associated cachexia, and was within the clinically relevant doses described in previous reports (2 ng-20 μg/ml) (Mattsby-Baltzer *et al.*, 1994). In addition, this dose is within the range of those previously reported to induce angiogenesis in the mesentery window assay and increase bladder tumourigenesis *in vivo* (Mattsby-Baltzer *et al.*, 1994; Kawai *et al.*, 1993). The enhanced tumour growth in response to LPS was reflected by increased proliferation and also by decreased apoptosis within the lung metastatic deposits. The consequence of these effects tip the balances governing tumour growth in favour of tumour expansion, resulting in a 50% increase in metastatic burden in mice following LPS injection.

There are many possible mechanisms accounting for the tumour-enhancing effect of LPS. Endotoxin has previously been shown to increase the incidence of tumours in the bladders of rats pre-treated with *N*-methyl-*N*-nitrosourea (Kawai *et al.*, 1993). In this study the enhanced effects were associated with increased oxidative stress by the reactive oxygen species H_2O_2 in the bladder lumen, and a proliferative response of the carcinogen-exposed urothelium to the inflammatory stimulation. LPS may also increase the proliferation of the tumour cells directly, through the phosphorylation and activation of several protein kinases such as protein tyrosine kinase (PTK) and protein kinase C (PKC), which have been implicated in cellular proliferation and differentiation (Weinstein *et al.*, 1992; Shapira *et al.*, 1994). It is also possible that the increased

proliferation observed in these tumour metastases is a consequence of cytokines produced in response to LPS. LPS can induce the release of cytokines such as IL-1, IL-6 and TNF-α, which have been shown to be involved in the proliferation of keratinocytes and subsequent pathogenesis of psoriasis (Nickoloff *et al.*, 1991; Kupper, 1988). Indeed, TNF-α has been associated with increased tumour metastases in murine and human tumour models (Malik *et al.*, 1990; Orosz *et al.*, 1993). In addition, the cytokines elicited by LPS, such as TNF-α may be angiogenic and result in marked vascular proliferation in the stroma of tumours (Leibovich *et al.*, 1987).

In this study, injection of LPS resulted in a six-fold increase in circulating levels of the potent pro-angiogenic cytokine VEGF. In addition a strong positive correlation between post-operative levels of LPS and serum VEGF was identified in previous experiments, described in chapter 3. Taken together these data suggest that LPS has a pro-angiogenic effect in 4T1 lung metastases through the induction of VEGF. On the other hand, levels of circulating VEGF may simply reflect the increased tumour burden observed in mice following LPS injection. However, the fact that a six-fold increase was observed in circulating levels, compared to only a 50% increase in metastatic burden, would suggest that tumour bulk alone is not responsible for the elevated levels of this angiogenic factor. These observations, coupled with the knowledge that LPS induces VEGF expression in human pulp cells (Matsushita *et al.*, 1999) suggest that LPS may regulate VEGF production by certain cell types that could influence tumour growth.

The cascade of biological events induced by LPS is composed of a large number of mediators interacting within a complex network. A number of therapeutic treatments targeted against LPS have sought to neutralise one or a combination of the cytokine mediators induced by LPS. Antibodies to TNF-α have been shown to protect mice from

the lethal effects of lipopolysaccharide and bacterial administration (Beutler *et al.*, 1985; Tracey et al., 1987). It is clear that a more complete effect might be seen by averting the harmful effects of LPS through the blockade of lipopolysaccharide itself, preventing the initiation of the cytokine cascade. The potential value of the novel anti-LPS agent, $rBPI_{21}$, in preventing LPS-induced tumour growth was evaluated. Previous studies have reported that the 25-kDa N-terminal portion of BPI possesses essentially all of BPI's LPS-neutralising activity (Ooi *et al.*, 1987), inhibiting LPS-induced cytokine release and oxygen free-radical generation from whole human blood (Mezaros *et al.*, 1993), as well as nitric oxide release from human macrophages (Betz-Corradin *et al.*, 1994). As all of these factors are potential mechanisms whereby LPS may influence tumour growth and angiogenesis, therefore $rBPI_{21}$, a recombinant form of BPI, was considered a strong candidate for the prevention of LPS-induced tumour growth. In the model of metastatic disease used in these studies, $rBPI_{21}$ therapy was capable of completely blocking LPS-induced metastatic tumour burden, at least in part by inhibiting LPS-induced proliferation within metastases and LPS-induced reductions in apoptosis within the same metastases. Treatment with $rBPI_{21}$ markedly reduces the pathological effects of LPS in endotoxemia and septic shock (Ammons *et al.*, 1996), however the data described here indicate that $rBPI_{21}$ is also capable of inhibiting the biological effects of LPS on tumour growth. To ensure that the observations made were specific for LPS-blockade, a group receiving therapy at each time point with a control peptide of similar size to $rBPI_{21}$, thaumatin, was included in each experiment. No protective effects were observed in any of the parameters examined relating to tumour growth, namely metastatic burden, mitosis or apoptosis within metastasis. In vivo, in addition to its bactericidal activity, BPI can also neutralise LPS released from bacteria. LPS-binding proteins such as LBP and BPI have been shown to function as a receptor/receptor-antagonist system to regulate the hosts

response to LPS (Marra *et al.*, 1992), as described in Fig. 1.4. The therapeutic administration of recombinant BPI should enhance the natural ability to control the bodies responses to endotoxin.

Previous data (described in chapter 3) demonstrated that surgery resulted in increased metastatic tumour growth. In these experiments elevated plasma endotoxin levels were detected in mice following surgery, with the highest levels detected in mice having received laparotomy with intestinal agitation as compared to anaesthetic controls. Abdominal contamination with airborne factors during surgery has been shown to result in immunological alterations and bacterial translocation in mice (Watson *et al.*, 1995). Treatment with $rBPI_{21}$ peri-operatively and post-operatively at 4 h intervals over a further 24 h blocked the surgically-induced metastatic growth observed following laparotomy. The effectiveness of the specific anti-LPS agent in blocking tumour growth clearly indicates that LPS is a major contributing factor to increased tumour growth observed following open surgery.

The lipid-A domain of endotoxin is thought to be the active component of the molecule (Galanos *et al.*, 1984). Antibodies directed against lipid A, such as the murine monoclonal antibody E5, have been developed and used in studies and clinical trials for sepsis. Although survival rates in some patients with gram-negative bacteremia and septic shock was reported, these studies failed to demonstrate adequate efficacy to warrant approved use because of insufficient binding potency of the antibodies (Greenman *et al.*, 1991; Schedel *et al.*, 1991; Greenberg *et al.*, 1992). There is also the added complication of allergic reactions to murine antibody therapy in some patients. In this study, therapy with E5 failed to show any protective effect against surgically-induced tumour growth. The likely reason for its lack of efficacy is through insufficient binding of LPS as previously described. There is another possible reason why $rBPI_{21}$ is more

effective at neutralising LPS-induced tumour growth than E5. It has been reported that BPIs inhibitory effects on bacterial cell growth diminished with increasing polysaccharide chain length (Jahr *et al.*, 1995). Long hydrophilic polysaccharide chains may impede access of the bulky BPI protein to the lipid A site that is embedded in the outer membrane of the gram-negative bacterial envelope, while the smaller recombinant BPI (21 KD) fragment can possibly penetrate more easily. The larger E5 antibody may also encounter steric hindrance preventing access binding to the lipid A site in endotoxin derived from endogenous bacteria. The failure of the E5 therapy in this study could therefore be as a result of the inability of the antibody to reach and neutralise the lipid A moiety of the bacteria. There is also the potential that the antibodies were cleared by the murine host defenses, which would be unlikely to occur following rBPI treatment because the protein is naturally occurring in the host.

It was also reported in the same study, that BPI binds to a site close to lipid A, with minimal binding observed to monophosphoryl lipid A (Jahr et al., 1995). Results indicated that rBPI had more neutralising activity on LPS with long polysaccharide chains and that it may have a higher affinity for smooth LPS than for the lipid A region, indicating that it may possibly interact with the polysaccharide part of the molecule. If this is the case, then anti-lipid A antibodies may be of little effect in controlling LPS induced tumour growth as the mechanisms controlling these effects may not be solely due to the lipid A region of the molecule.

$rBPI_{21}$ therapy also prevented increased serum VEGF levels in response to open surgery, and appeared to lower VEGF levels following LPS injection. In the case of mice receiving LPS injection, $rBPI_{21}$ failed to significantly reduce VEGF levels. However, treatment with $rBPI_{21}$ resulted in a 50% reduction in serum VEGF and it is highly likely that the failure to reach significance is due to the lower numbers of mice in the study,

compared to other experiments. This is confirmed by the fact that injection of 10 μg of LPS failed to significantly increase serum VEGF in this experiment, while the same treatment led to significance with more numbers. Endotoxin has previously been reported to be angiogenic in a number of experimental models (Li *et al.*, 1991; BenEzra *et al.*, 1993; Mattsby-Baltzer *et al.*, 1994). Increased angiogenesis within tumour stroma has also been reported in bladder tumorigenesis associated with endotoxin-induced urinary tract infection (Johansson *et al.*, 1987; Kawai *et al.*, 1993). By reducing VEGF, $rBPI_{21}$ may act as a potential anti-angiogenic agent by blocking LPS-induced induction of angiogenic cytokines in the post-operative period. E5 therapy failed to reduce surgically induced serum VEGF levels.

In clinical situations many patients undergo extensive antibiotic therapy in response to severe bacterial infections. Anti-LPS agents such as $rBPI_{21}$ could be most beneficial in these patients because, although antibiotics kill the organisms, the bacteria destroyed during such therapy release breakdown products that include lipopolysaccharide that could influence tumours that would otherwise be in a state of clinical dormancy. Similarly, it may be used in the post-operative treatment of patients to prevent the pro-metastatic and pro-angiogenic environment that is created in response to LPS released from endogenous bacteria or introduced from air.

Chapter 5

Regulation of VEGF and angiogenesis by LPS.

5.1 Introduction

Tumours are composed of a heterogenous population of both host and tumour cells. In addition to endothelial cells, which form the basic unit of blood vessels within tumours, there are other host cells present including fibroblasts and a variety of immune cells. These host cells may actually contribute to the growth of the tumour through the release of factors which regulate proliferation and angiogenesis within the tumour.

Macrophages form a major component of the immune infiltrate of many solid tumours, and in the case of breast tumours they may account for up to 50% of the tumour mass (O'Sullivan and Lewis, 1994). Macrophages can mediate tumour cytotoxicity but many tumour cells are capable of suppressing this effect through the secretion of factors such as TGF-β1, interleukin-10 (IL-10) and prostaglandin E_2 (PGE_2) (Elgert *et al.*, 1998). Macrophages have also been associated with multiple steps in the metastatic process, forming aggregates with tumour cells in the circulation they could enable the cells to avoid immune recognition and aid invasion (Van Netten *et al.*, 1993). They can also stimulate angiogenesis through their secretory activity (Leibovich *et al.*, 1987; Sunderkotter *et al.*, 1991; Sunderkotter *et al.*, 1994). Conditioned medium of tumour associated macrophages, isolated from experimental murine fibrosarcomas, induced endothelial cell proliferation *in vitro* and angiogenesis *in vivo* (Polverini and Leibovich, 1984). Furthermore, experimental murine fibrosarcomas were shown to be less vascularised when mice were depleted of monocytes (Evans, 1977). The angiogenic activity of macrophages requires the specific activation of the cells. Lipopolysaccharide (LPS) or endotoxin is the most potent stimulator of macrophages known, inducing the release of a number of factors with potential angiogenic activity including TNF-α, TGF-β, IL-6 and bFGF (O'Sullivan *et al.*, 1993; Leibovich *et al.*, 1987; Lewis *et al.*, 1995;

Koch., 1998). Endotoxin has been reported to be angiogenic and it has been suggested that its angiogenic activity may be mediated through monocyte activation and cytokine production (Mattsby-Baltzer *et al.*, 1994).

Macrophages may also regulate angiogenesis through the production of angiogenesis inhibitors. They are the source of metalloelastase which cleaves plasminogen into the anti-angiogenic fragment angiostatin (Dong *et al.*, 1997). LPS stimulation of macrophages suppresses the release of metalloelastase, resulting in reduced production of angiostatin (Kumar *et al.*, 1996; Dong *et al.*, 1997). By reducing anti-angiogenic molecules, LPS may tip the balance in favour of angiogenesis. In addition to downregulating anti-angiogenics, LPS stimulates VEGF production by a variety of cell types including human pulp cells, ventricular myocytes, polymorphonuclear leukocytes and 4T1 murine tumour cells (Matsushita *et al.*, 1999; Sugishita *et al.*, 2000; Scapini *et al.*, 1999; McCourt *et al.*, 1999; Pidgeon *et al.*, 1999). In endometriosis patients, LPS stimulation of peritoneal macrophages increased VEGF secretion by these cells (McLaren *et al.*, 1996). A similar effect, namely increased VEGF secretion in response to LPS, has also been reported in macrophages isolated from cirrhotic patients (Perez-Ruiz, 1999). More recently, a separate study reported that LPS activation of monocytes from healthy volunteers increased VEGF mRNA, but not bFGF expression (Torisu *et al.*, 2000). This study also reported that anti-TNF-α monoclonal antibodies inhibited LPS-induced VEGF expression by monocytes, suggesting that in this case, the effects of LPS are mediated by TNF-α. There is also evidence that macrophage mediated angiogenesis is due, at least in part, to TNF-α production by these cells (Leibovich *et al.*, 1987).

LPS could potentially stimulate angiogenesis by acting directly on the endothelial cells. It is known that endotoxin, and its associated cytokines, have profound effects on

the vascular endothelium during sepsis, resulting in vascular injury and hyperpermeability (Morrison and Ryan, 1987; Pober and Cotran, 1990). Whereas LPS mediates its effects on monocytes, macrophages and neutrophils through the CD14 receptor (Wright *et al.*, 1990), endothelial cells do not express the CD14 receptor on their surface. LPS induced vascular endothelial cell injury and activation, an effect that could be blocked by the anti-endotoxin agent rBPI (Arditi *et al.*, 1994). A recent study reported that LPS enhanced VEGF production by human endothelial cells (Marx *et al.*, 1999). Thus LPS has multiple effects on host cells, inducing macrophage and neutrophil mediated angiogenesis, suppressing the production of angiogenesis inhibitors and altering the host vasculature.

From the literature, it was unclear whether the angiogenic effect of LPS was mediated directly or indirectly through its repertoire of cytokines. The aim of this chapter was to investigate the mechanisms through which LPS exerts its angiogenic activity. As macrophages are the major immune infiltrate in human tumours, the effect of LPS on the expression of the angiogenic factors, VEGF, bFGF and TGF-β was examined. As LPS has been shown to be angiogenic in a number of model systems, the direct effect of LPS on endothelial cell proliferation and apoptosis was investigated. The effect of LPS on tumour cell proliferation was also examined. An in-vivo Matrigel™ model of angiogenesis was used to establish whether LPS is directly angiogenic.

5.2 Results

5.2.1 LPS upregulates VEGF and TGF-β mRNA in human monocyte-derived macrophages (MDMs).

Monocytes isolated from healthy volunteers were matured to macrophages, activated with interferon-γ and treated with LPS (1 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml) for 18 h. VEGF, TGFb-1 and bFGF expression were examined by reverse transcriptase PCR (RT-PCR) with commercial PCR primers. RT-PCR resulted in a 204 bp product from all VEGF isoforms. LPS treatment resulted in a dose dependent increase in VEGF expression by macrophages (lane 7 - 4) compared to untreated controls shown in lane 8 (Fig. 5.1A). VEGF expression is increased above the dose of 10 ng/ml (lane 6), while treatment with 1 ng/ml (lane 7) has little effect. The absence of a band in lane 3 indicates that amplification of this sample didn't occur, due to the omission of PCR primers.β-actin was also amplified by PCR to serve as a control on loading differences (Fig. 5.1D). VEGF mRNA levels were normalised against the constitutively expressed β-actin and expressed as the ratio between VEGF and β-actin PCR products (Fig 5.1E). Treatment with LPS also resulted in a dose dependent increase in TGF-β (Fig 5.1B), however the increase was not as strong as that noted with VEGF. No increase in bFGF expression was noted in response to LPS treatment in human macrophages (Fig. 5.1C).

5.2.2 Effect of LPS on VEGF protein expression in human MDMs.

The effect of LPS on VEGF protein production by human monocyte-derived macrophages was examined by ELISA (n=4). MDMs were activated with interferon-γ and treated with LPS (1 ng/ml, 10 ng/ml, 100 ng/ml, 1 μg/ml and 10 μg/ml) for 24h. As observed at the mRNA level, LPS resulted in a statistically significant increase in VEGF protein

producton by macrophages at concentrations of 1 μg/ml (42.66±2.91 pg/μg protein) and 10 μg/ml (64.91±5.79 pg/μg protein) compared to untreated cells (13.61±2.83 pg/μg protein, $p<0.05$) (Fig 5.2).

5.3.3 Effect of LPS on tumour cell proliferation.

As LPS increased metastatic tumour growth in the *in-vivo* 4T1 tumour model (chapter 4), the direct effect of LPS on the proliferation of both 4T1 and the human mammary adenocarcinoma MDA-MB-231 cells was examined. Two proliferation assays were used and compared in 4T1 cells, described in section (2.2.12). The results from both assays were similar with LPS causing a dose-dependent increase in tumour cell proliferation relative to untreated controls. This increase in proliferation was statistically significant at 1 μg/ml LPS (114%±2.98) or 10μg/ml LPS (133.65%±3.60) relative to controls ($p<0.01$) with the BrdU based assay (Fig. 5.3A). Similarly, in the tetrazolium dye-based proliferation assay (MTT) concentrations of 1 μg/ml LPS (109%±0.71) or 10 μg/ml LPS (124.68%±1.96) resulted in significantly higher proliferation relative to controls ($p<0.01$) (Fig. 5.3B).

As the metabolic based proliferation assay was more economical and provided similar results, it was used for all subsequent proliferation assays. In human MDA-MB-231 cells, LPS also increased cell proliferation reaching statistical significance at 100 ng/ml (116.2 ± 4.16%), 1 μg/ml (121.1±0.645%) and 10 μg/ml LPS (128.8±0.484%) ($P<0.05$, Fig. 5.4).

