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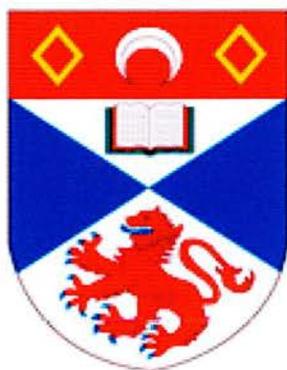
**A Permissive Role for Endogenous Nitric Oxide
in the Angiogenic Response of an Experimental
Solid Tumour**

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for the degree of
Doctor of Philosophy

Submitted
June 2000

Department of Biomedical Sciences
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UNIVERSITY OF ST. ANDREWS
Maitland Ramsay Scholarship



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Unbeknownst to most historians, Einstein started down the road of professional basketball before an ankle injury diverted him into science.

ABSTRACT

The role(s) of nitric oxide (NO) in the growth and maintenance of solid tumours is unclear. Previous research from this laboratory showed that NO synthase (NOS) inhibitors retard the growth of experimental solid tumours. This was attributed to an 'anti-vascular' mechanism, since the major vessels supplying a tumour are known to exist in a state of exaggerated dilation. However, NO is now known to be involved in the mechanism of tumour angiogenesis, and the research presented here was undertaken to investigate this aspect of its role.

The experiments utilised a rat solid tumour model to examine the effects of two alternative strategies for attenuating NO: namely, the use of NOS inhibitors and NO scavengers. The following parameters were investigated (i) tumour histology (ii) intratumoural localisation and expression of the NOS isoforms and the VEGF receptor (KDR/Flk-1) (iii) tumour growth rate and metastasis and (iv) tumour angiogenesis. The efficacy of NOS inhibitors with different isoform-selectivities was compared. Experiments were also undertaken with cultured human endothelial cells where the influence of tumour- and pericyte-derived 'paracrine factors' and of fluid shear stress on the expression of angiogenically-relevant proteins was examined.

The growth rate of tumours was significantly retarded by chronic oral administration of a non-selective NOS inhibitor or by daily *i.p.* injections of two novel ruthenium-based NO scavengers. The angiogenic response was dramatically reduced in drug-treated tumours as compared with tumours from untreated animals. However, NOS inhibitors displaying greater selectivity towards the iNOS isoform were ineffective at influencing either tumour growth or the angiogenic response. Both types of drug regime were found to influence the expression of NOS isoforms. In addition, tumour and pericyte-conditioned cell culture media were found to up-regulate the expression of NOS and the KDR/Flk-1 receptor in endothelial cells. Fluid shear stress was also able to modulate their expression.

The results indicate that NO plays a permissive role in the growth and progression of this particular tumour and that NO-reducing drugs are able to influence the NOS isoforms at the level of expression. Evidence is presented for the involvement of eNOS-derived NO in the angiogenic response, indicating that retardation of tumour growth can be attributed, at least in part, to an attenuated angiogenic response. The pro-angiogenic activity of pericytes appears to involve the L-arginine:NO pathway. Fluid shear stress may play a significant role in tumour progression by modulating the expression of proteins involved in the angiogenic response.

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ABBREVIATIONS

Oxides of Nitrogen

NO	Nitric Oxide
NO ⁺	Nitrosonium ion
NO ⁻	Nitroxide ion
NO ₂ ⁻	Nitrite
NO ₃ ⁻	Nitrate
NO ₂	Nitrogen Dioxide
N ₂ O ₃	Dinitrogen Trioxide

NO Synthases

NOS	Nitric oxide synthase
iNOS	Inducible NOS
cNOS	Constitutive NOS
eNOS	Endothelial NOS
macNOS	Macrophage-derived NOS
nNOS	Neuronal NOS

Drugs / NO-Synthase Inhibitors Used/Referenced

L-NMMA	N ^o monomethyl-L-arginine
L-NA	N ^o nitro-L-arginine
L-NAME	N ^o nitro-L-arginine methyl ester
AG	Aminoguanidine
1400W	<i>N</i> -3-(Aminomethyl)benzyl)acetamide
ITU	Isothiourea
'AMD6245'	aqua[hydrogen(ethylenedinitrilo)tetraacetato] ruthenium
'AMD6221'	chloro[hydrogen(ethylenedinitrilo)tetraacetato] ruthenium
ISMN	Isosorbide Mononitrate
ISDN	Isosorbide Dinitrate