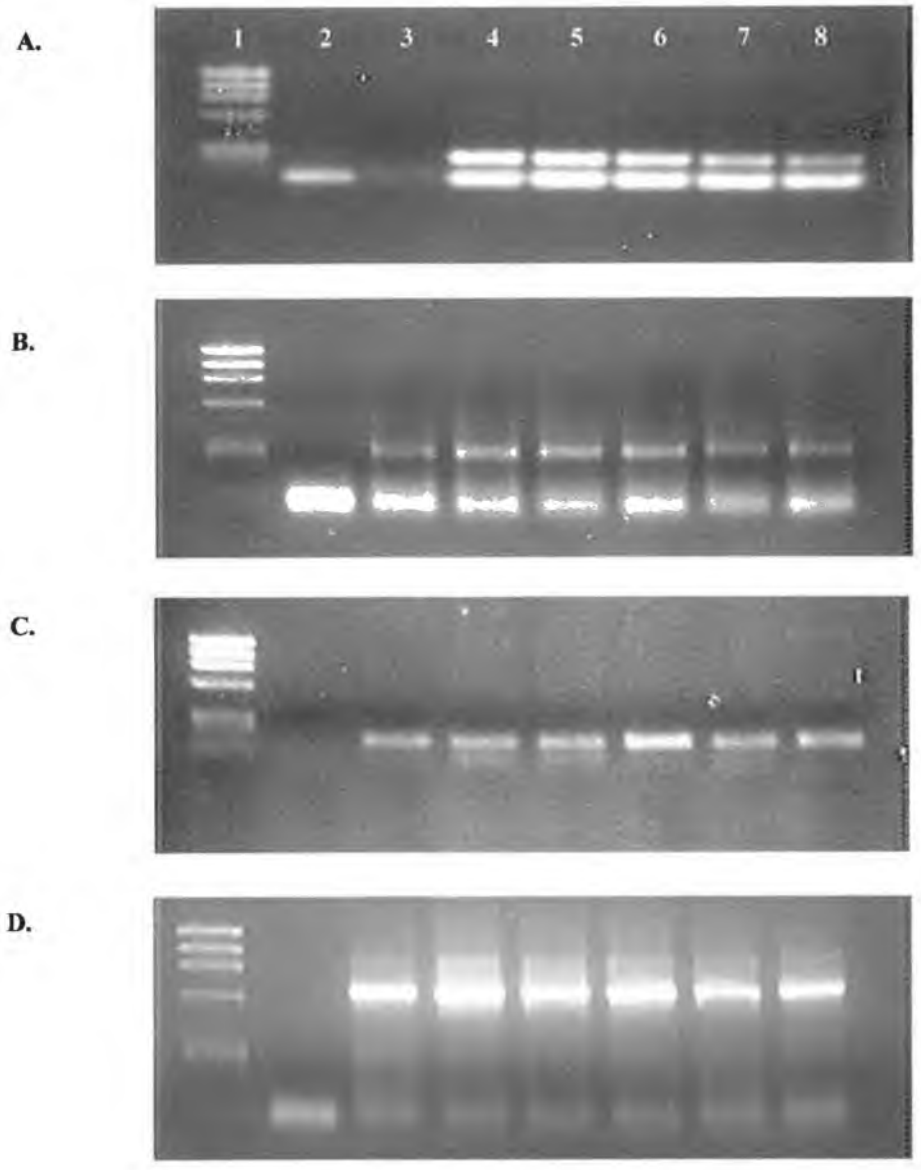


Fig. 5.1. VEGF, TGF-b and bFGF expression in MDMs in response to LPS. Cells were incubated for 18 h and expression assessed by RT-PCR. Lane 1 shows molecular weight markers ϕX174DNA digested with *Hae*III, lane 2 is a negative control; lanes 3-8 are 200, 100, 50, 10, 1 and 0 ng/ml LPS respectively. Positions of PCR products are indicated. **A.** VEGF mRNA expression. **B.** TGF-β mRNA expression. **C.** bFGF mRNA expression. **D.** β-Actin mRNA expression. **E.** Densitometric analysis of VEGF relative to β-actin.

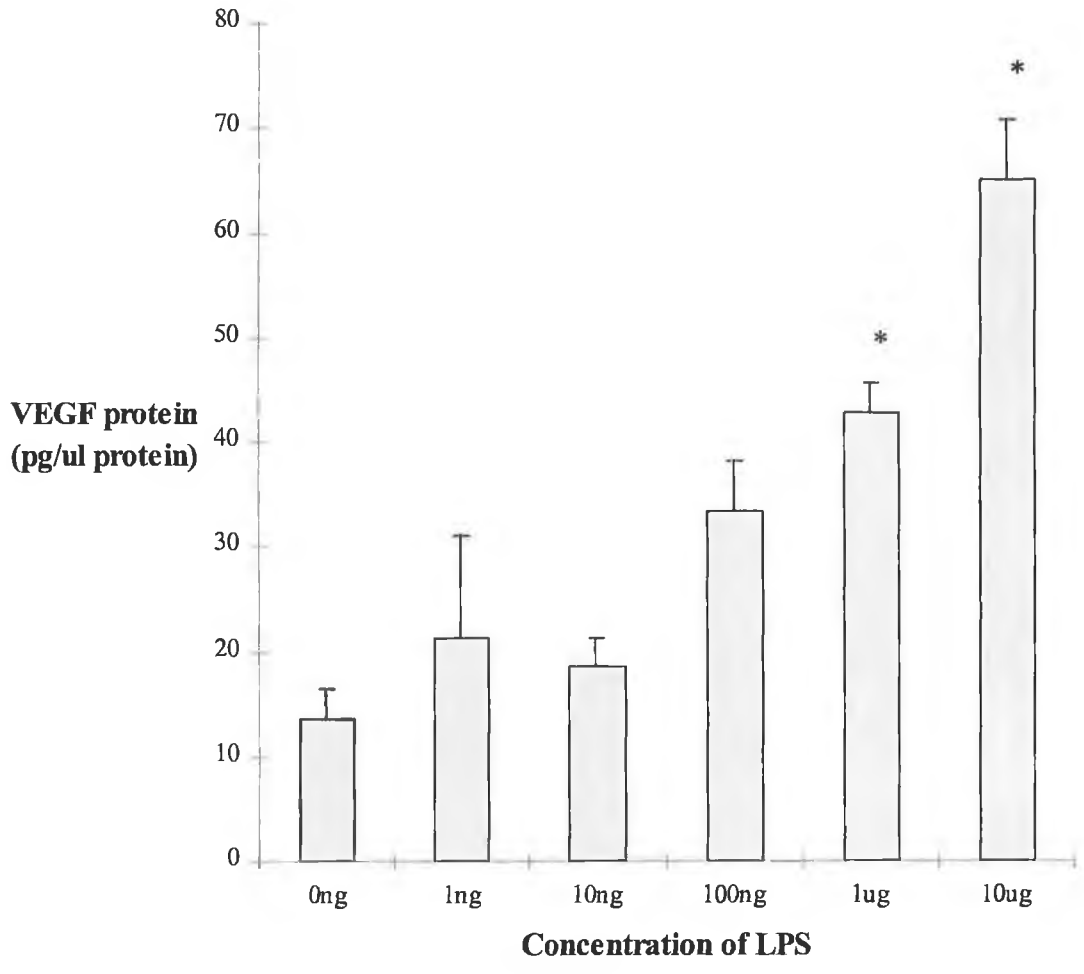


Fig. 5.2 VEGF protein levels in human macrophages exposed to LPS. Human monocytes were isolated, matured to macrophages, activated with IFN_{γ} and stimulated with LPS (1 ng/ml-10 μg/ml) for 24h. VEGF protein production was assayed by ELISA and total cell protein by the BCA assay. Results are expressed as pg VEGF/ mg total protein and shown as mean $\pm$ S.E.M (n=4). Statistical analysis is by ANOVA with scheffe post-hoc correction * ($p<0.05$, vs control)

5.3.4 Effect of TNF-α on tumour cell proliferation.

A number of studies suggest that LPS may mediate its tumourigenic effect, inducing angiogenesis, via the induction of TNF-α (Leibovich *et al.*, 1987; Torisu *et al.*, 2000). To examine if increased tumour cell proliferation in response to LPS could be due to TNF-α secretion, the effect of TNF-α (100 pg/ml - 1 μg/ml) on proliferation of the MDA-MB-231 cells was examined (n=4). These levels of TNF-α are within the physiological levels observed in Balb/C mice (1522±97.1 pg/ml) following LPS injection induced by LPS (unpublished observations, Harmey). In direct contrast to LPS stimulation of proliferation, TNF-α resulted in a dose-dependent decrease in proliferation at 100 ng/ml (89±3.0%) or 1 μg/ml (79±3.7%) ($p<0.02$) relative to untreated controls (Fig. 5.5). This reduction in cell numbers may be due to the cytotoxic effects associated with TNF-α rather than a reduction in the proliferation rate of the cells *per se*, as the metabolic based tetrazolium dye assay does not distinguish between a reduction in proliferation and cytotoxicity. This is supported by the fact that the cells looked stressed at these concentrations, losing their characteristic morphology.

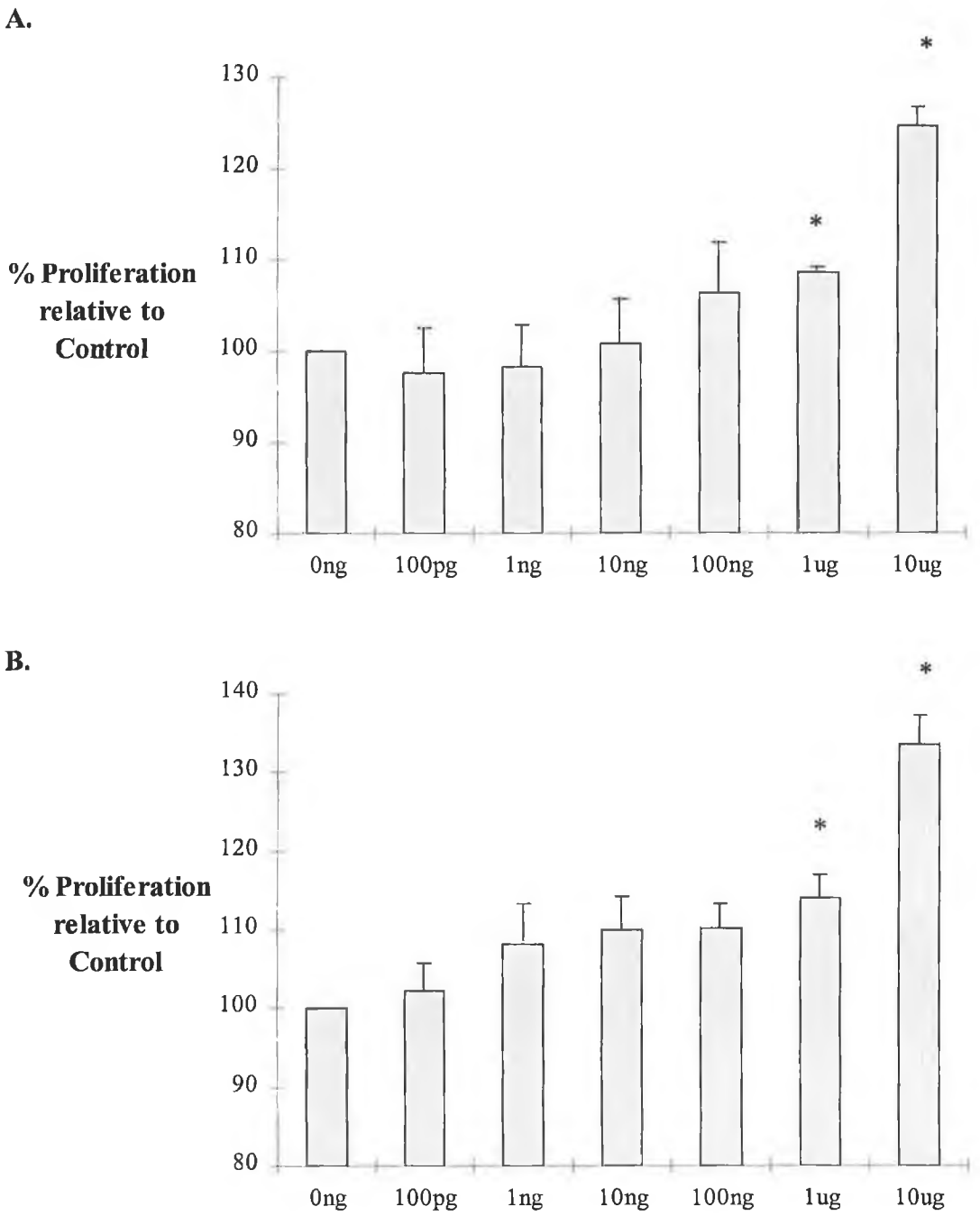


Fig. 5.3 Effect of LPS on 4T1 cell proliferation. Cells were stimulated with LPS (100 pg/ml-10$_{\mu}$g/ml) for 48 h. Tumour cell proliferation was assessed by a metabolic tetrazolium dye assay (**A**) and a BrdU based assay (**B**). Data is representative of 4 independent experiments and expressed as mean $\pm$ S.E.M where control cells are taken as 100%. Statistical analysis is by ANOVA with scheffe post-hoc correction * ($p<0.05$ vs control).

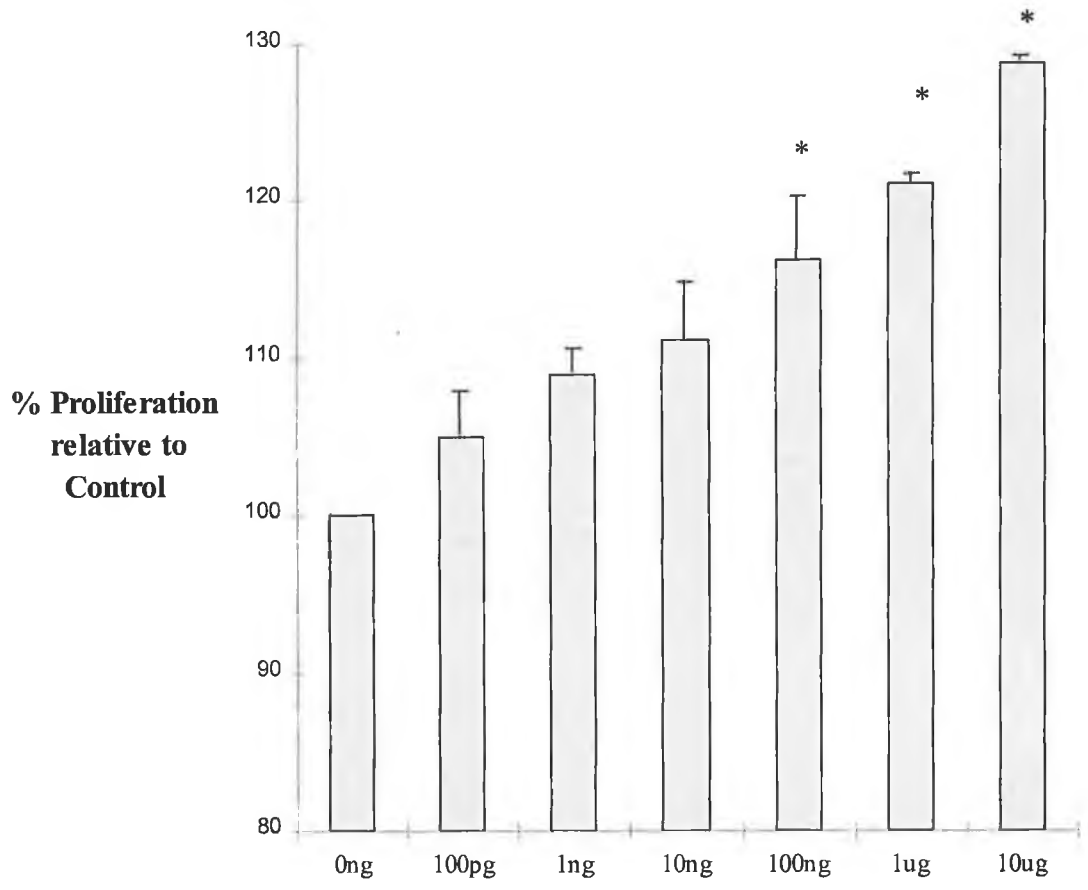


Fig. 5.4 Effect of LPS on proliferation of MDA-MB-231 cells. Cells were stimulated with LPS (100 pg/ml-10μg/ml) for 48 h. Tumour cell proliferation was assessed by the metabolic tetrazolium dye assay. Data is representative of 4 independent experiments and expressed as mean $\pm$ S.E.M where control cells are taken as 100%. Statistical analysis is by ANOVA with scheffe post-hoc correction * ($p<0.05$ vs control).

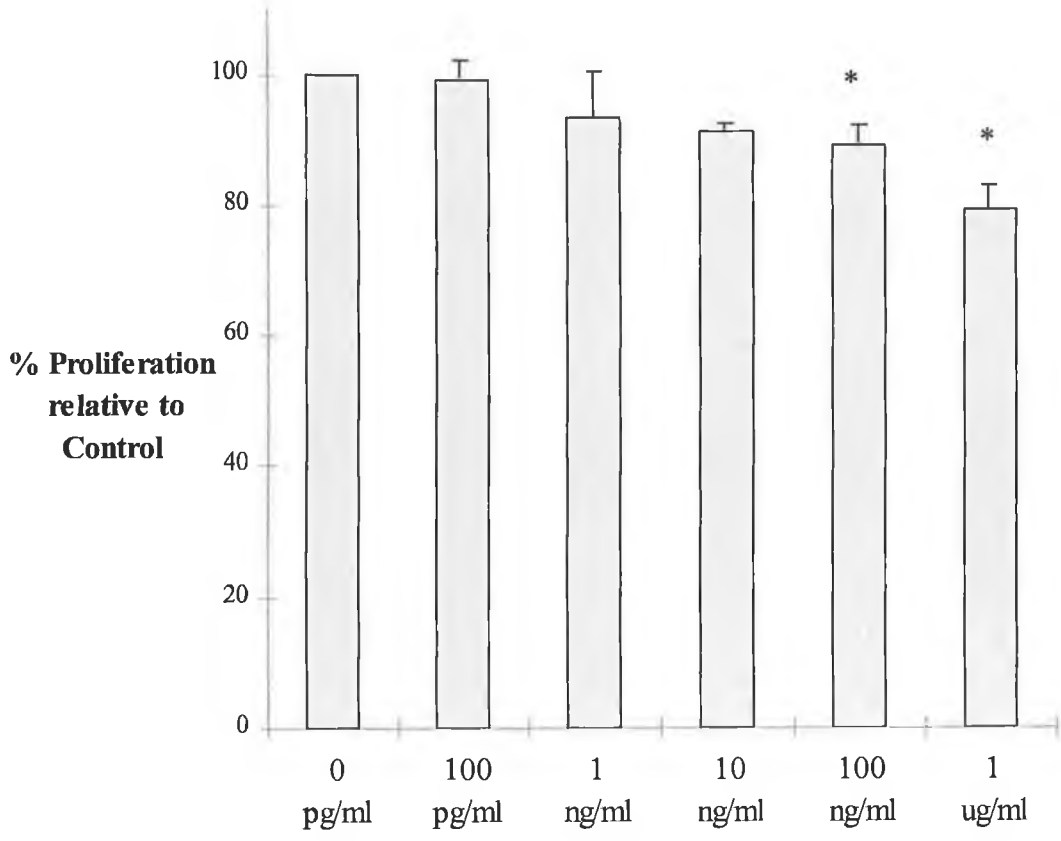


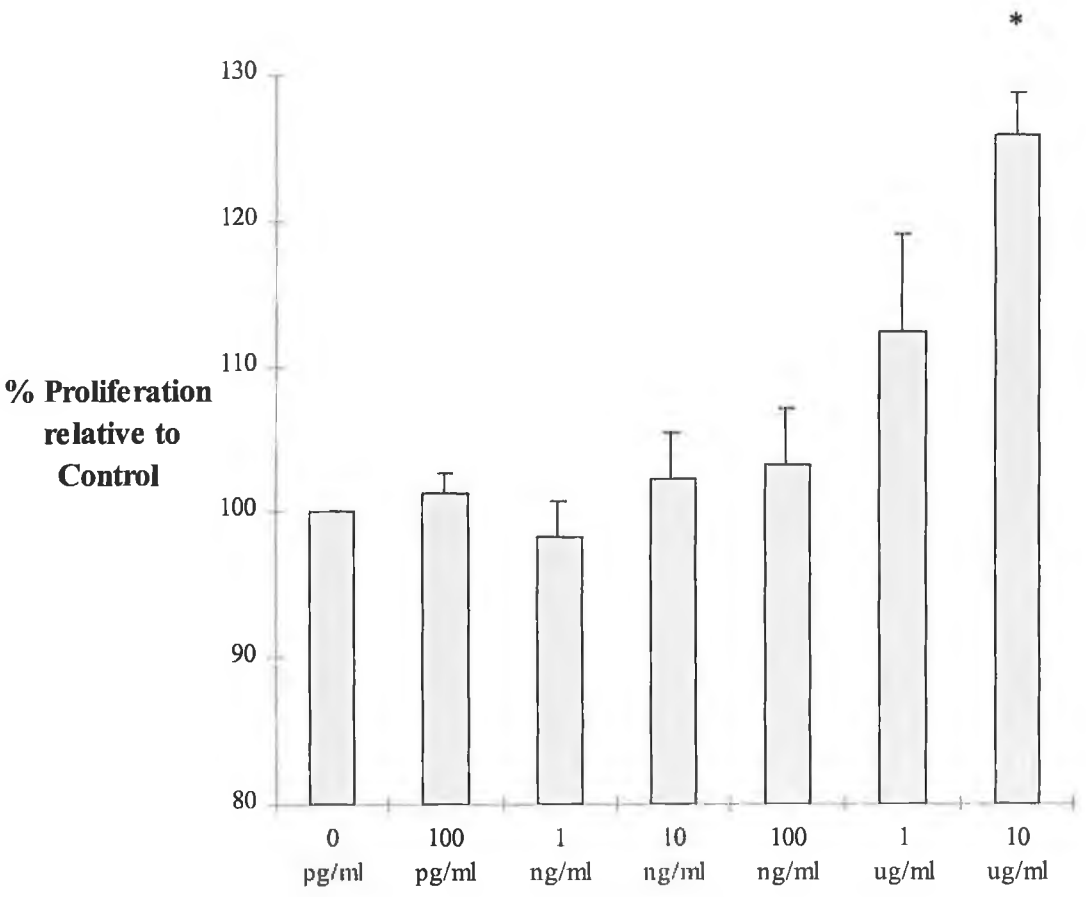
Fig. 5.5 Effect of TNF-α on proliferation of MDA-MB-231 cells. Cells were treated with TNF-α (100 pg/ml-1 μg/ml) for 48 h. Tumour cell proliferation was assessed by the metabolic tetrazolium dye assay. Data is representative of 4 independent experiments and expressed as mean $\pm$ S.E.M where control cells are taken as 100%. Statistical analysis is by ANOVA with scheffe post-hoc correction * ($p<0.02$ vs control).