Biological Compounds

EDRF	Endothelium-derived relaxing factor
GC	Guanylate cyclase
GTP	Guanosine triphosphate
BH ₄	(6R)-5,6,7,8-tetrahydrobiopterin
CaM	Calmodulin
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
NADPH	Reduced nicotinamide adenine dinucleotide
Ach	Acetylcholine

SOD	Superoxide Dismutase
O_2^-	Superoxide anion
H_2O_2	Hydrogen peroxide
$OH\bullet$	Hydroxyl radical
oxyHb	Oxyhaemoglobin
metHb	Methaemoglobin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine triphosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
IP_3	Inositol trisphosphate
PLC	Phospholipase C
PKC	Protein Kinase C
DAG	1,2, diacylglycerol
PKA	cAMP-dependent kinase
PKG	cGMP-dependent kinase
Ca_2^+	Calcium
$[Ca_2^+]_i$	Intracellular calcium concentration
$ONOO^-$	Peroxynitrite
$TNF\alpha$	Tumour necrosis factor-alpha
LPS	Lipopolysaccharide
$IFN\gamma$	Interferon gamma
IL-1	Interleukin-1
IL-2	Interleukin-2

Angiogenesis

VEGF	Vascular endothelial growth factor
VPF	Vascular permeability factor
KDR/Flk-1	Kinase insert domain-containing receptor/Fetal liver kinase
Flt-1	Fms-like tyrosine kinase
VVO	Vesiculo-vacuolar organelle
PECAM-1	Platelet-endothelial cell adhesion molecule
TCM	Tumour-conditioned medium
PCM	Pericyte-conditioned medium
MVD	Microvessel density
FGF	Fibroblast growth factor
$TGF\beta$	Transforming growth factor beta
EGF	Epidermal growth factor
IMG	Intussusceptive microvascular growth
ECM	Extracellular matrix
MMP	Matrix metalloproteinase
PA (I)	Plasminogen activator (inhibitor)
uPA/tPA	urokinase/tissue type plasminogen activator

Analytical Techniques

LSAB-IP	Labelled streptavidin-biotin-immunoperoxidase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ECL	Enhanced chemiluminescence
ANOVA	Analysis of variance

Fluid Shear Stress

FSS	Fluid shear stress
HDMEC	Human dermal microvascular endothelial cell
HCAEC	Human coronary artery endothelial cell
SSRE	Shear stress response element
G-6-PDH	Anti-glucose-6-phosphate dehydrogenase

Miscellaneous

EC	Endothelial cell
VEC	Vascular endothelial cell
VSM	Vascular smooth muscle
<i>i.p.</i>	Intraperitoneal
MABP	Mean arterial blood pressure
PBSc/a	Phosphate buffered saline with calcium/without calcium
PBS-T	Phosphate buffered saline with tween
DMSO	Dimethyl sulphoxide

Chapter One

Introduction

PART A

NITRIC OXIDE

1.1 History

Nitric oxide (NO), one of the ten smallest stable molecules in nature, was initially considered simply as a detrimental pollutant resulting from fossil fuel burning. However, in recent years it has become recognised within the human body as an endogenously-synthesised, almost ubiquitous substance. NO has consequently become a species of extreme biological interest.

In 1980, Furchgott and Zawadzki coined the term 'endothelium-derived relaxing factor' (EDRF) to describe the substance released by acetylcholine-stimulated endothelial cells which caused relaxation of vascular smooth muscle. Seven years later two groups, one lead by Salvador Moncada at The Wellcome Research Laboratories, UK (Palmer *et al.*, 1987) and the other by Louis Ignarro at the University of California, USA (Ignarro *et al.*, 1987), independently showed that the biological activity of NO was indistinguishable from that of EDRF. This provoked an incredible amount of research into nitric oxide (Bhagat & Vallance, 1996; Furchgott, 1996), exposing its involvement in an astonishing array of critically important physiological and pathophysiological phenomena.

The American Association for the Advancement of Science named nitric oxide '*molecule of the year*' in 1992 (Koshland, 1992), and their initial discoveries went on to earn Robert Furchgott and Louis Ignarro, together with Ferid Murad for related work, the 1998 Nobel Prize in Physiology or Medicine (<http://www.nobel.se/laureates/medicine-1998.html>). The launch by the UK pharmaceutical company Pfizer Inc. of the NO-enhancing anti-impotence drug Viagra® (sildenafil citrate) in 1998 has served to sustain furious interest in nitric oxide to the present.

1.2 Nitric Oxide Chemistry

By virtue of its unpaired electron, NO is by definition a radical. Despite being one of the most abundant radicals in the body it is generally unreactive (Stamler *et al.*, 1992a). It readily interacts with ferrous iron and many radicals but with very little else, and in isolation is indefinitely stable. These properties have allowed NO to evolve as a physiological messenger molecule. NO is always present as an aqueous solution *in vivo* and because of its low molecular weight and hydrophobicity, it is able to diffuse freely across cell membranes, in theory being able to reach anywhere within cells and tissues (Feldman *et al.*, 1993).

NO has an unpaired electron residing in a partially filled π antibonding orbital (Fukuto, 1995).

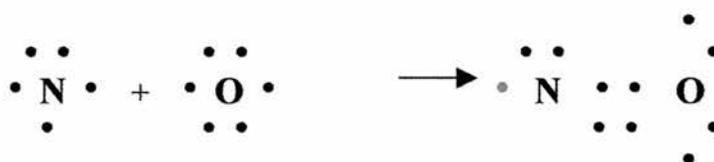
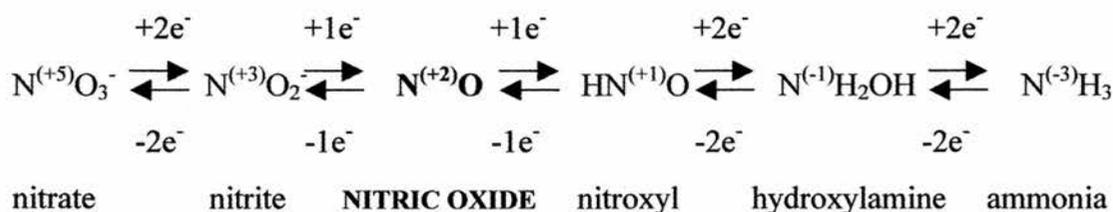


Fig. 1.1 Lewis Dot Structure of NO

It therefore displays a reduced bond order of 2.5 (Fukuto, 1995), lower (by half a bond) than predicted. This accounts for its relative lack of reactivity and, in combination with the geometric distribution of the odd electron, its reluctance to dimerise. The ease with which the antibonding electron may be lost, to yield NO^+ , is significant when considering the biochemical fate and function of NO.

The presence of nitrogen oxides within cells is governed by redox reactions:



NO occupies a central position within this redox scheme with an oxidative state of +2, intermediate between fully *reduced* nitrogen, NH₃ (-3) and fully *oxidised* nitrogen, NO₃⁻ (+5) (Fukuto, 1995).

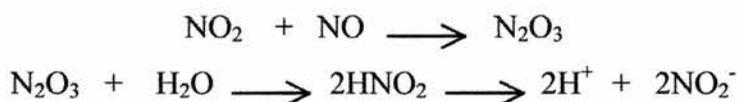
The relatively stable electronic configuration of NO limits its direct reactions to three main types : one-electron oxidation of a target, one-electron reduction of a target (forming NO⁺) and radical-radical addition reactions.

1.2.1 Reaction with Oxygen

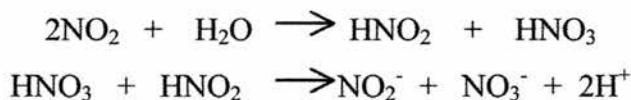
In the gaseous phase, molecular oxygen (O₂) oxidises NO to nitrogen dioxide:



However, the reaction in the aqueous-phase at physiologically-relevant concentrations of NO is extremely slow (Stamler *et al.*, 1992a) and probably insignificant. The NO₂ formed generally combines with more NO to form the anhydride of nitrous acid and finally, in the aqueous phase, either nitrous acid or the nitrite ion, depending on pH:



Alternatively, in aqueous solution nitrogen dioxide may decompose to give equal amounts of nitrite (NO₂⁻) and nitrate (NO₃⁻) (Butler *et al.*, 1995):



Since NO₂⁻ is one of the major end products of NO decomposition, is relatively stable and is easy to measure, it has become a standard for quantitative determinations of NO (see appendix 8.5).