5.3.5 Effect of LPS on HUVEC proliferation.

LPS has been shown to be angiogenic in a number of animal experimental model systems (Mattsby-Baltzer *et al.*, 1994; Li *et al.*, 1991). Like tumour growth, the growth of blood vessels is governed by both the rate of proliferation and cellular death (apoptosis) of the endothelial cells (Duke *et al.*, 1996). Human umbilical vein endothelial cells (HUVECs) were freshly isolated, grown in 96-well plates and treated with various concentrations of LPS (100 pg/ml - 10 μg/ml) for 48 h. Thereafter the percentage proliferation relative to untreated control cells was examined using the tetrazolium dye assay based on metabolic activity of the cells. Treatment with 10 μg/ml (125.8±2.82 %) resulted in significantly higher proliferation compared to controls ($p<0.02$) (Fig. 5.6). This result indicates that LPS induces the proliferation of human endothelial cells over a 48 h period, either directly or through the release of other cytokines by the cells.

5.3.6 Effect of LPS on HUVEC apoptosis.

Having illustrated that LPS increases proliferation of HUVECs, the effect of LPS on the other side of the balance governing cell numbers, namely apoptosis, was examined by TUNEL staining. HUVEC cells were freshly isolated, grown in culture chamber slides and treated with various concentrations of LPS (100 ng/ml, 1 μg/ml and 10 μg/ml) for 18 h, and stained thereafter. Treatment with 1 μg/ml (19.68±1.7%) and 10 μg/ml (16.41±1.7%) significantly reduced HUVEC apoptosis relative to controls ($p<0.01$, 44.46±2.2%) (Fig. 5.7) indicating that LPS confers a survival advantage on endothelial cells, which would contribute to the angiogenic nature of LPS. Thus LPS is directly pro-angiogenic, affecting both sides of the balance, increasing proliferation and decreasing apoptosis of endothelial cells.



0**Fig. 5.6** Effect of LPS on proliferation of HUVECs. Cells were stimulated with LPS (100 pg/ml-10 μg/ml) for 48 h and proliferation assessed by the metabolic tetrazolium dye assay. Data is representative of 4 independent experiments and expressed as mean $\pm$ S.E.M. Statistical analysis is by ANOVA with scheffe post-hoc correction * ($p<0.02$ vs control).

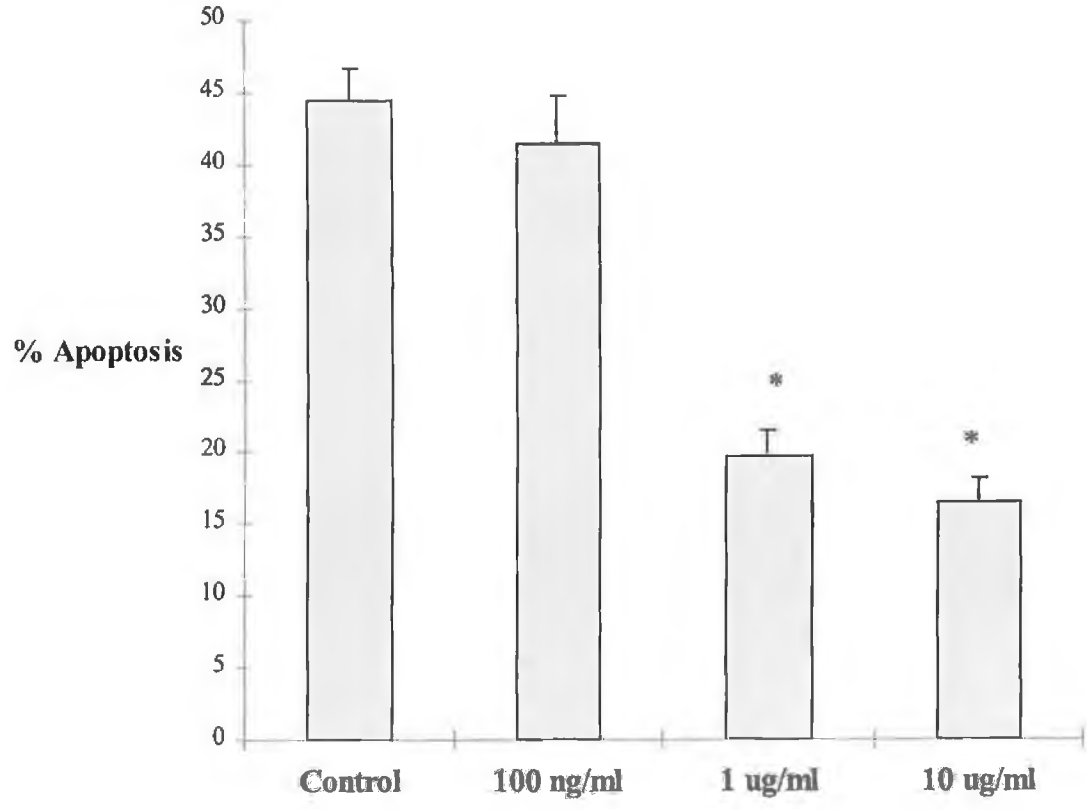


Fig 5.7 Effect of LPS on HUVEC apoptosis on culture chamber slides. Cells were treated with LPS for 18 h and apoptosis was assessed *in situ* by TUNEL staining Data is representative of 3 independent experiments and expressed as mean ± s.e.m. Statistical analysis is by ANOVA with scheffe post-hoc correction * ($p<0.01$).

5.3.7 Effect of TNF-α on HUVEC proliferation.

A number of reports have suggested that much of the angiogenic effects of LPS have been attributed to its induction of TNF-α secretion (Torisu *et al.*, 2000). There have also been reports that macrophage mediated angiogenesis is mediated by TNF-α (Leibovich *et al.*, 1987). To examine if the proliferative effect of LPS on endothelial cells is mediated by TNF-α secretion, the effect of treatment with TNF-α (100 pg/ml - 1 μg/ml) on proliferation of HUVECs was examined (n=4). TNF-α had no effect on HUVEC proliferation at any of the doses tested (Fig. 5.8). Unlike the results observed for tumour cells, there was no cytotoxic effect or growth inhibition observed at any dose. This indicates that TNF-α does not mediate LPS-induced proliferation of HUVEC or tumour cells but can inhibit the growth of MDA-MB-231 cells at high levels. The effect of TNF-α on HUVEC or tumour cell apoptosis was not examined because TNF-α, as its name suggests, causes death by necrosis and not by apoptosis, which is the mode of death favoured by tumour cells.

5.3.8 Effect of LPS on HUVEC and tumour cell proliferation in the absence of FCS.

It is recognised that LPS mediates most of its biological effects through its binding to LPS binding protein (LBP) and the binding of the resulting complex to the CD14 receptor on the cell surface (Tobias *et al.*, 1989). To establish if the proliferative effect of LPS on tumour cells was mediated by a serum factor such as LBP, the effect of LPS on MDA-MB-231 and HUVEC proliferation in the absence of Fetal Calf Serum (FCS) was examined (n=4). MDA-MB-231 cells were cultured in 96-well plates and then treated for 48 h with various concentrations of LPS (10 ng/ml - 10 μg/ml) in the absence of FCS.

Although 1 μg and 10 μg LPS/ml stimulated the proliferation of MDA-MB-231 cells (see Fig. 5.4), this stimulatory effect was lost in the absence of FCS (Fig. 5.9A).

To examine the effect of LPS on endothelial cell proliferation in the absence of serum factors, HUVECs were grown in medium containing endothelial growth factor (EGF) only, with various concentrations of LPS (10 ng/ml - 10 μg/ml) in the absence of FCS. Similarly, although 10 μg LPS/ml stimulated the proliferation of endothelial cells previously (see Fig. 5.6), this stimulatory effect was not observed when FCS was omitted from the medium (Fig. 5.9B). These data implicate a serum factor, most likely LBP, in LPS-induced proliferation of tumour cells and endothelial cells.

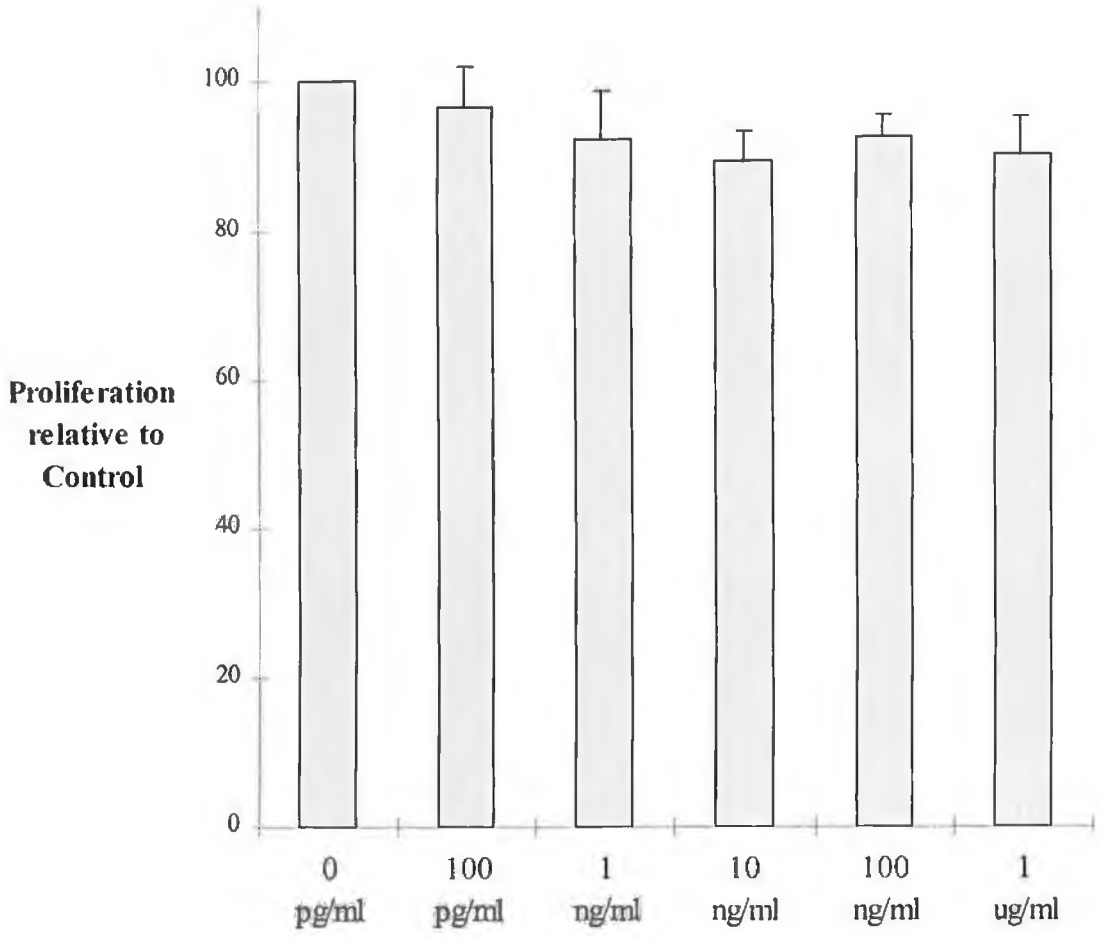


Fig. 5.8 Effect of TNF-α on proliferation of HUVECs. Cells were stimulated with TNF-α (100 pg/ml-1μg/ml) for 48 h. HUVEC proliferation was assessed by the metabolic tetrazolium dye assay. Data is representative of 4 independent experiments and expressed as mean $\pm$ S.E.M. Statistical analysis was by ANOVA with Scheffe post-hoc correction (p=n.s).

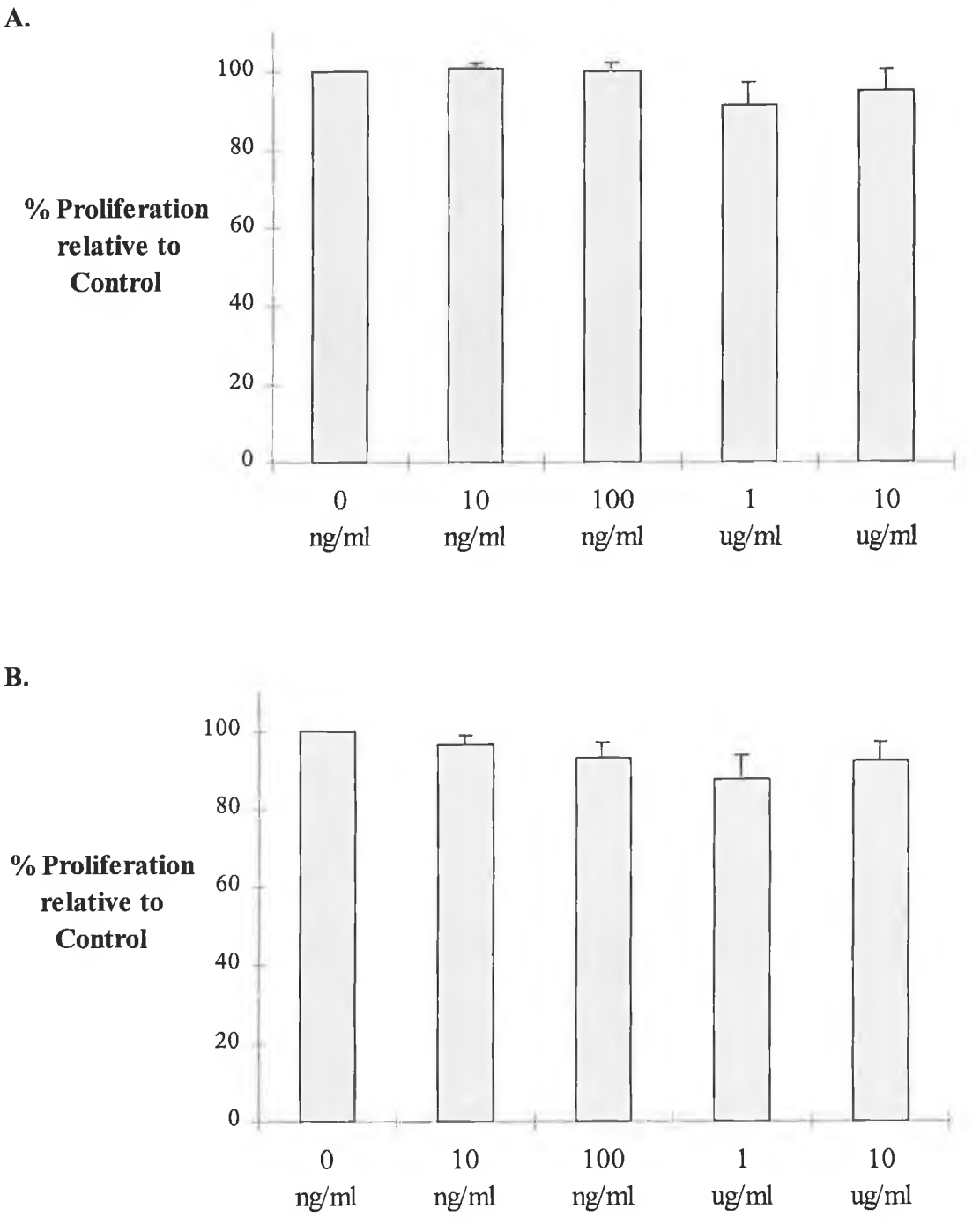


Fig. 5.9 Effect of LPS on proliferation of MDA-MB-231 cells (A) and HUVECs (B) in the absence of FCS. Cells were treated with LPS (10 ng/ml-10μg/ml) for 48 h in medium containing no FCS. Data is representative of 4 independent experiments, expressed as mean $\pm$ S.E.M where proliferation of control cells is taken as 100%. Statistical analysis was by ANOVA with Scheffe post-hoc correction (p=n.s).

5.3.9 Effect of LPS on angiogenesis *in vivo* in a matrigel model.

In the earlier half of this decade a number of investigators reported that endotoxin was angiogenic in many different animal models (Li *et al.*, 1991; BenEzra *et al.*, 1993; Mattsby-Baltzer *et al.*, 1994). In none of these reports was the mechanism of LPS-induced angiogenesis elucidated. To establish whether LPS was directly angiogenic in BALB/c mice, a subcutaneous Matrigel™ model was used. With no tumour present, matrigel impregnated with sterile PBS (negative control), VEGF (100 ng/ml, positive control) or LPS (10 μg/ml) were implanted subcutaneously in mice (n=3). 5 days later the gels were excised and infiltrating endothelial cells and vessels stained by PECAM/CD31 specific immunostaining. The number of positively stained cells or vessels in various regions of the gel was examined by counting 5 high power fields of vision (magnification X 400).

Gels containing VEGF (35.72±2.02) had significantly ($p<0.004$) more endothelial cells at the edge of the gel compared to those containing PBS (7.87±1.18), showing that VEGF impregnated matrigels stimulated angiogenesis (Fig.5.10). Similarly, gels containing LPS (25.08±4.92) contained significantly ($p<0.004$) higher numbers of endothelial cells compared to the PBS controls. There was no difference in the numbers of infiltrating non-endothelial cells at the edge of the gel between any groups, however gels containing LPS (28.62±5.35) showed a trend toward more of these cells compared to either the PBS gels (11.58±3.87) or VEGF gels (15.83±4.73) (Fig. 5.11). An increase in immune cell infiltrate would be anticipated due to the pro-inflammatory agent LPS in the gels. Failure to reach significance is likely due to the small numbers used in the study (n=3). Representative sections from the edge of the gel in each group are shown in panels (A-E) in Fig. 5.12.

It has been suggested that a more accurate measure of angiogenesis is observed by taking

the endothelial cell counts at the centre of the gels. These counts represent cells that have migrated inward. Gels containing VEGF (35.42±2.38) and LPS (25.47±2.74) resulted in significantly higher numbers of endothelial cells at the centre of the gels compared to gels containing PBS (6.36±1.18; $p<0.001$ vs VEGF, $p<0.007$ vs LPS). The number of formed blood vessels (containing a lumen) was also examined, and gels containing VEGF (10.7±0.25) or LPS (4.97±0.49) contained significantly higher numbers of blood vessels compared to PBS gels (1.03±0.4; $p<0.001$ vs VEGF, $p<0.05$ vs PBS) (Fig. 5.15). This would indicate that LPS appears to be directly angiogenic, inducing the infiltration of endothelial cells at the edge of the gels and also the migration of the cells to the centre of the gels to form blood vessels.