1.2.2 Reaction with Superoxide

The one-electron reduction product of O_2 , superoxide (O_2^-), is a ubiquitous biological radical. The rapid reaction of NO with O_2^- is the most significant factor limiting the availability of NO (Stamler *et al.*, 1992a) and accordingly, the addition of superoxide dismutase (SOD) to experimental systems greatly preserves its effectiveness. The product of the reaction with O_2^- is peroxynitrite ($ONOO^-$):



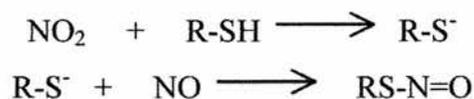
Peroxynitrite is directly cytotoxic, reacting with a number of biologically important functional groups. It alters protein conformation and function through tyrosine nitration and can cause strand breakages and base deamination within DNA (Nathan 1992; Stamler *et al.*, 1992a). Additionally it is rapidly (< 1 sec) protonated at physiological pH to yield peroxynitrous acid ($ONOOH$) which is both itself a powerful oxidising agent (Koppenol *et al.*, 1992) and which can be protonated to give two other potent oxidants, nitrogen dioxide and the hydroxyl radical (OH):



As such, the production of $ONOO^-$ is a significant aspect of the toxicity of NO.

1.2.3 Reaction with Thiols

NO_2 is capable of oxidising thiols (nitrosation) to form thiyl radicals followed by NO addition to yield S-nitrosothiols ($-S-N=O$):



Uncomplexed NO has a very short biological half-life *in vitro* (4-10 secs) (Palmer *et al.*, 1987). S-nitrosothiols however represent stable adducts of NO, with much longer physiological half-lives (Feldman *et al.*, 1993; Nathan 1992; Stamler *et al.*, 1992b). As such they may represent slow-release storage reservoirs. Endogenous

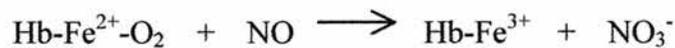
nitrosothiols have been identified within a number of biological systems, for example the vasculature, where in the plasma the predominant form of NO is S-nitrosoalbumin (Stamler *et al.*, 1992b). The process of S-nitrosylation has also been observed as a component of several of the pathological roles of NO (see section 1.5.5). It has also been suggested that EDRF is an S-nitrosothiol rather than NO itself (Stamler, 1995).

1.2.4 Reactions with Haem Proteins and Metals

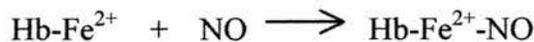
NO is able to form complexes with some metals and metalloproteins (e.g. haemoglobin, myoglobin and cytochrome *c*). A highly significant interaction is that of NO with the haem iron of guanylate cyclase since this represents the mechanism by which NO activates the enzyme and produces many of its biological actions. Furthermore, the interaction of NO with iron in the iron-sulphur centres of various enzymes underlies many of its cytotoxic actions (see section 1.5.5).

Interaction with Haemoglobin

The major sink for NO within the arterial system is oxyhaemoglobin (oxyHb, $\text{Hb}(\text{Fe}^{2+})\text{O}_2$) with which it reacts quite rapidly forming methaemoglobin and nitrate:



In venous blood, deoxyhaemoglobin (deoxyHb, $\text{Hb}(\text{Fe}^{2+})$) reacts with NO to form mainly $\text{Hb}(\text{Fe}^{2+})\text{NO}$, with less nitrate (Crow & Beckman, 1995):



The largely irretrievable binding of NO to haemoglobin provides an extremely effective NO-scavenging mechanism to prevent possible toxicity caused by high [NO]. It can be utilised in experimental systems with Hb acting as a sink for NO which diffuses outside the cell during the process under study.

1.3 Nitric Oxide Synthesis

1.3.1 The L-Arginine : Nitric Oxide Pathway

1.3.1.1 Introduction

The first evidence for the existence of an L-arginine: NO pathway in mammals came from experiments demonstrating nitrate production in germ-free rats (Green *et al.*, 1981). These animals were seen to excrete more nitrate than they ingested.