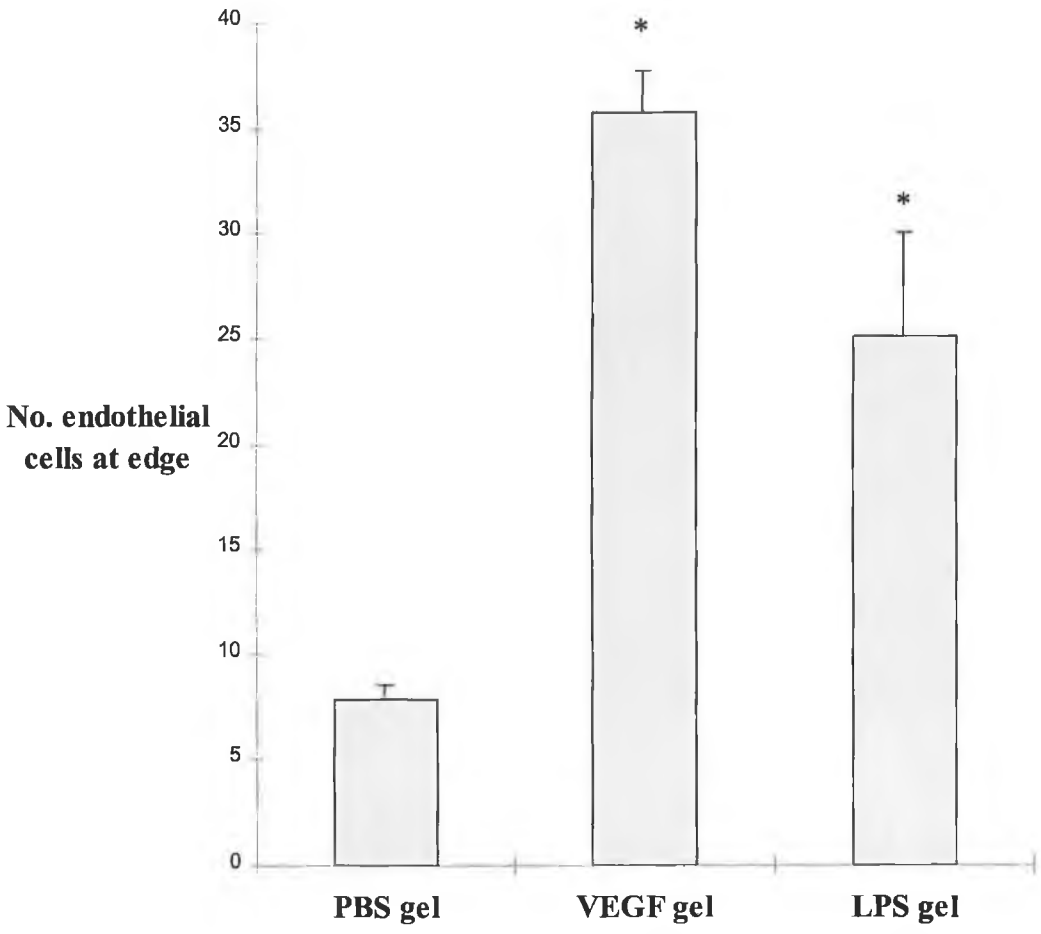


Fig. 5.10 Numbers of infiltrating endothelial cells at the edge of impregnated matrigels. Matrigel containing PBS, VEGF (100 ng/ml) or LPS (10 μg/ml) were implanted subcutaneously. 5 days later gels were recovered and endothelial cells were stained with CD31. The numbers of positively stained cells were averaged in 5 high power fields (X 400) for each gel. Data is representative of 3 mice/groups, expressed as mean number of cells $\pm$ S.E.M. Statistical analysis is by ANOVA with Scheffe post-hoc correction * ($p<0.004$ vs PBS gel).

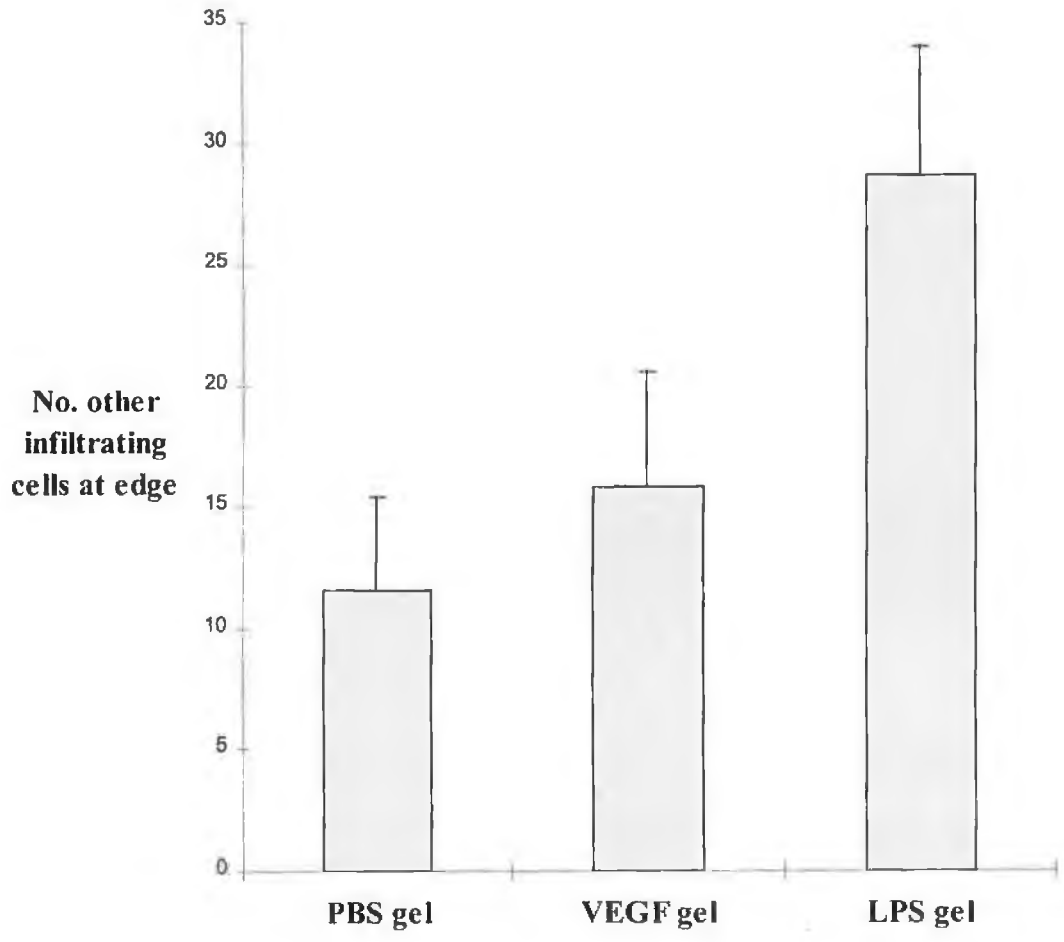


Fig. 5.11 Numbers of other cells infiltrating the edge of matrigels. Non-endothelial cells stained with haemotoxylin in CD31/pecam sections and were averaged over 5 fields of vision (X 400). Data is representative of 3 mice/groups, expressed as mean number of cells ± S.E.M. Statistical analysis is by ANOVA with Scheffe post-hoc correction (p=n.s).

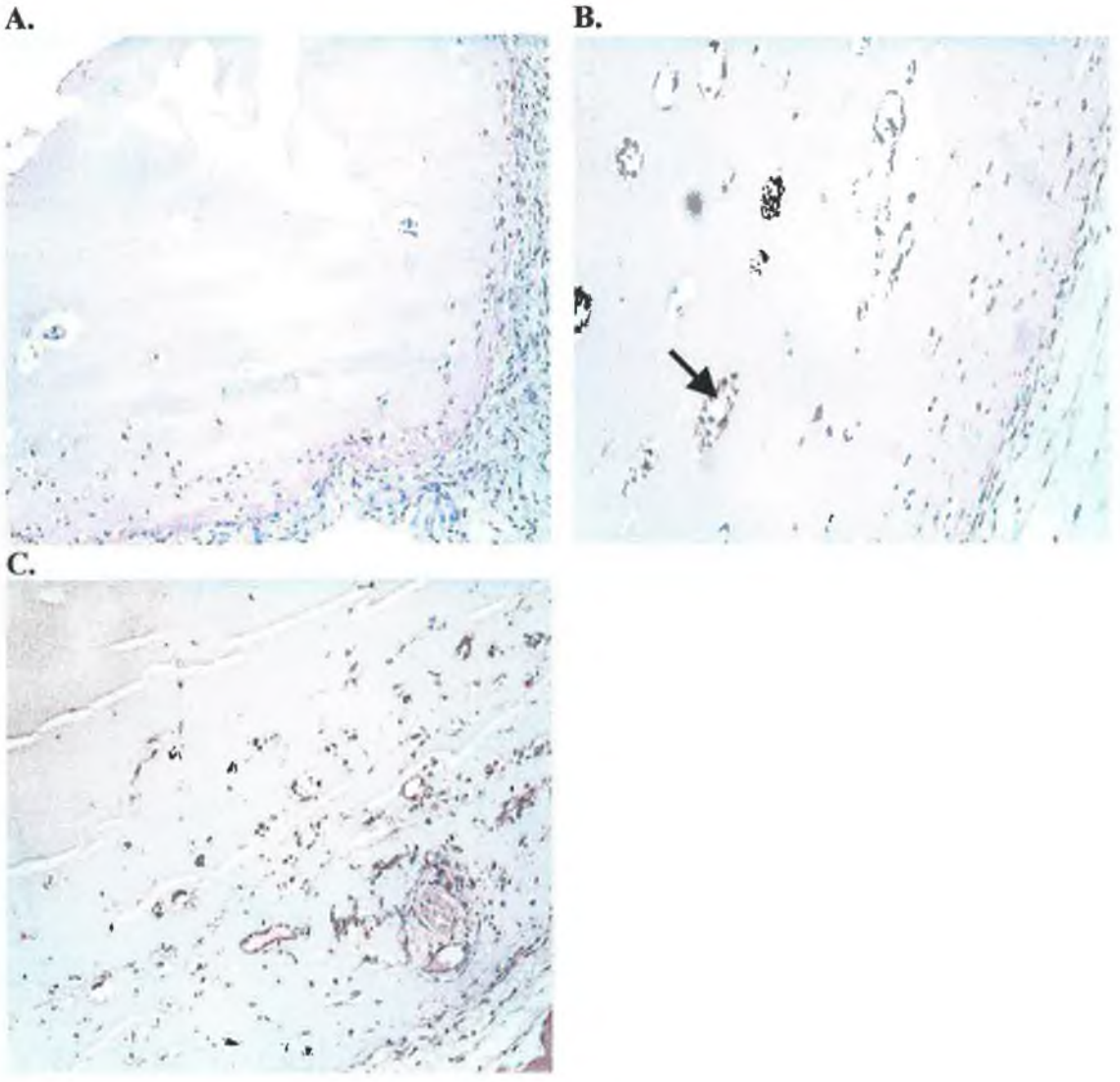


Fig. 5.12 Representative sections from the edge of matrigels stained for the endothelial-cell-specific CD31 marker and counterstained with haemotoxylin and eosin (X 400). Immune cells (CD-31 negative) stain blue and endothelial cells (CD31-positive) stain brown and are indicated by the arrow in panel B. Panels are as follows: **A.** PBS gel, **B**. VEGF gel, **C.** LPS gel.

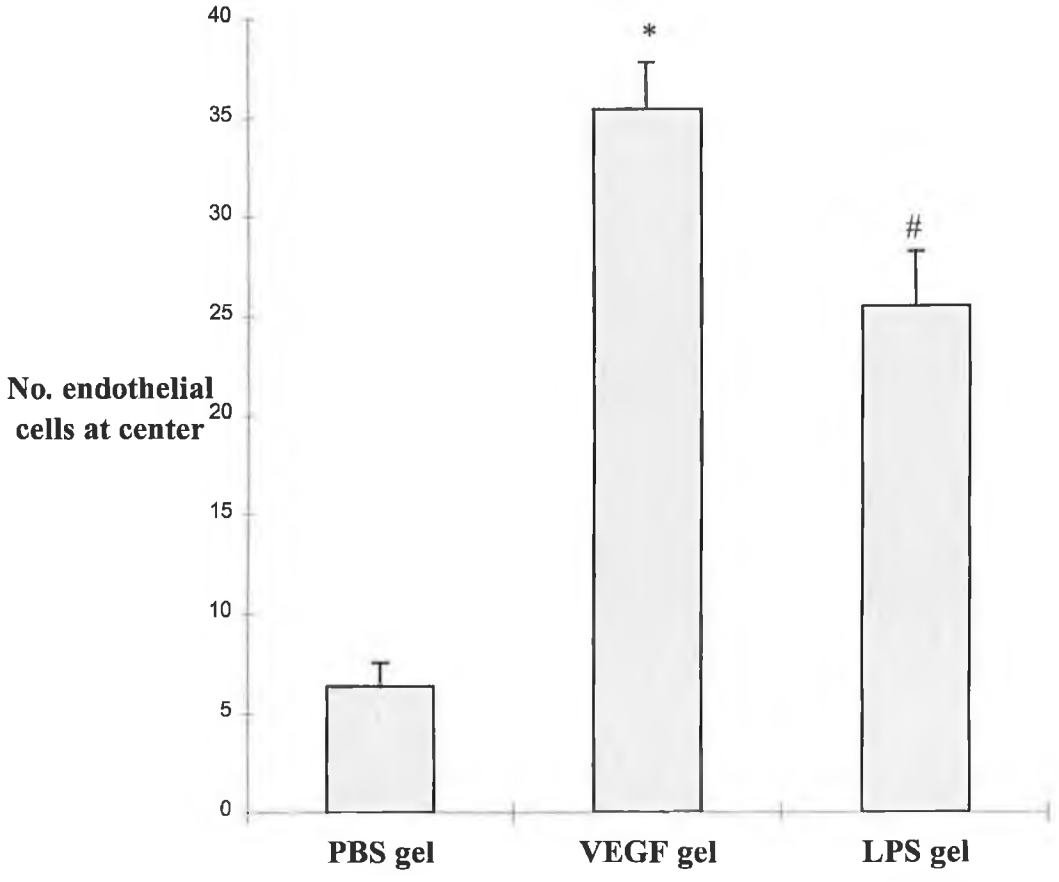


Fig. 5.13 Numbers of endothelial cells in the centre of impregnated matrigels. Endothelial cells were stained with CD31 and the numbers of positively stained cells were averaged in 5 high power fields (X 400) for each gel. Data is representative of 3 mice/groups, expressed as mean number of cells $\pm$ S.E.M. Statistical analysis is by ANOVA with Scheffe post-hoc correction *0 ($p<0.001$ vs PBS gel), # ($P<0.007$ vs PBS gel).

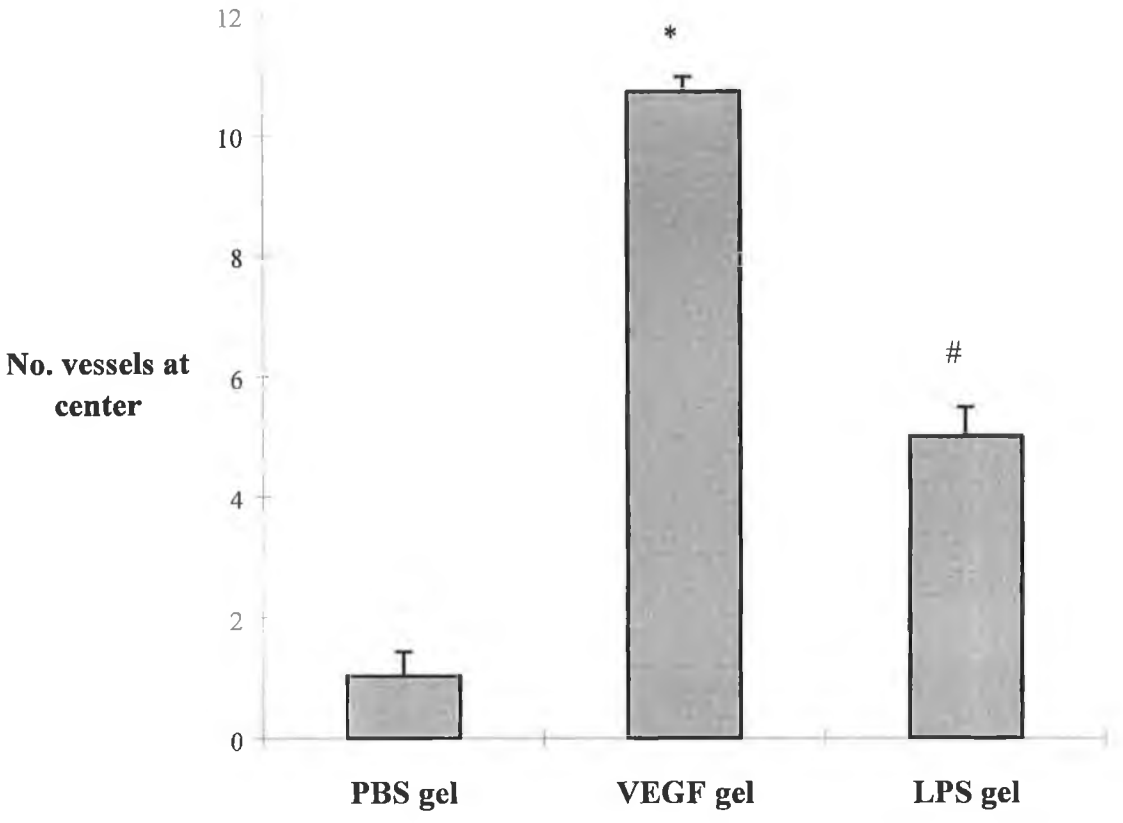


Fig. 5.14 Numbers of stained blood vessels in the centre of impregnated matrigels. . Endothelial cells were stained with CD31 and the numbers of positively stained cells were averaged in 5 high power fields (X 400) for each gel. Data is representative of 3 mice/groups, expressed as mean number of cells ± SEM. Statistical analysis is by ANOVA with Scheffe post-hoc correction * ($p<0.001$ vs PBS gel), # ($p<0.05$ vs PBS gel).

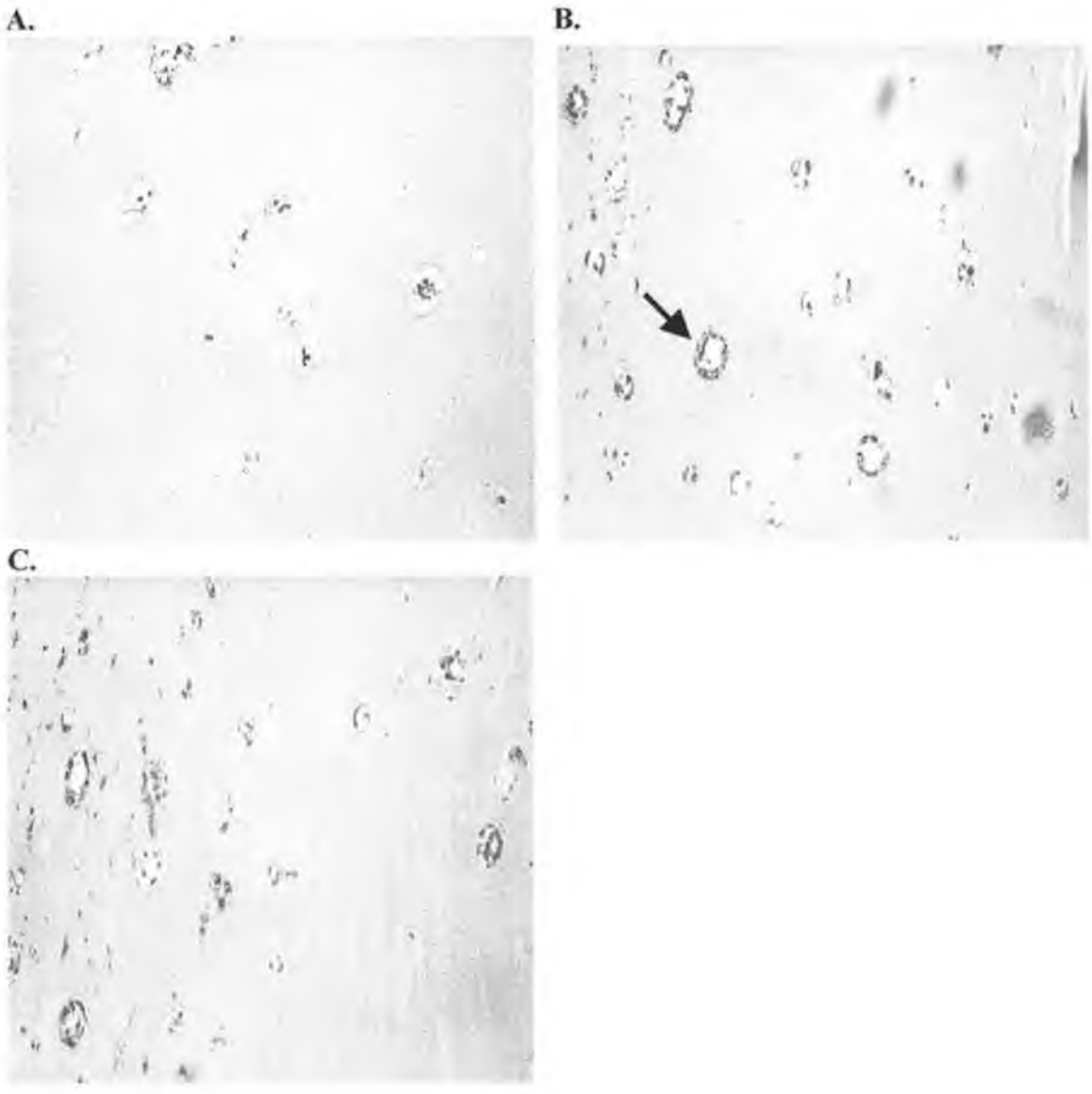


Fig. 5.15 Representative sections from the centre of matrigels stained for the endothelial-cell-specific CD31 marker and counterstained with haemotoxylin (X 400). Endothelial cells (CD31-positive) stain brown and a formed vessel is indicated by the arrow. Panels are as follows: **A.** PBS gel, **B.** VEGF gel, **C.** LPS gel.

5.3 Discussion

As VEGF plays a central role in tumour growth promoting angiogenesis and facilitating metastasis by increasing vessel permeability, factors regulating its production and cells secreting it are therefore critical in disease progression. There is much controversy as to the prognostic significance of tumour infiltrating macrophages, which appears to depend on the tumour type. However, with respect to breast tumours, there is overwhelming evidence that the infiltration of immune cells supports tumour growth. Previous chapters demonstrated increased LPS in the circulation following surgery; it is therefore possible that LPS stimulates the release of VEGF from host macrophages contributing to angiogenesis at a distant site. The data presented in this chapter shows that LPS upregulates VEGF expression in human macrophages at both the mRNA and protein level. LPS also resulted in increased expression of TGF-β mRNA, though to a lesser extent than VEGF. However, TGF-β can also increase the production of VEGF from both macrophages and tumour cells (Harmey *et al.*, 1998; Donovan *et al.*, 1997).

LPS has been implicated in cellular proliferation and differentiation, possibly through the phosphorylation and activation of several protein kinases (Weinstein *et al.*, 1992; Shapira *et al.*, 1994). In vivo, LPS increased the mitotic index in experimental metastases (Pidgeon *et al.*, 1999). LPS increased the proliferation of both tumour cells (murine 4T1 and human MDA-MB-231 cells) and endothelial cells (HUVECs) *in vitro*. At the highest concentration used (10 μg/ml), proliferation of the cells was increased by 24.7% in 4T1 cells and by 28.8% in MDA-MB-231 cells relative to controls. When HUVEC or MDA-MB-231 cells were treated with LPS in the absence of FCS, no proliferative effect was observed. This implicates a serum factor in LPS-mediated cell proliferation. In macrophages and other cells expressing CD14, the serum factor LPS-binding protein (LBP) is required for the interaction of the LPS molecule with the

receptor. It would appear that in this tumour cell line a similar mechanism is necessary to allow LPS to interact with the cells.

A potent cytokine produced by macrophages upon activation with LPS is tumour necrosis factor alpha. TNF-α has been implicated in tumorigenic activities related to LPS (Torisu *et al.*, 2000, Leibovich *et al.*, 1987). In contrast to the results observed when cells were treated with LPS, TNF-α significantly inhibited tumour cell proliferation in a dose-dependent manner. At the highest concentration used (1 μg/ml) proliferation was inhibited by 21%. At the lower doses, which are more physiologically relevant, TNF-α had no effect on tumour cell proliferation. This clearly indicates that TNF-α secretion is not responsible for the increased cellular proliferation in response to LPS. There is evidence that TNF-α plays a role in macrophage mediated angiogenesis in response to LPS (Leibovich *et al.*, 1987) and anti-TNF-α monoclonal antibodies blocked LPS induced VEGF expression in monocytes (Torisu *et al.*, 2000). It therefore appears that LPS-induced VEGF and cell proliferation occur by independent mechanisms.

Endotoxin has been reported to induce de-novo angiogenesis in adult, mammalian, normally vascularised tissues at doses (20 ng/ml - 20 μg/ml) that appear realistic in the clinical setting (Mattsby-Baltzer *et al.*, 1994). The majority of investigators suggest that the angiogenic effects observed following LPS treatment are probably mediated by cytokines released by macrophages (Li *et al.*, 1991; Mattsby-Baltzer *et al.*, 1994). LPS resulted in a dose-dependent increase in HUVEC proliferation; 10 μg LPS/ml resulted in a 25.8% increase in proliferation relative to controls. At the same dose, LPS inhibited HUVEC apoptosis from 44.5% in control cells to 16.4%. Therefore LPS affects both proliferation and apoptosis of the endothelial cells, altering the balance on both sides to favour blood vessel growth. It is possible that LPS mediates

these effects on endothelial cells through VEGF production. It has previously been reported that LPS induces the expression of VEGF in human endothelial cells at the concentrations used in this study (Marx *et al.*, 1999). In that study apoptosis was rarely seen following LPS treatment, but simultaneous treatment with anti-VEGF antibodies resulted in increased apoptosis. It has also been shown that VEGF acts as a survival factor for endothelial cells by inhibiting apoptosis (Alon *et al.*, 1995; Nor *et al.*, 1999). It is therefore possible that the inhibition of HUVEC apoptosis by LPS increased VEGF production, which acted as a survival factor for the cells.

The *in vivo* angiogenesis assay used in this study demonstrated that LPS is directly angiogenic at the concentration tested (10 μg/ml). Although the angiogenic response was not as dramatic as that seen with VEGF implants, LPS gels contained significantly more endothelial cells at both the edge of the gels and in the centre of the gels, suggesting that LPS stimulated cell migration. VEGF and LPS also resulted in increased numbers of blood vessels, which were denoted by an intact lumen. Within the assay there was some degree of inflammation and endothelial cell infiltrate in gels impregnated with PBS control probably due to an inflammatory reaction caused at the site of the injection. During an inflammatory response, cells migrate toward the source of inflammation. Indeed, there was a high degree of non-endothelial cell infiltrate in all gels at the edge. Gels containing LPS had significantly higher amounts of these non-endothelial cells, supporting the fact that these cells were most probably immune infiltrates in response to inflammation. This is in agreement with the findings by other investigators, that in the models of angiogenesis used, namely corneal implant and mesentery window, induction of angiogenesis is secondary to an inflammatory reaction (Li *et al.*, 1991; Mattsby-Baltzer *et al.*, 1994).

Taken together this data clearly shows that LPS is directly angiogenic, inhibiting

endothelial cell apoptosis, increasing the proliferation of the cells, and increasing endothelial cell migration into matrigel and the subsequent formation of vessels.

Chapter 6

LPS and VEGF as Survival Factors in Tumour Cells

Submitted : Pidgeon G. P., Barr M.P., Harmey J.H., Foley D.A. and Bouchier-Hayes D.J. (2000) Vascular endothelial growth factor (VEGF) upregulates Bcl-2 and inhibits apoptosis in human and murine adenocarcinoma cells. *Br. J. Cancer.*

6.1 Introduction

Under normal physiological conditions, a delicate balance between cellular proliferation and cell death ensures that the overall numbers of cells are maintained within an appropriate range (Reed, 1997). Cancer progression may result from disturbances to either of these mechanisms, such as a relative increase in cell proliferation or a decrease in cell death, and in extreme cases both mechanisms may be effected (Thompson, 1995). The selective process whereby cells are discretely removed from populations without affecting surrounding cells is termed apoptosis or 'programmed cell death'. Disturbances to this process may confer a growth advantage to neoplastic tissues, resulting in clonal expansion of the tumour (Reed, 1994; Thompson, 1995).

The Bcl-2 family of apoptosis-regulating proteins function to either promote or suppress cell death (Oltvai *et al.*, 1993). The function of several members of the Bcl-2 family is regulated by their ability to form homo- and heterodimers with each other (Oltvai *et al.*, 1993; Sato *et al.*, 1994). Some of the pro-apoptotic Bcl-2 family members preferentially form dimers with subsets of the pro-survival members, in which apoptosis proceeds. For instance, the pro-apoptotic protein Bax will preferentially dimerise with the anti-apoptotic protein Bcl-2 to form a heterodimer, rather than with a second Bax protein to form a homodimer. These differences in dimerisation partners suggests that the expression of various members of the Bcl-2 family is important in the regulation of apoptosis or proliferation within a cell at any time. Increased expression of Bcl-2 has been reported in many cancers and is associated with a reduction in the level of apoptosis (Lipponen *et al.*, 1999). Paradoxically, some reports suggest that increased expression of Bcl-2 is a good prognostic indicator in breast cancer correlating with response to therapy and overall survival (Shabaik *et al.*, 1994; Krajewski *et al.*, 1999). A possible explanation for this paradoxical finding in breast cancer may be that Bcl-2 is induced by estrogen and

as such may represent a marker of less aggressive or poorly differentiated tumours (Krajewski *et al.*, 1999). Alternatively the anti-proliferative effect of Bcl-2 may be involved (Borner, 1996).

The induction of anti-apoptotic proteins is a possible mechanism whereby tumour cells escape the cytotoxic effects of many chemotherapeutic and radiation regimes. Indeed, in the case of one neoadjuvant chemotherapy trial in breast cancer, a long-term treatment benefit was observed in patients whose tumours displayed features favouring apoptosis, ie. no or weak Bcl-2 expression, and Bax overexpression (Diadone *et al.*, 1999). In this case Bcl-2 and Bax expression predicted the cellular response to genotoxic damage. Overexpression of another of the anti-apoptotic members of the Bcl-2 family, Bcl-XL, suppressed chemotherapy induced apoptosis of mammary tumours in a murine model (Liu *et al.*, 1999).

Angiogenesis, the development of new blood vessels, is an essential requirement for both primary and metastatic tumour growth (Morgan *et al.*, 1996). It is well documented that in the absence of angiogenesis, tumours are restricted to a limited size of 1-2 mm^3 in a dormant-like state (Folkman., 1994). The ability of tumours to stimulate neovascularisation is governed by the net balance of angiogenic stimulators and inhibitors in the tumour micro-environment (Hanahan and Folkman, 1996). Tumours secrete or mobilise a variety of angiogenic factors that tip the balance in favour of angiogenesis.

By inducing blood vessels within tumours, the angiogenic factor, VEGF facilitates metastasis by providing an escape route for metastatic tumour cells. Recently, it has been shown that VEGF acts as a survival factor for endothelial cells by inhibiting apoptosis (Alon *et al.*, 1995; Nor *et al.*, 1999). In one of these studies, VEGF protected endothelial cells from apoptosis induced by growth factor withdrawal by up-regulating Bcl-2

expression (Nor *et al.*, 1999). Furthermore, VEGF has been shown to protect leukemia cells and haematopoetic stem cells from radiation induced apoptosis (Katoh *et al.*, 1995). In MCF human breast cancer cells, bcl-2 and hypoxia acted synergistically to modulate the expression of VEGF in bcl-2 transfected cells, and tumours transfected with bcl-2 produced more VEGF and were more highly vascularised than controls (Birroccio *et al.*, 2000). Therefore there is a growing body of evidence to suggest an inter-relationship between VEGF and the anti-apoptotic protein, bcl-2.

In the experimental metastasis model used in this thesis, LPS exposure resulted in elevated circulating VEGF and decreased apoptosis with lung metastases relative to control mice (Pidgeon *et al.*, 1999). Increased expression of VEGF in response to LPS has also been reported in human pulp cells (Matsushita *et al.*, 1999).

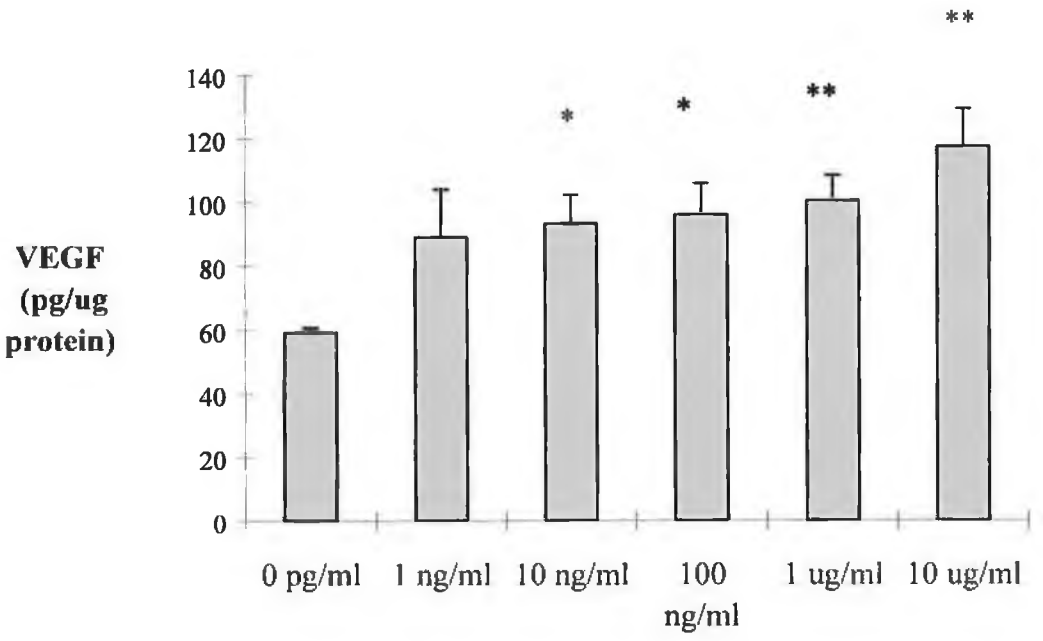
The aim of this chapter was to examine the effect of VEGF and LPS as survival factors for tumour cells. The effect of LPS treatment on VEGF protein expression by human MDA-MB-231 and murine 4T1 mammary carcinoma cells was examined *in vitro*. As Bcl-2 has previously been shown to be upregulated in response to VEGF, the effect of VEGF or LPS treatment on Bcl-2 expression in both cell lines was examined. LPS was used to stimulate VEGF in this set of experiments and anti-VEGF antibodies were used to specifically block VEGF. As it is alterations in these proteins that ultimately regulate tumour cell apoptosis, the effect of these treatments on tumour cell apoptosis was investigated.

6.2 Results

6.2.1 LPS upregulates VEGF production by 4T1 and MDA-MB-231 tumour cells.

4T1 and MDA-MB-231 cells were treated with LPS (1ng/ml, 10ng/ml, 100ng/ml, 1μg/ml and 10μg/ml) and VEGF protein expression was analysed by ELISA (n=3). VEGF production was expressed as pg VEGF/μg total cell protein. LPS significantly (*$p<0.05$, **$p<0.01$ v's control) up-regulated VEGF protein at a concentration of 10ng/ml (89.06 ± 14.98 pg/μg protein), 100ng/ml (93.27 ± 9.07 pg/μg protein), 1μg/ml (95.94 ± 9.9 pg/μg protein) and 10μg/ml (117.22 ± 11.89 pg/μg protein) of LPS in the 4T1 tumour cells and at 10μg/ml (28.32 ± 2.05 pg/μg protein) in the MDA tumour (Fig. 6.1A and Fig. 6.1B respectively).

A.



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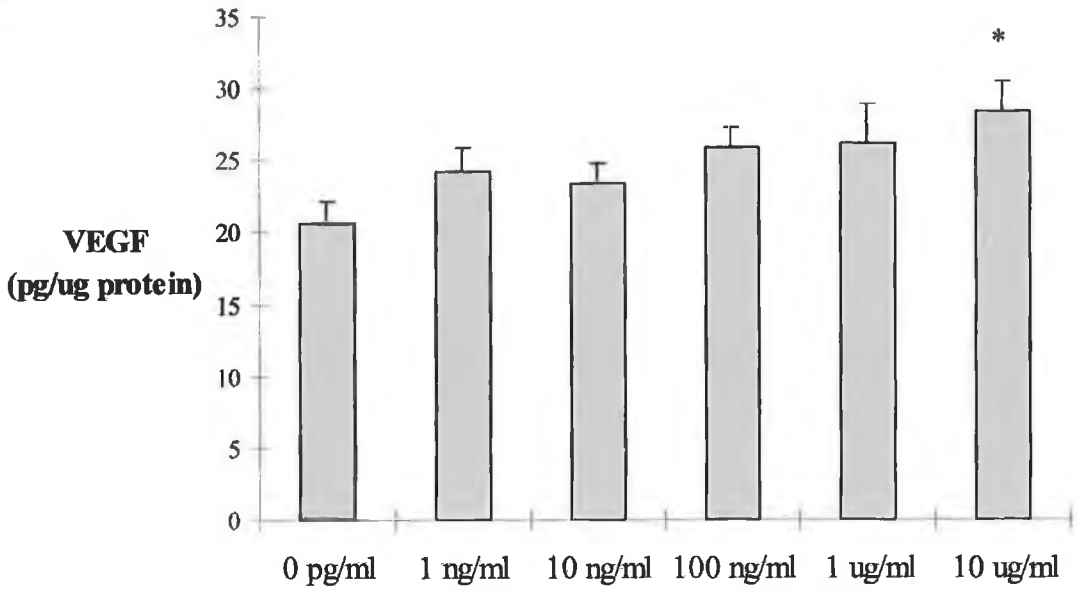


Fig. 6.1. LPS upregulation of VEGF protein. 4T1 (A) and MDA (B) cell cultures were stimulated with LPS (1ng/ml-10μg/ml) for 24 h. VEGF protein was assayed by ELISA and total cell protein by the BCA assay. VEGF levels were significantly increased relative to controls at a concentration of 10ng/ml, 100ng/ml, 1μg/ml and 10μg/ml of LPS in the 4T1 tumour cells and at 10μg/ml in the MDA tumour cells (*$p<0.05$,**$p<0.01$ vs controls).

6.2.2 Effect of VEGF, LPS and anti-VEGF antibodies on Bcl-2 protein expression.

The level of Bcl-2 protein expression was examined by Western Blot analysis following treatment with LPS, VEGF and/or anti-VEGF antibodies (n=3). Total protein was isolated and separated on denaturing polyacrylamide gels before transfer to nitrocellulose membranes. Fig. 6.2 shows coomassie stained SDS-PAGE of cell lysates demonstrating the integrity of the proteins and equal loading. VEGF (100 ng/ml) (lane 5) and LPS (10 μg/ml) (lane 2) resulted in increased Bcl-2 expression in both cell lines examined relative to controls (lane 1) (Fig. 6.3A for 4T1, and Fig. 6.4A for MDA-MB-231). β-actin expression was examined to control for loading differences (Fig. 6.3B and Fig. 6.4B respectively). Densitometry confirmed that relative Bcl-2 expression by 4T1 and MDA-MB-231 cells was significantly increased following treatment with either LPS (4T1, 140$\pm$7.5%; MDA-MB-231, 138$\pm$13.4%) or VEGF (4T1, 136$\pm$7.6%; MDA-MB-231, 166$\pm$31.2%) compared to controls ($p<0.02$, Fig. 6.3C and $p<0.05$, Fig. 6.4C respectively). Neutralising anti-VEGF antibody (1μg/ml) (lane 4) decreased Bcl-2 protein expression in both cell lines (4T1, 46$\pm$10.2%; MDA-MB-231, 18$\pm$3.5% relative to controls). In this case, the antibody is blocking basal VEGF expressed by the tumour cells. Densitometric analysis illustrated that this blockade of endogenous VEGF was significant relative to controls ($p<0.03$). Anti-VEGF antibodies also blocked LPS-induced Bcl-2 expression (lane 3), resulting in a significant decrease in Bcl-2 protein levels relative to LPS treated cells (4T1, 51$\pm$11.1%; MDA-MB-231, 48$\pm$6.6%) ($p<0.03$). These data demonstrate that anti-VEGF antibodies reduce endogenous and LPS-induced Bcl-2 expression in both 4T1 and MDA-MB-231 cells.