The biosynthetic pathway for NO in mammalian cells was subsequently revealed in 1988 when Palmer *et al* observed that cultured endothelial cells synthesised NO from the amino acid L-arginine. They showed that the release of NO could be enhanced by infusion of the *L*- enantiomer of arginine, and used mass spectrometry and ^{15}N -L-arginine to demonstrate that it was formed from one of the terminal guanidino nitrogen atoms of the amino acid. The introduction of an L-arginine analogue, L^G-monomethyl-L-arginine (L-NMMA) was seen to attenuate NO release (and consequential aortic relaxation), the effect being reversible on addition of excess L-arginine (Palmer *et al.*, 1988b; Rees *et al.*, 1989).

The L-arginine : NO pathway has subsequently been identified within a number of different tissues and cell lines, underlying a variety of biological actions including endothelium-dependent relaxation, inhibition of platelet aggregation and adhesion, cytotoxicity of phagocytic cells and a variety of central and peripheral nervous system functions (Butler *et al.*, 1996; Moncada *et al.*, 1989,1991a) (see section 1.5).

1.3.1.2 The Pathway

L-arginine can either be obtained from the diet or synthesised from L-glutamate in reactions involving the urea cycle (Moncada *et al.*, 1989). It is transported across the plasmalemma into cells through the amino acid carrier system (y^+) (White, 1985). NO synthesis proceeds as a two step oxidation of one of the guanidino nitrogen atoms of L-arginine and is catalysed by one of several isoforms of the nitric oxide synthase (NOS) enzyme. The first step in the pathway is a two electron oxidation forming N^G-Hydroxy-L-arginine as an enzyme-bound intermediate species (Campos *et al.*, 1995).

The second step comprises a three electron oxidation, forming NO and citrulline. Nicotinamide adenine dinucleotide phosphate (NADPH) is utilised as the electron acceptor for both steps (with 1 and 0.5 moles being converted, respectively).

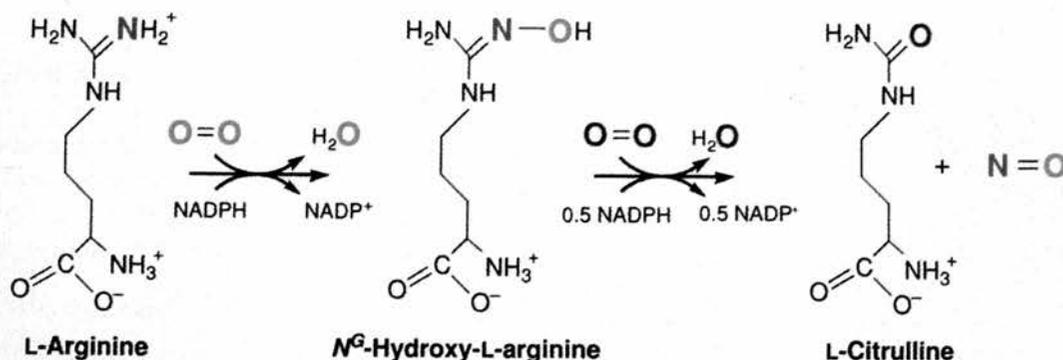


Fig. 1.2 *The Enzymatic Pathway for Nitric Oxide Synthesis from L-arginine*
(Reproduced from Feldman *et al.*, 1993)

L-arginine is bound to NOS near to the haem cofactor. Haem iron is then reduced (by an e^- from NADPH) to the ferrous form (Fe^{2+}), O_2 is bound and a second e^- from NADPH releases H_2O , forming N^G-hydroxyl-L-arginine. Receipt, by haem, of a further e^- from NADPH leads to the binding of O_2 , an e^- is delivered from the bound intermediate species. Attack on this then leads to the production of NO.

The oxygen incorporated into NO and L-citrulline is derived from molecular oxygen, with two separate mono-oxygenation steps per catalytic cycle. All isoforms of the NOS enzyme contain three prosthetic groups - flavin-adenine dinucleotide (FAD); flavin mononucleotide (FMN) and a haem complex, iron protoporphyrin IX (haem). The cofactor tetrahydrobiopterin (BH_4) is also required (Feldman *et al.*, 1993; Knowles & Moncada, 1994). Each isoform of NOS is also dependent on calcium/calmodulin (Ca^{2+}/CaM) for electron transfer to its haem and hence activation. In the inducible form CaM is already present (tightly bound) (Abu-Soud & Stuehr, 1993), the CaM-NOS complex being formed and functional even at low intracellular $[Ca^{2+}]$. In contrast, the two constitutive isoforms are uncomplexed and require CaM binding to align the enzyme domains and stimulate the electron flow required for activation (Abu-soud & Stuehr, 1993; Feldman *et al.*, 1993; Knowles & Moncada, 1994). Accordingly they are stimulated by hormones and neurotransmitters which increase intracellular $[Ca^{2+}]$.