6.3.3 Effect of VEGF, LPS and anti-VEGF antibodies on tumor cell apoptosis.

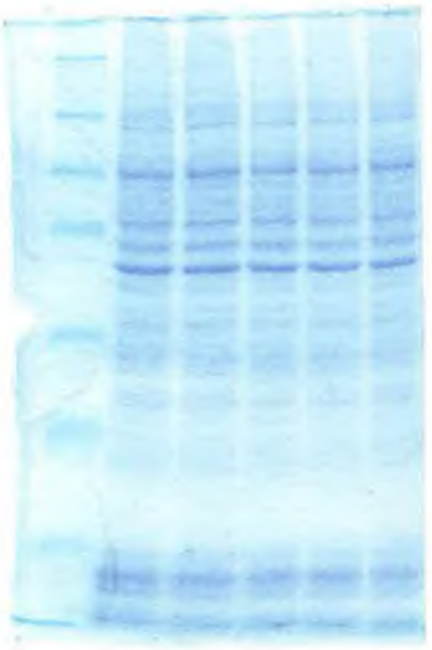
Having shown that LPS or VEGF upregulate the expression of the anti-apoptotic protein, Bcl-2, in human and murine tumor cells and that Bcl-2 expression can be blocked with anti-VEGF antibodies, we studied their effect on tumor cell apoptosis directly by TUNEL staining on culture chamber slides. Due to the low basal rate of apoptosis in tumor cells, apoptosis was induced to a level of 10.33±1.41% in 4T1 and 8.4±0.47% in MDA-MB-231 cells by growth factor withdrawal (Fig. 6.5A and 6.6A, respectively). Treatment with LPS (4T1, 5.39±0.66%; MDA-MB-231, 3.4±0.18%) or VEGF (4T1, 4.07±0.44%; MDA-MB-231, 4.2±0.39%) resulted in a significant decrease in the rate of apoptosis compared to untreated cells (4T1, 10.33±1.41%; MDA-MB-231, 8.4±0.47%) ($P<0.05$, Fig. 6.5A and 6.6A, respectively).

Treatment of cells with anti-VEGF antibodies resulted in a significant increase in the level of apoptosis in both cell lines (24.2±0.90%, $p<0.01$ in 4T1 cells and 22±2.84%, $p<0.02$ in MDA-MB-231 cells) compared to controls. In both cell lines, treatment with anti-VEGF antibodies prevented LPS-mediated inhibition of apoptosis compared to cells treated with LPS alone (15.12±1.98% v's 5.39±0.66%, $p<0.01$ in 4T1) and (11.8±2.23% v's 3.4±0.18%, $p<0.005$ in MDA-MB-231).

Typical stained MDA-MB-231 cells from each treatment are shown in Fig. 6.6B. Increased numbers of TUNEL-positive cells are clearly visible in samples treated with anti-VEGF (panel IV) relative to untreated controls (panel I), where endogenous VEGF is active. Blocking either endogenous VEGF produced by tumour cells (panel IV), or exogenous (in this case induced by LPS exposure) (panel III) leads to increased apoptosis, indicating that VEGF increases tumour cell survival by inhibiting apoptosis.

Treatment with either LPS (panel II) or recombinant VEGF (panel V) decreases apoptosis in both cell lines.

A.



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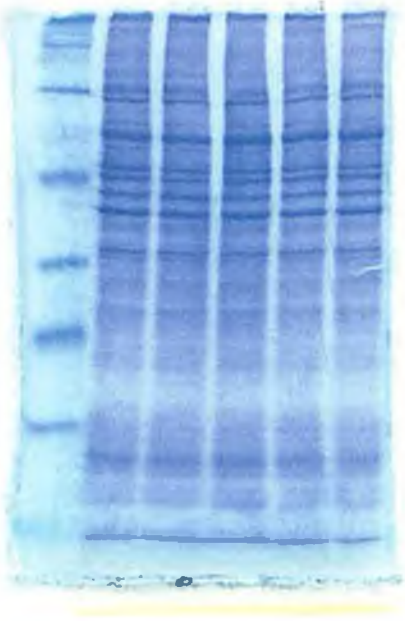


Fig. 6.2 Total proteins isolated from 4T1 (A) and MDA-MB-231 (B) cells and separated on 12% denaturingpolyacrylamide gels by electrophoresis.

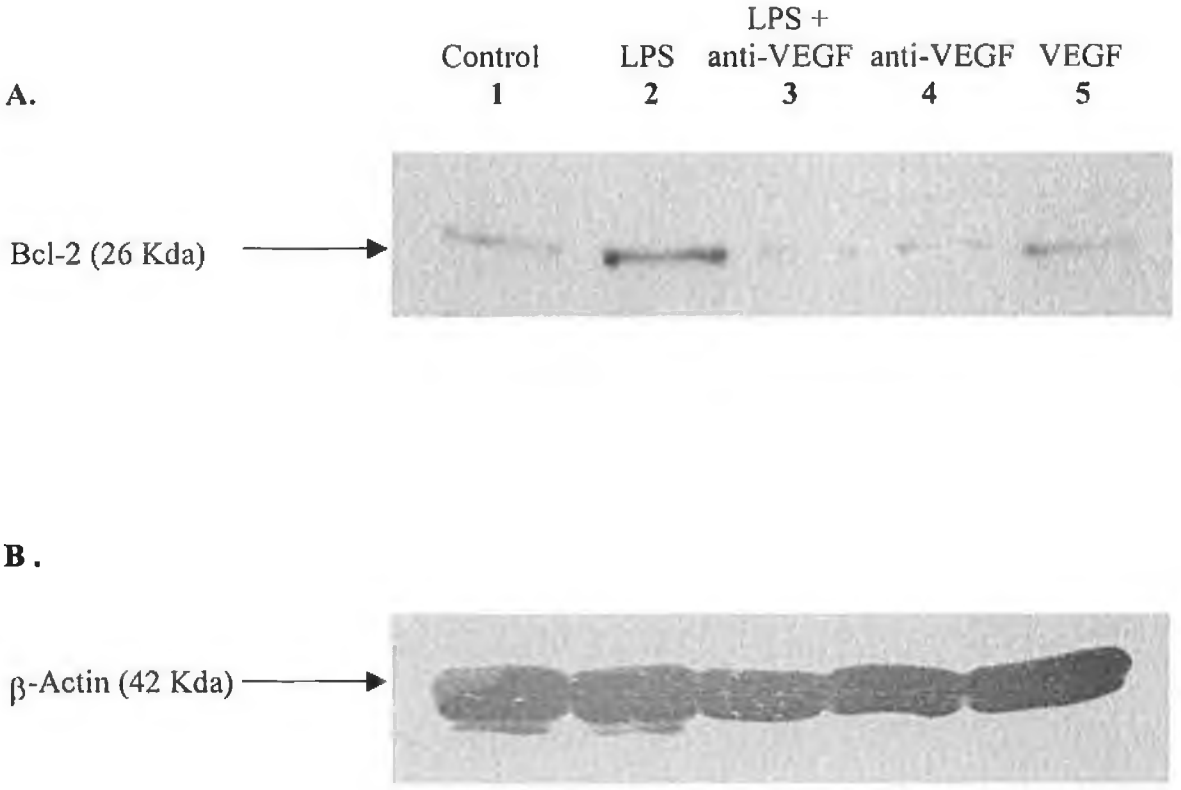
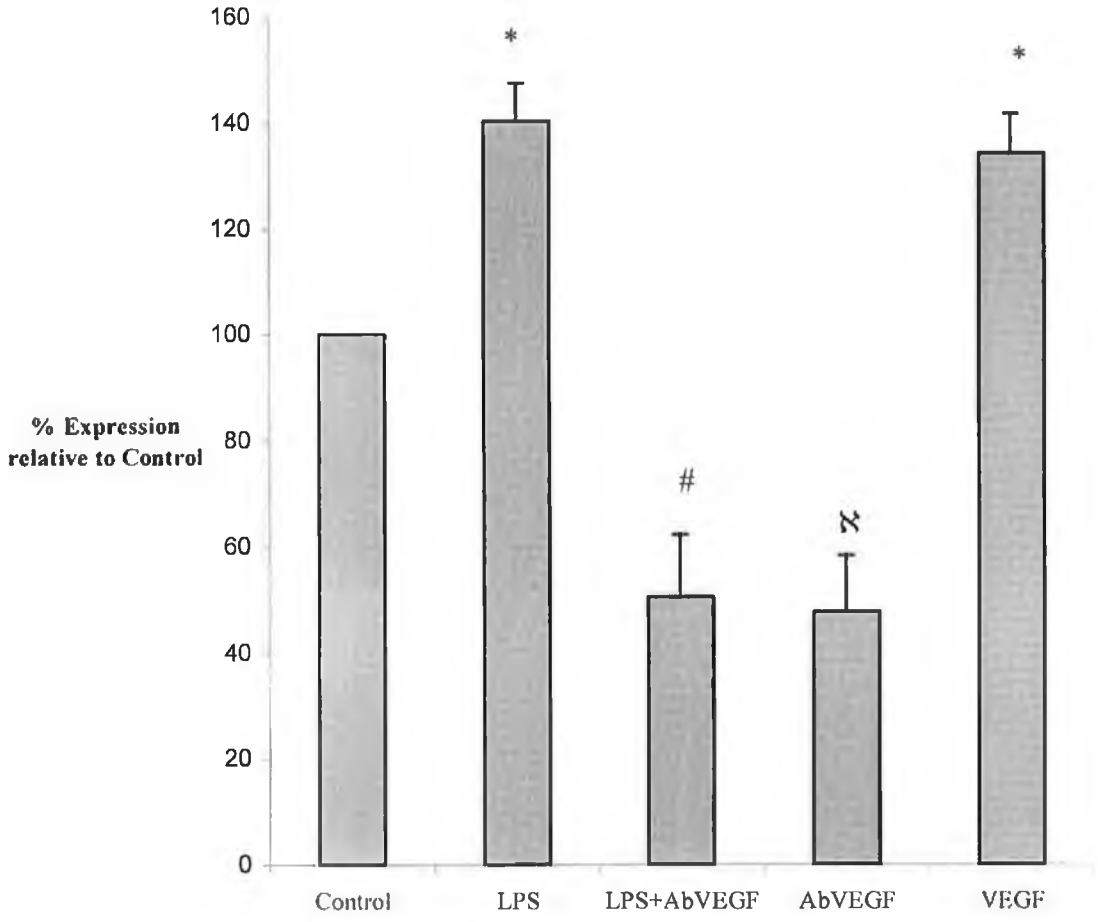


Fig. 6.3 Expression of Bcl-2 in 4T1 cells by western blot analysis. Blots shown are representative of 3 independent experiments. (A) Bcl-2 protein expression in 4T1 cells. Position of Bcl-2 is indicated by arrow. (B) B-actin expression. Position of b-actin indicated by arrow. (C) Densitometric analysis of Bcl-2 protein relative to B-actin. Values are expressed as expression relative to control cells (100%). * ($p<0.02$ vs control), # ($p<0.003$ vs LPS), ℵ ($p<0.03$ vs control).

C.


160
140
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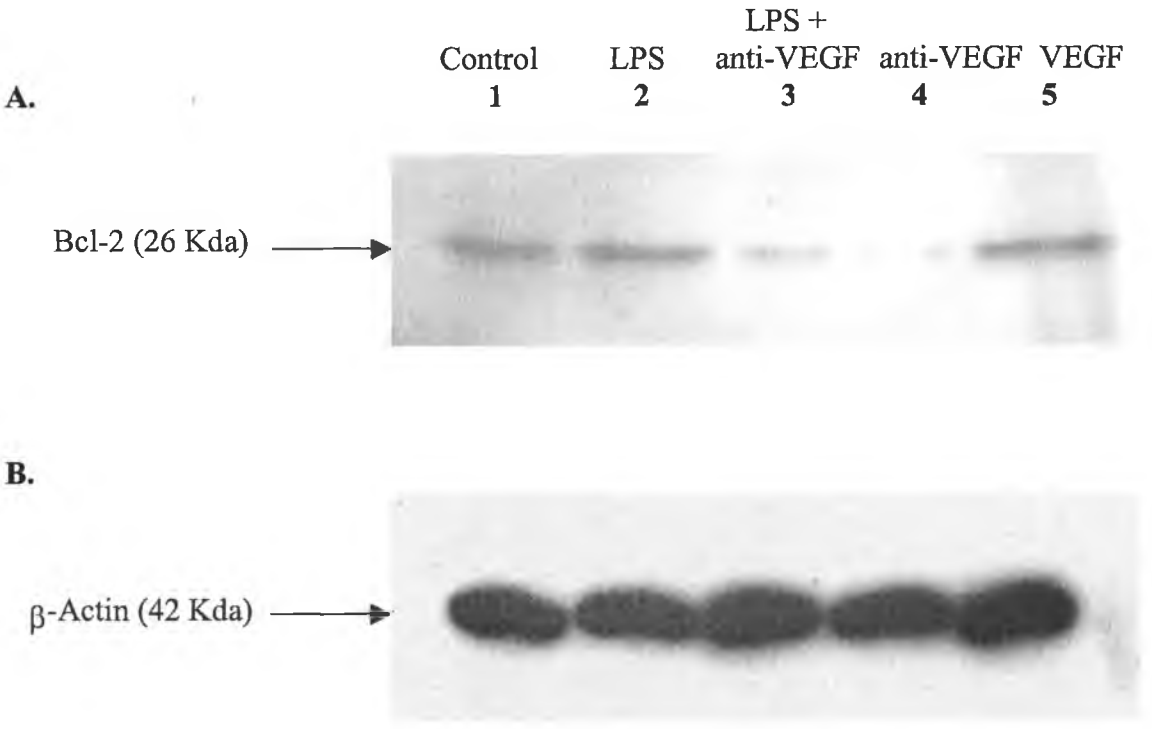
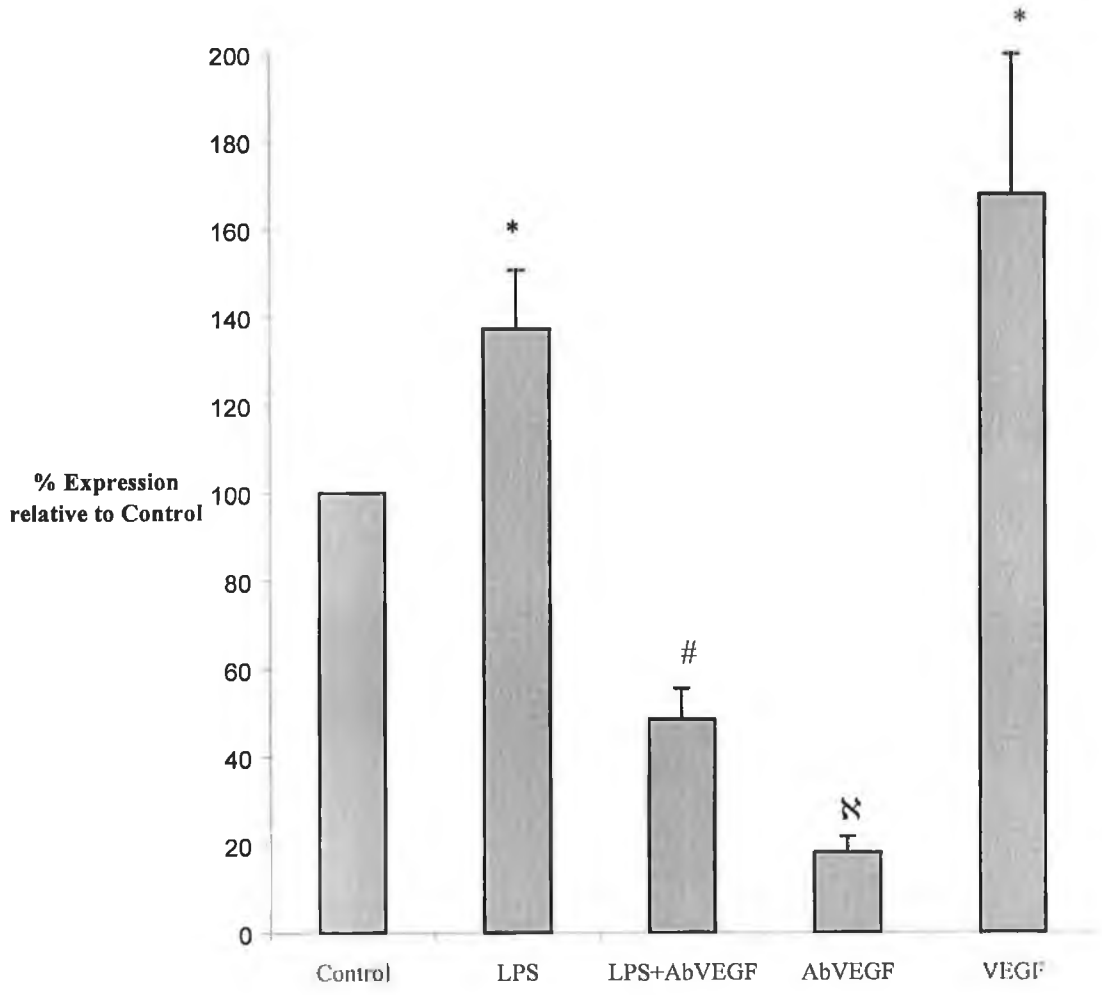


Fig. 6.4 Expression of Bcl-2 in MDA-MB-231 cells by western blot analysis. Blots shown are representative of 3 independent experiments. (A) Bcl-2 protein expression in 4T1 cells. Position of Bcl-2 is indicated by arrow. (B) B-actin expression. Position of b-actin indicated by arrow. (C) Densitometric analysis of Bcl-2 protein relative to B-actin. Values are expressed as expression relative to control cells (100%). * ($p<0.05$ vs control), # ($p<0.01$ vs LPS), ℵ ($p<0.02$ vs control).

C.