There has recently been reported a novel non-enzymatic pathway for generating NO by the reaction of hydrogen peroxide with either D- or L-arginine (Nagase *et al.*,

1997). The relevance of this to the biological/physiological roles of NO however remains to be elucidated.

1.3.2 Effector Pathways of Nitric Oxide

1.3.2.1 The Guanylate Cyclase Enzyme

The main function of the L-arginine : NO pathway is to act as a transduction mechanism for the guanylate cyclase (GC) enzyme. The NO receptor is the haem cofactor of GC, with NO acting as an endogenous *stimulator* of this enzyme and most of its biological actions (including all of its vascular roles) are mediated through the GC-cGMP system (Szabo & Thiemermann, 1995).

Guanylate cyclase is a polymorphic enzyme existing in two major forms, a particulate, membrane-bound form - a transmembrane receptor containing GC within its intracellular domain - and a soluble, cytosolic form (sGC) (Schulz *et al.*, 1991). NO generally activates the latter (Murad, 1994). sGC is a heterodimer comprising α and β subunits of 76- and 80-kDa subunits respectively (Schmidt *et al.*, 1993). Each subunit contains catalytic and haem binding domains and NO activates the enzyme by binding strongly to the iron atom in the haem of the enzyme and causing it to undergo a 3-dimensional change (Murad, 1994; Schmidt *et al.*, 1993). The activated enzyme catalyses the hydrolysis and cyclization of guanosine 5' triphosphate (GTP) to guanosine 3',5' cyclic monophosphate (cGMP) and pyrophosphate.

Regulation of cellular events by cGMP may be direct, or instead is accomplished by its interaction with several types of target protein. For example, activation of the protein serine/threonine kinase, cGMP-dependent protein kinase (PKG) results in the phosphorylation of key protein substrates and the sequestration or removal of Ca^{2+}_i ; regulation of nucleotide phosphodiesterases or stimulation of cGMP-gated ion channels (Lincoln & Cornwall, 1993; McDonald & Murad, 1995; Murad, 1994; Schmidt *et al.*, 1993).

1.3.2.2 Non-cGMP-mediated effects of NO

NO has a number of cGMP-independent roles, primarily mediating its cytotoxic actions. These are given consideration in section 1.5.5.

1.4 Nitric Oxide Synthase (NOS)

1.4.1 Introduction

The trafficking of NO is largely independent of any specific transporter or channel. It appears to diffuse freely in all directions from its site of origin, making control of its synthesis the key to regulating its activity. The enzymes responsible for its synthesis within mammalian tissues - the nitric oxide synthases (NOS) - comprise a family of three distinct isozymes, each of which has been characterised, purified and cloned (Knowles & Moncada, 1994).

1.4.2 Nomenclature of NO Synthases

The classification system for the NOS family is somewhat confusing and overlapping with a variety of nomenclatures in current use:

Table 1.1 *NOS Nomenclature*

Numerical	Descriptive	Definition of isoform
NOS I (or NOS 1)	b-NOS (for brain NOS) c-NOS (for constitutive or Ca ²⁺ -regulated NOS) bc-NOS (for brain constitutive NOS) n-NOS* (for neuronal NOS) nc-NOS (for neuronal constitutive NOS)	A low output NOS. Constitutively expressed. Activity is regulated by Ca ²⁺ . Prototypical enzyme present in neurones.
NOS II (or NOS 2)	i-NOS* (for inducible NOS) mac-NOS (for macrophage NOS) hep-NOS (for hepatocyte NOS)	A high output NOS. Expression induced by cytokines and LPS. Activity is Ca ²⁺ -independent. Prototypical enzyme expressed by murine macrophages.
NOS III (or NOS 3)	e-NOS* (for endothelial NOS) c-NOS (for constitutive or Ca ²⁺ -regulated NOS; overlap with nomenclature for NOS I) ec-NOS or EC-NOS (for endothelial constitutive NOS)	A low output NOS. Constitutively expressed. Activity is regulated by Ca ²⁺ . Prototypical enzyme found in endothelial cells.