% Expression relative to Control
200
180
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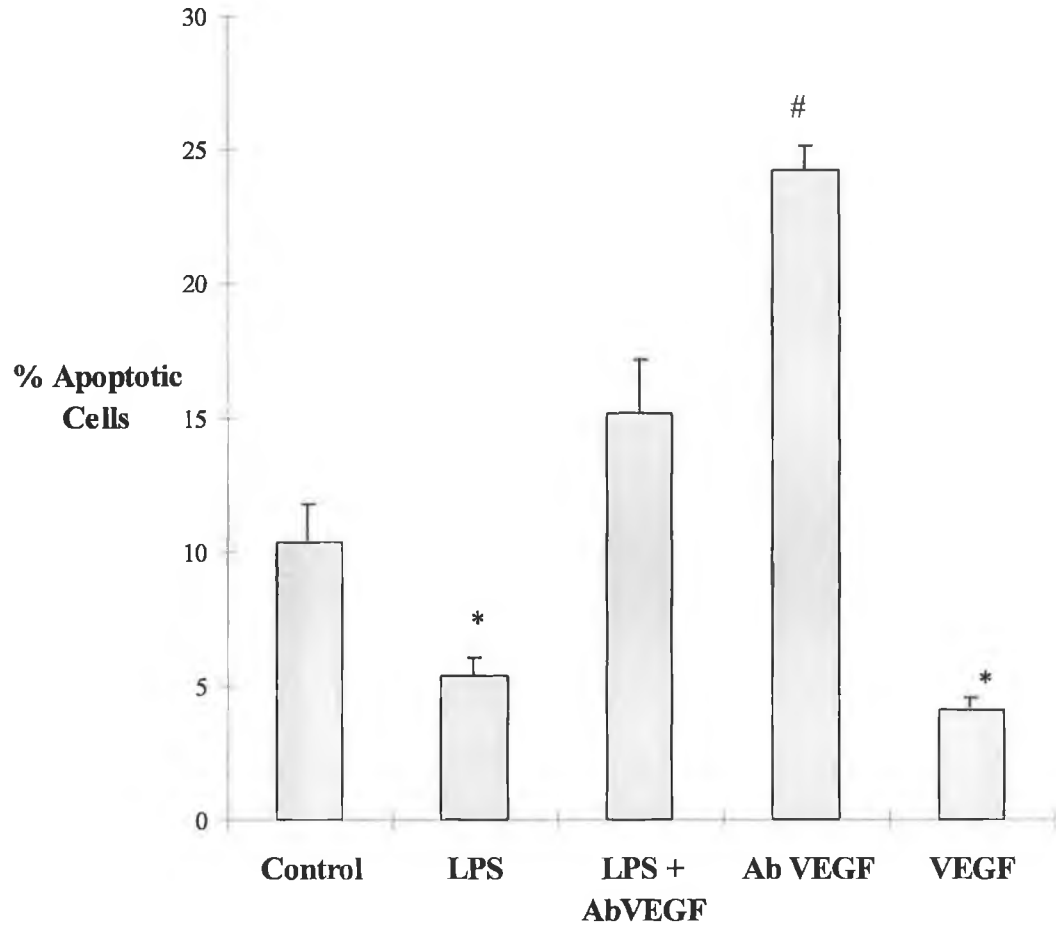


Fig 6.5 Levels of apoptosis in 4T1 cells were examined in chamber slides by TUNEL staining (n=3). Apoptosis was induced to a level of 10.04% in control cells by growth factor depletion. Percentage apoptosis in treatment groups * ($p<0.03$ vs control) #($p<0.05$ vs control).

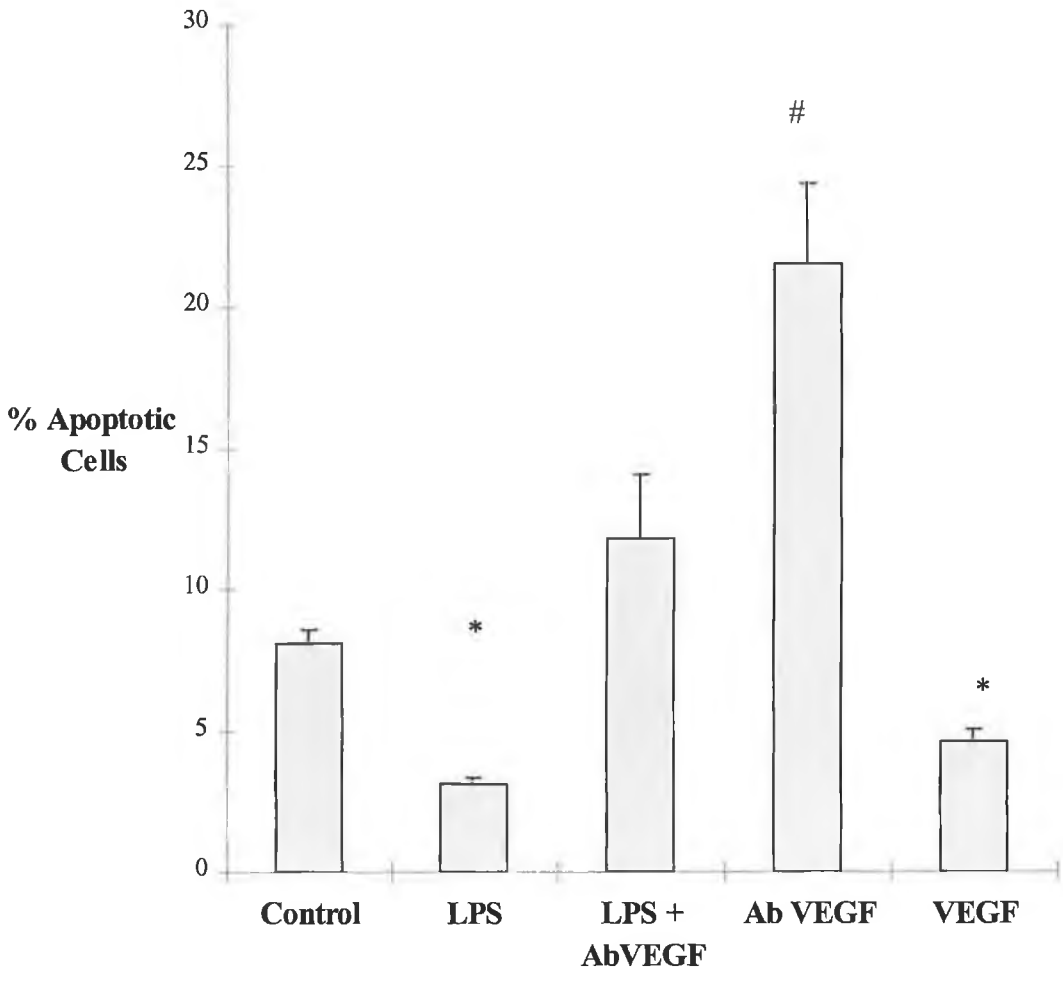
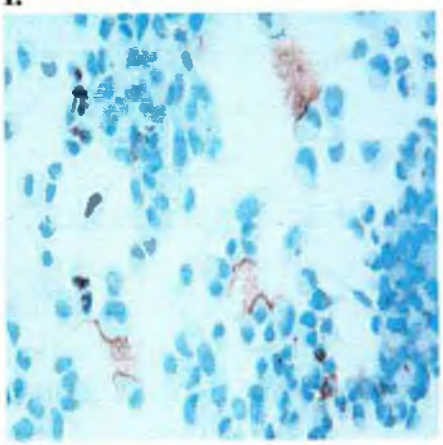


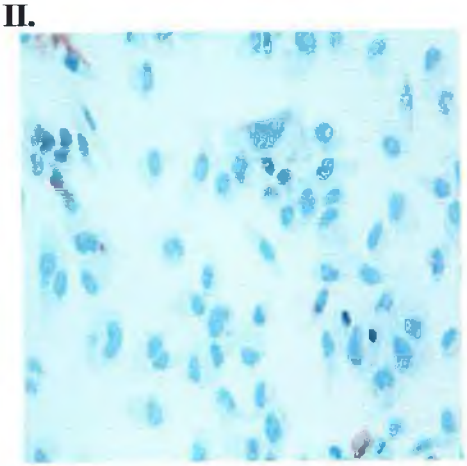
Fig 6.6 Levels of apoptosis in MDA-MB-231 cells were examined in chamber slides by TUNEL staining (n=3). Apoptosis was induced to a level of 8.4% in control cells by growth factor depletion. **A.** Percentage apoptosis in treatment groups * ($p<0.03$ vs control) #($p<0.05$ vs control) **B.** Representative MDA-MB-231 cells from culture chamber slides following treatment as follows: (A) untreated controls, (B) 10μg/ml LPS, (C) 10μg/ml LPS and 1μg/ml VEGF neutralising antibody, (D) 1μg/ml VEGF neutralising antibody, (E) 100ng/ml rhVEGF.

B.

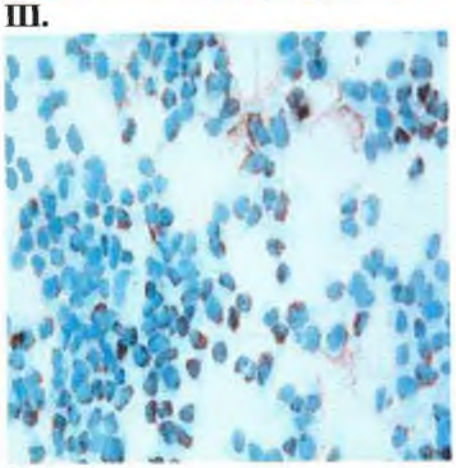
I.



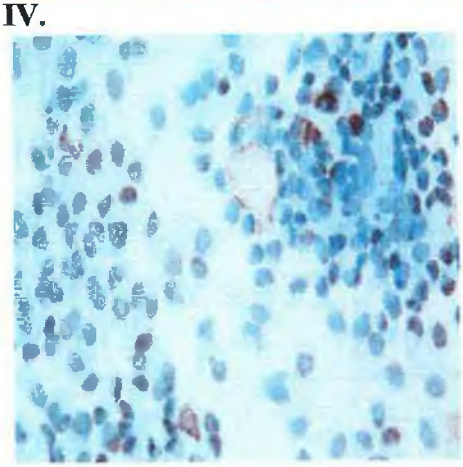
II.



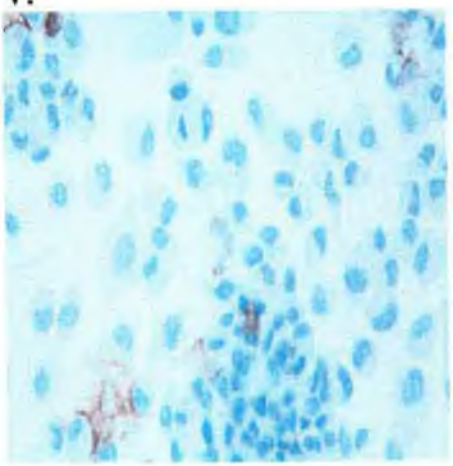
III.



IV.



V.



6.3 Discussion

A number of studies have shown that anti-angiogenic strategies result in increased tumour cell apoptosis (Holmgren *et al.*, 1995; Parangi *et al.*, 1996; O'Reilly *et al.*, 1997). To date, this effect has been attributed to blood vessel regression. However, the angiogenic factor VEGF inhibits radiation and chemotherapy induced apoptosis of haematopoeitic cells, the latter effect being achieved by the induction of MCL1, a member of the Bcl-2 family (Katoh *et al.*, 1998; Gorski *et al.*, 1999), showing that it may mediate effects in other non-endothelial cells. VEGF is known to act as a survival factor for endothelial cells, inducing the expression of the anti-apoptotic protein Bcl-2 (Nor *et al.*, 1999). We previously showed that LPS increased circulating VEGF and decreased tumour cell apoptosis in the 4T1 murine model of experimental metastases (Pidgeon *et al.*, 1999). In that study, groups with high levels of circulating VEGF displayed lower levels of apoptosis within experimental tumour metastases, and subsequently higher ratios of mitosis/apoptosis.

The potent inflammatory mediator endotoxin / lipopolysaccharide (LPS) has been shown to be angiogenic in a number of experimental systems (Mattsby-Baltzer *et al.*, 1994; Li *et al.*, 1991; Kenyon *et al.*, 1996). However, the mechanism whereby LPS induces angiogenic activity has to date, remained unknown. Treatment of human or murine metastatic breast carcinoma cell lines with 10 μg/ml endotoxin resulted in increased VEGF expression relative to controls. This observation demonstrates that LPS directly upregulates VEGF and is in agreement with previous work that demonstrated a strong positive correlation between post-operative plasma LPS and serum VEGF levels in 4T1 tumour bearing mice (Chapter 3).

LPS has been shown to upregulate the expression of VEGF in human pulp cells

at similar concentrations, via a soluble CD14 mechanism (Matsushita *et al.*, 1999). The link between LPS and VEGF expression has not been fully characterised. However, TNF-α, an important inflammatory mediator induced by LPS, has been shown to strongly enhance VEGF mRNA in a glioma cell line (Ryuto *et al.*, 1996). This has been attributed to the transcription factor SP-1. LPS activates a number of MAP kinases such as c-Jun and c-Fos (Paul *et al.*, 1997), resulting in the over-expression of the transcription factors AP-1, AP-2 and SP-1. The VEGF promoter contains binding sites for these transcription factors (Shima *et al.*, 1996). Thus, the induction of these factors by LPS could result in increased VEGF transcription. These suggestions are supported by the fact that LPS increased the expression of the transcription factors AP-1, SP-1 and NF-$_\kappa$B in pulp cells (Matsushita *et al.*, 1999). Inhibitors to AP-1 were capable of blocking LPS induced VEGF expression, while inhibiting SP-1 or NF-$_\kappa$B were inactive. In this study it was also observed that the protein synthesis inhibitor, cycloheximide, was capable of blocking the VEGF protein accumulation in response to LPS, indicating that *de novo* protein synthesis is required for this induced VEGF expression. It would appear that at least in pulp cells, VEGF induction by LPS is mediated by an ongoing synthesis of c-jun and c-fos protein which produce AP-1. This is not to say that VEGF production by other cell types may be regulated by different factors, as in the case of TNF-α and SP-1.

In this chapter the link between LPS-induced VEGF and LPS-mediated inhibition of tumour cell apoptosis was investigated. VEGF has previously been shown to induce the expression of the anti-apoptotic protein, Bcl-2, in endothelial cells. This set of experiments demonstrates a similar effect in murine and human metastatic tumour cells. Following stimulation with either LPS or VEGF, tumour cell expression of Bcl-2 was increased in both the human and murine tumour cells. Inclusion of a neutralising

antibody to VEGF resulted in a marked reduction in LPS-induced Bcl-2 protein expression. The data demonstrates that LPS induced Bcl-2 expression in tumour cells is via a VEGF mediated mechanism.

Folkman and co-workers have shown that anti-angiogenic therapy inhibits the growth of tumours through increased tumour cell apoptosis in the absence of alterations in tumour cell growth rates (Holmgren *et al.*, 1995; Parangi *et al.*, 1996; O'Reilly *et al.*, 1997). In these studies, the induction of apoptosis within the tumours had been attributed to vessel regression and consequent depletion of nutrients. The data presented in this chapter showed that LPS or VEGF inhibited apoptosis of tumour cells *in-vitro*, determined by TUNEL immunostaining. As the basal rate of apoptosis was very low in both cell lines (<4%) apoptosis was induced by growth factor withdrawal. This induction of apoptosis was most probably mediated through alterations in Bcl-2, as serum depletion potentiates the effects of VEGF on Bcl-2 transcription in endothelial cells (Gerber *et al.*, 1998). Treatment of tumour cells with anti-VEGF antibodies resulted in almost a three-fold increase in the rates of apoptosis in both 4T1 and MDA-MB-231 tumour cells, presumably due to blocking endogenous VEGF production by the tumour cells. This result alone, indicates that the removal of VEGF from tumour cells results in increased tumour cell death directly. Anti-VEGF antibodies also blocked LPS-induced reductions in tumour cell apoptosis, providing further evidence that the LPS-induced survival effect on tumour cells is mediated through VEGF. There is a growing body of evidence to support these observations. VEGF null fibrosarcomas were found to have an 8.5-fold increase in apoptosis compared to VEGF +/+ tumours (Grunstein *et al.*, 1999). The results in this chapter suggest that loss of VEGF, or its blockade in certain tumour types inhibits tumour growth in part by affecting tumour cell survival. Therefore, in addition to

its direct angiogenic effects on endothelial cells, VEGF has a role as a survival factor in tumour cells.

The signalling pathway through which VEGF acts on tumour cells remains to be clarified. However, recently a novel third receptor, neuropilin 1, specific for VEGF165, has been identified on a number of tumour cells including MDA-MB-231 (Soker *et al.*, 1998). In previous studies in our laboratory, western blot analysis has failed to identify either KDR/Flk-1 or Flt1, the VEGF receptors expressed by endothelial cells, in 4T1 or MDA-MB-231 cells (unpublished observations, Siobhan Griffen). A role for VEGF in preventing tumour cell apoptosis is further supported by recent reports that overexpression of soluble neuropilin 1 (sNRP1), which prevents VEGF165 binding to cell surface receptors, in tumour cells was associated with increased prostate tumour cell apoptosis *in vivo* (Gagnon *et al.*, 2000). These authors also demonstrated that sNRP1 prevented VEGF165 binding to rat prostate carcinoma cells, although the consequences of VEGF binding to these tumour cells was unknown. VEGF has been shown to upregulate the expression of the KDR receptor on endothelial cells, which may be a positive feedback mechanism for VEGF action (Shen *et al.*, 1998). Thus strategies that block VEGF activity may reduce VEGF receptor expression. Decreased VEGF receptor expression may therefore lead to an overall reduction in VEGF expression.

Anti-angiogenic therapy has received a lot of attention in the last decade and has resulted in a number of promising results (Baillie *et al.*, 1995; Parangi *et al.*, 1996; O'Reilly *et al.*, 1998). The results presented here suggest that anti-angiogenics, in particular, those directed against VEGF, may have multiple anti-tumour effects. Firstly, they inhibit endothelial cell growth, inducing endothelial cell apoptosis and vessel regression. Secondly, by blocking or reducing angiogenic molecules they may have a

direct anti-tumour effect by increasing tumour cell apoptosis. It would suggest that anti-VEGF strategies, as part of combination therapy, may improve the efficacy of conventional treatments aimed at inducing tumour cell apoptosis, such as chemotherapy and/or radiation therapy.

Chapter 7

Concluding Discussion

In these studies a number of factors critical in regulating tumour growth were examined, including the balance of mitosis and apoptosis within tumours and VEGF as an index of angiogenesis. The results presented demonstrate that open surgery results in the increased growth of lung metastases in a murine model of experimental metastasis. Metastatic burden was increased following an open laparotomy compared to animals which received anaesthetic alone. The increase in tumour burden was associated with increased proliferation and decreased apoptosis within lung metastases. The tumour enhancing effect of surgery has been demonstrated previously, however the mechanisms underlying the effect have not been fully elucidated. It has been suggested that the surgical removal of a primary tumour may lead to the rapid recurrence of the disease, as a consequence of removing anti-angiogenic factors produced by the primary (O'Reilly *et al.*, 1994; O'Reilly *et al.*, 1997). In this series of experiments there was no primary tumour present, therefore the increased tumour growth observed could not be attributed to a similar effect. Open surgery had previously been shown to induce the translocation of endotoxin from the peritoneum into the systemic circulation. Endotoxin has been implicated in bladder tumour growth and shown to be angiogenic in the corneal implant and rat mesentery models of angiogenesis. Circulating levels of endotoxin were therefore examined post-operatively and were found to be elevated following open surgery or laparoscopy with air. More significantly, these levels correlated with post-operative VEGF levels, which were an indication of angiogenesis within the tumours.

Interestingly, when laparoscopy was performed with sterile CO_2, containing no endotoxin, the critical parameters governing tumour growth, namely metastatic burden, mitosis, apoptosis and serum VEGF, were comparable to those of mice receiving anaesthesia alone. However, when the procedure was performed with room air, which

does contain endotoxin, a similar response to that of an open surgical procedure was observed. This implicated some air-borne factor in the tumour growth. The fact that the air laparoscopy procedure resulted in similar findings to open surgery implied that the laparotomy wound was not the causative factor in the tumour growth observed. This is supported by the fact that the tumour growth was observed 5 days post-operatively, while it has been reported that wound factors peak between 1 and 2 weeks after surgery. Therefore it may be of interest to extend the study to examine the growth of the tumours over a longer period of time.

As endotoxin was elevated following surgery and correlated with serum VEGF, the effect of endotoxin injection on metastatic tumour growth was examined. Endotoxin resulted in a similar response to that observed following open surgery, increasing metastatic burden, tumour cell proliferation and decreasing apoptosis. Intra-peritoneal injection of endotoxin also resulted in increased circulating levels of VEGF. These experiments confirmed the findings of the surgical experiments, and suggests that endotoxin, introduced into the circulation during the surgical procedure augments metastatic tumour growth by altering the balances governing tumour expansion and angiogenesis. These findings may be of particular relevance to patients with a previous history of cancer undergoing surgery for an unrelated complaint, as it would appear that LPS may stimulate the growth of previously dormant cells, possibly leading to recurrence.

There have been many anti-endotoxin strategies designed to limit the effect of LPS, and most of these have been designed as therapeutic strategies in the treatment of sepsis. Of all of the strategies reviewed to date, the most promising agents appear to be the BPI proteins. Recombinant BPI ($rBPI_{21}$) has been shown to prevent, in a dose-dependent manner, a) the LBP-mediated binding and internalisation of LPS to

monocytes and b) LPS-induced TNF-α release from monocytes (Heumann *et al.*, 1993). Therefore, the effect of $rBPI_{21}$ on endotoxin-induced metastatic tumour growth was investigated *in-vivo*. Treatment with $rBPI_{21}$ during and after LPS injection inhibited LPS-induced metastatic tumour growth. This was reflected in the balances governing tumour expansion, with proliferation and apoptosis ratios similar to mice receiving a saline injection. More importantly, peri-operative $rBPI_{21}$ blocked surgically-induced metastatic tumour growth, and decreased circulating levels of VEGF observed following surgery. This is the first study to demonstrate that administration of an anti-endotoxin agent following surgery blocks metastatic tumour growth. The monoclonal antibody E5 was ineffective at blocking surgically-induced tumour growth. This is most probably due to weaker binding of the monoclonal antibody to LPS, and the fact that $rBPI_{21}$ binds to both smooth and rough LPS, as well as the lipid-A moiety of the LPS molecule.

These results indicate that the endotoxin-neutralising activity of $rBPI_{21}$ may be of therapeutic benefit in many other endotoxin-related disorders, such as SIRS and MODS. It would also be of interest to examine the usefulness of this agent in limiting other LPS-induced effects, such as vascular injury, permeability and angiogenesis, all of which create a favourable environment for tumour growth. Although encouraging, these experiments were by no means conclusive that $rBPI_{21}$ will work in the clinical setting. Further study over a longer period would be necessary to examine the effect of the compound at inhibiting tumour recurrence, when other factors such as wound elements would come into play. However, the study does highlight a potential therapeutic window in the peri- and early post-operative period for reducing a risk factor that may be inadvertently imposed upon every surgical / oncology patient.

As LPS was found to be elevated in mice following surgery, and LPS induced tumour growth *in vivo*, the mechanism whereby LPS enhanced tumour growth was investigated. A number of cell types, both host and tumour derived are involved in tumour progression. Thus, the effect of LPS on macrophages, endothelial cells and tumour cells was investigated. LPS increased macrophage VEGF expression at both the mRNA and protein levels. However, higher levels of VEGF protein were induced in murine tumour cells following treatment with LPS. This would suggest that the majority of circulating VEGF found in tumour bearing mice following surgery or endotoxin injection is most probably derived from the tumour metastases. This is supported by the fact that serum VEGF was highest in groups with larger tumour bulk. However, whether increased VEGF is responsible for the increase in tumour bulk, or simply a consequence of it, remains to be determined. As increased proliferation and decreased apoptosis was observed in the metastases of mice following open surgery or endotoxin injection, the direct effect of LPS on proliferation and apoptosis of tumour and endothelial cells was examined. LPS increased proliferation of both murine and human mammary adenocarcinoma cells as well as HUVECs. This effect was not observed when cells were grown in the absence of FCS, indicating that a serum factor, probably LBP was required for LPS binding and activation of the cells. Future work could focus on the mechanisms regulating proliferation in response to LPS. As endotoxin induces the secretion of a large number of cytokines from a variety of cell types, it would be of interest to examine whether any of these factors could be responsible for the increased proliferation observed in these experiments. TNF-α is one of the most potent factors secreted in response to the stimulation of cells with LPS, and has been implicated as an angiogenic factor *in-vivo*. However, TNF-α was not responsible for the increased proliferation of tumour cells observed in response to LPS,

and was in fact, cytotoxic to the cells at doses above 100 ng/ml. As it is likely that the effects of LPS are mediated through its binding to its cell surface receptor, it would be interesting to investigate the effect of using anti-CD14 antibodies to block the biological effects of LPS. In a similar manner, examination of the downstream activation of different protein kinases or signalling molecules in response to LPS could provide more information into the mechanism whereby LPS induces VEGF production and proliferation in cells.

Endotoxin has been reported to be angiogenic in a number of systems. However, the mechanism has not been elucidated. In an *in vivo* matrigel model of angiogenesis, LPS was directly angiogenic, inducing endothelial cell infiltration at both the edge and centre of the gel, as well as the formation of blood vessels at the gel core. Further investigations could prove useful in determining whether LPS is angiogenic through the stimulation of other cytokines, using gels impregnated with a variety of factors elicited in response to LPS. However, the evidence presented in this thesis suggests that the angiogenic effect of LPS observed in the matrigel model is a direct effect.

Tumour dormancy is a major clinical concern in cancer patients. Dormant metastases provide a constant risk of recurrence throughout a patient's life-time. Interestingly the growth of metastasis following the removal of primary tumours has been attributed to alterations in apoptosis levels in tumours and not in their proliferation. As VEGF has been shown to be a survival factor for endothelial cells, and decreased apoptosis was observed in the metastases of mice with elevated serum VEGF, the effects of VEGF and LPS on tumour cell apoptosis were examined *in-vitro*. VEGF and LPS were found to increase the expression of the anti-apoptotic protein bcl-2 in both the murine and human breast cancer cell lines examined. Furthermore, anti-

VEGF antibodies were capable of blocking LPS-induced bcl-2 expression, indicating that the induction of bcl-2 was via a VEGF mechanism. This is similar to the situation reported in endothelial cells, where VEGF is a survival factor by inducing bcl-2. Since VEGF and LPS were shown to induce bcl-2, the effect of these treatments on tumour cell apoptosis was investigated. Either LPS or VEGF resulted in a decrease in tumour cell apoptosis, and again anti-VEGF antibodies blocked LPS-mediated inhibition in apoptosis. These experiments suggest that VEGF is not solely an angiogenic factor, but perhaps more importantly is also a survival factor for tumour cells through the upregulation of bcl-2. Therefore, it implies that VEGF may make tumour cells resistant to chemotherapy and radiotherapy, and that blocking VEGF in combination with chemotherapy may prove useful in the treatment of cancer.

Collectively, this thesis has examined the role of endotoxin in many aspects of metastatic disease. It has shown that LPS regulates VEGF production by a number of cell types, and is directly angiogenic *in vivo*. It highlights a serious issue with regard to tumour recurrence following surgery, and provides direct evidence that LPS is at least partly responsible for this effect. Furthermore, a potentially promising therapeutic approach for the limitation of post-operative metastatic disease was investigated. Further study is required to examine the mechanisms involved in these LPS-mediated effects and the long-term usefulness of anti-endotoxin therapy, with the ultimate goal of producing more therapeutic agents that may inhibit tumour growth.

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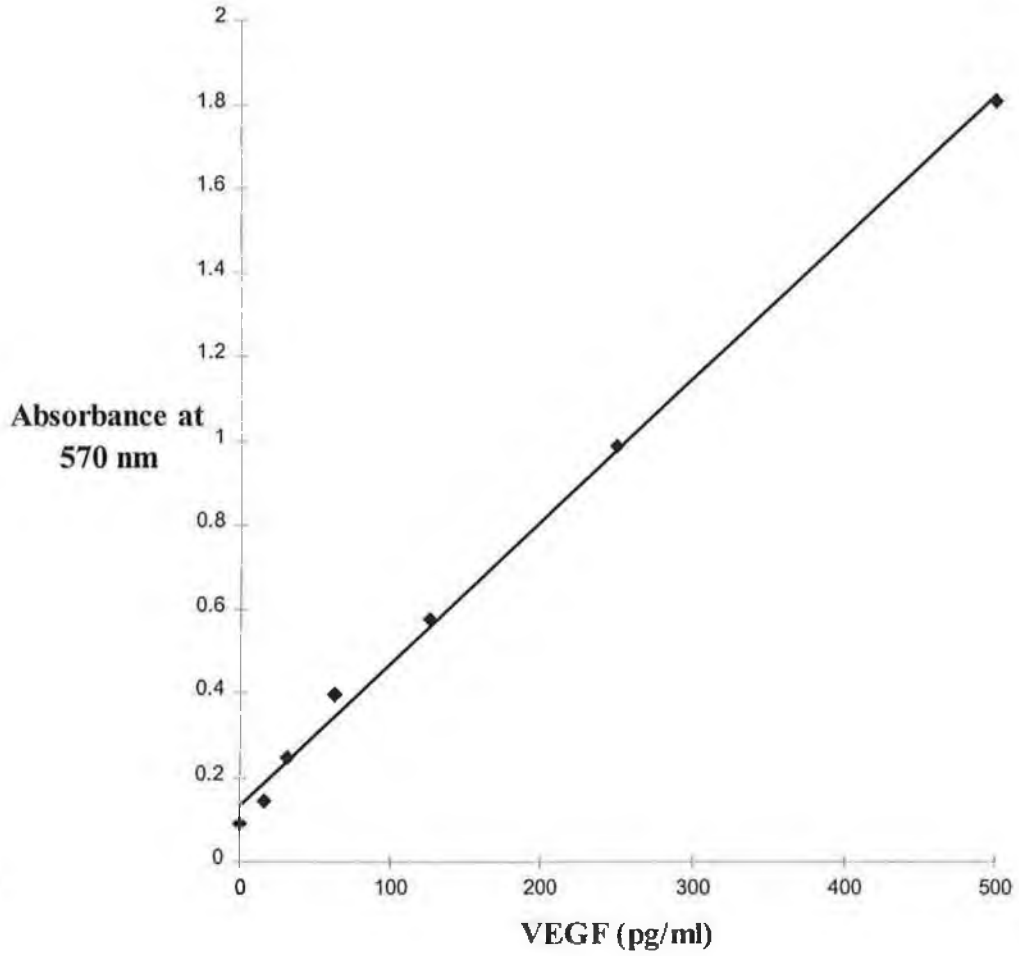
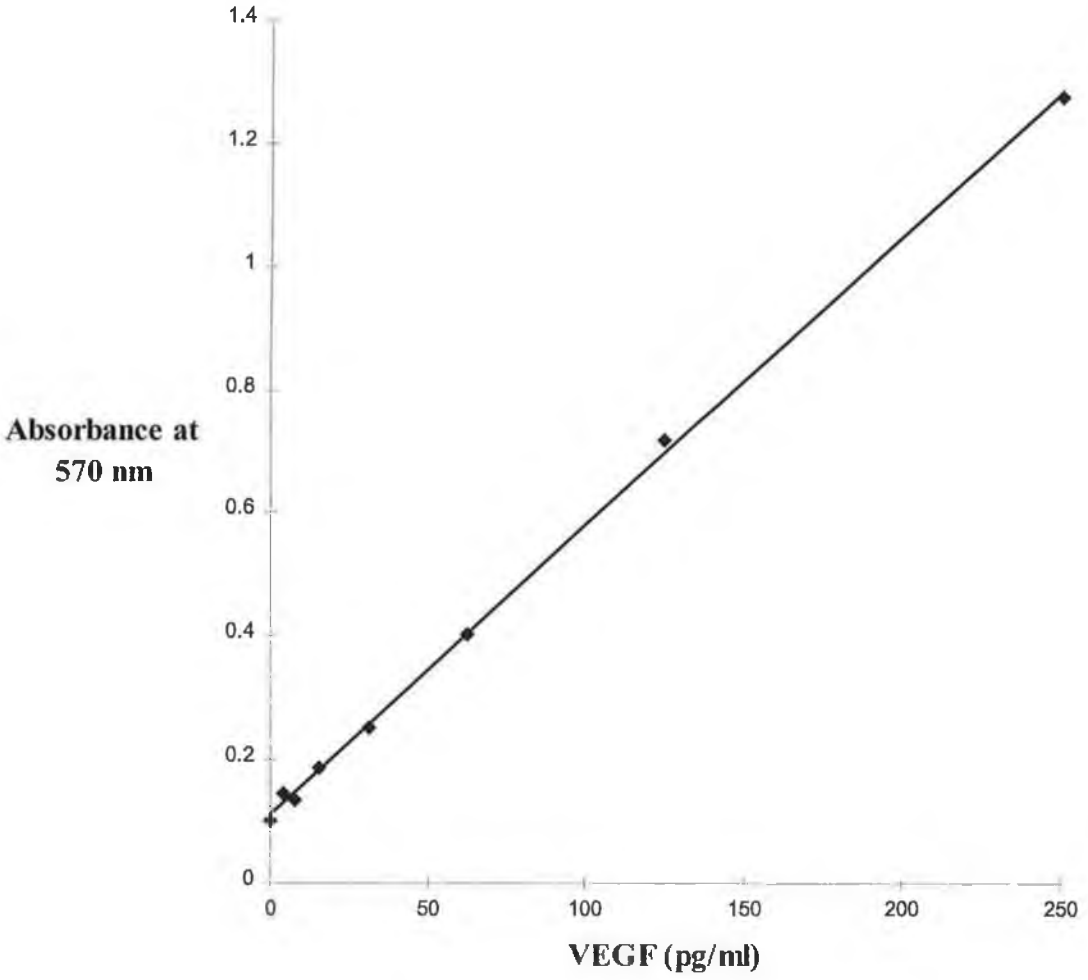
Appendix

Appendix I - Representative Standard curves



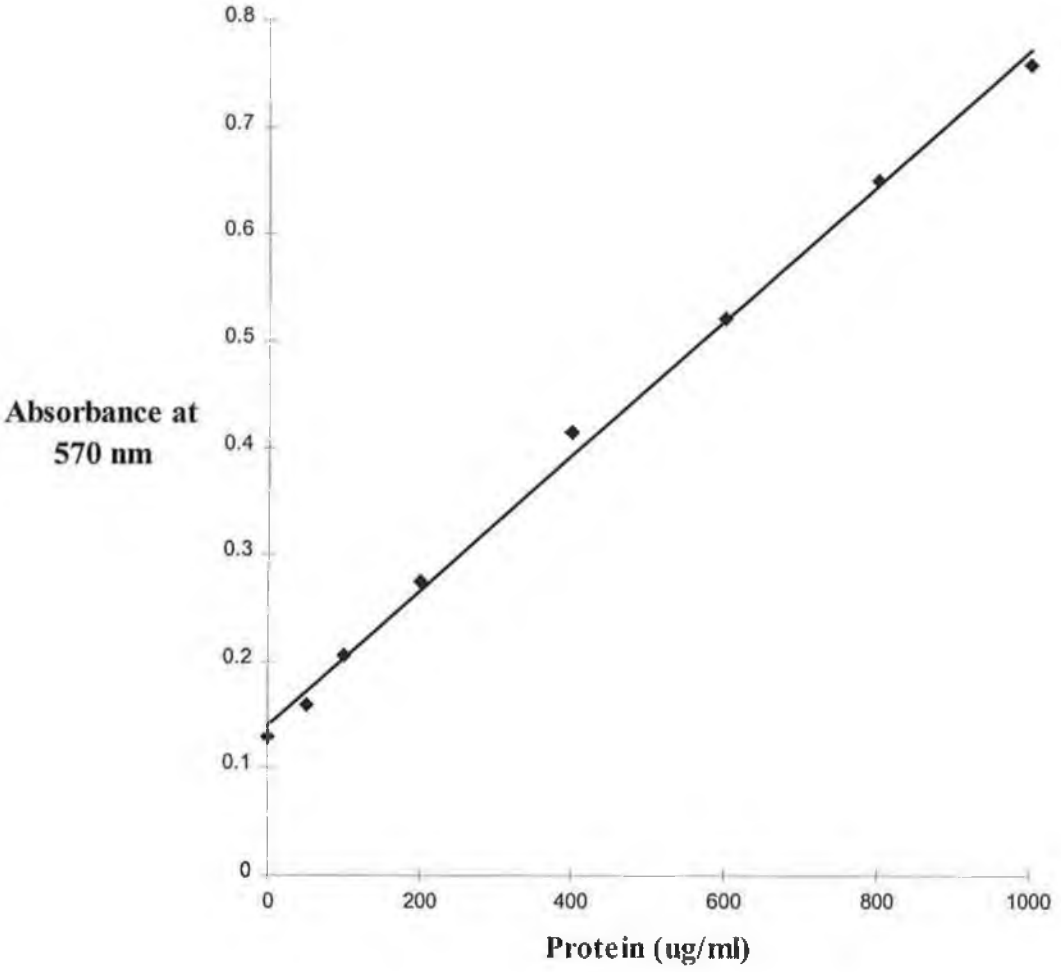
A.

Representative standard curve from ELISA used for human VEGF measurements in conditioned medium. Regression coefficient, $r^2 = 0.997$.



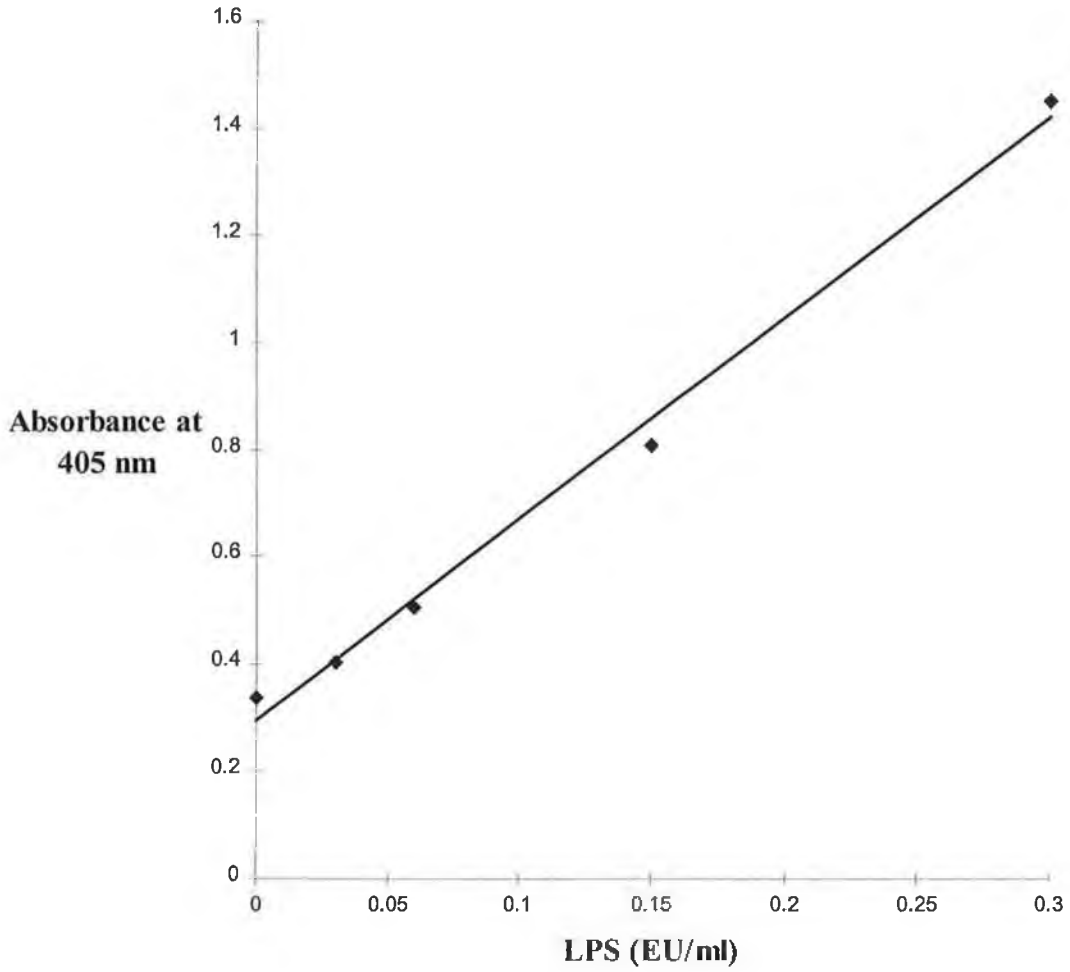
B.

Representative standard curve from ELISA used for murine VEGF measurements in conditioned medium. Regression coefficient, $r^2 = 0.999$.



C.

Representative standard curve from BCA assay used for protein measurements in conditioned medium and cell lysates. Regression coefficient, $r^2 = 0.997$.



D.

Representative standard curve from LAL assay used for endotoxin measurements in murine plasma samples. Regression coefficient, $r^2 = 0.993$.