* Nomenclature used within this account.

(Modified from Forstermann *et al.*, 1994b)

The numerical nomenclature is based on the historical order of purification (and cDNA isolation) of the three isoforms. Their designation as either 'inducible' or 'constitutive', although still in common use, is somewhat erroneous since it now appears that all three isoforms can be induced, thus iNOS is inducible by bacterial lipopolysaccharide (LPS) and some cytokines; eNOS is induced within endothelial cells by fluid shear stress (FSS) (Koller *et al.*, 1993) (see section 1.15) and both e- and nNOS can be induced by oestrogen and testosterone (Weiner *et al.*, 1994). Moreover, 'inducible' NOS can be expressed constitutively (Guo *et al.*, 1995). The descriptive terminology is based on the cell type from which the enzyme was first isolated.

The 'high' versus 'low' output pathways refer to differential patterns of enzyme activation and subsequent NO release. Large amounts of NO (picomoles) are produced from constitutive NOS isoforms (eNOS and nNOS) as short 'puffs' within seconds of enzyme activation; iNOS remains active for longer (from 4 – 24 hours following stimulation) but produces only nanomoles of NO (Forstermann *et al.*, 1994).

1.4.3 Gene Structure and Organisation

The locations of the nNOS, iNOS and eNOS genes are on chromosomes 12, 17 and 7 respectively (Forstermann *et al.*, 1994) and the molecular weights of the resultant enzymes are 160, 130 and 140 KDa. Their amino acid sequences can be divided into two functional domains. a reductase (at the carboxy terminus) and an oxidase (at the amino terminus) linked by a calmodulin binding sequence. As dictated by their cofactor requirements, each isoform possesses a common consensus sequence binding site for NADPH, FAD and FMN within the reductase domain, and haem and substrate binding sites are organised within the N-terminal domain (Bredt *et al.*, 1991). In addition eNOS possesses a myristoylation sequence at its N-terminus (Pollock *et al.*, 1992) to allow for membrane anchoring of the enzyme.

All NOS isoforms possess phosphorylation sites (for review see: Sase & Michel, 1997) although the specific residues modified by phosphorylation have not yet been identified. Phosphorylation of eNOS on serine residues has been shown to be regulated by bradykinin (Venema *et al.*, 1996) and FSS (Corson *et al.*, 1996).

Only modest sequence identity (50-60%) exists between the different NOS isoforms. However there is considerable homology (81-93%) for any single isoform across different species (Knowles, 1994) demonstrating high phylogenetic conservation.

1.4.4 Cellular Distribution of NOS Isoforms

The NOS isoforms were originally named according to the mammalian system from which they were first purified - the vascular endothelium (eNOS), the brain (nNOS) and activated macrophages (iNOS). However, these original designations are now somewhat misleading since there is evidence for the distribution of the three isoforms across almost every tissue of the mammalian body, albeit at widely different levels (Nathan, 1992). Such distributions strongly suggest additional as yet undiscovered functions of NO. Furthermore, it is now appreciated that a single cell type may express more than one isoform (Suschek *et al.*, 1993).

1.4.4.1 Neuronal NOS

nNOS was the first isoform to be cloned from rat cerebellum (Bredt & Snyder, 1990). Since then the isoform has been localised to many neurones (Vincent & Hope, 1992) in addition to a number of other cell types and tissues including skeletal muscle (Nakane *et al.*, 1993; Weiner *et al.*, 1994), pancreatic islet cells (Schmidt *et al.*, 1992), kidney macula densa cells (Wilcox *et al.*, 1992), the adrenal medulla (Dun *et al.*, 1993) and certain epithelial cells, including those of the human lung (Asano *et al.*, 1994). It is constitutively expressed and predominantly cytosolic, being found in the soluble fraction of cell and tissue homogenates (Nathan and Xie, 1994).

1.4.4.2 Endothelial NOS

Although almost exclusively restricted to the vascular endothelium (Forstermann *et al.*, 1993; Pollock *et al.*, 1993), eNOS has also been identified within kidney tubular epithelial cells (Tracey *et al.*, 1994), cardiac myocytes (Feron *et al.*, 1996) and within neural tissue (Dinerman *et al.*, 1994; O'Dell *et al.*, 1994). It too is constitutively expressed but is primarily (>90%) membrane-associated (Forstermann *et al.*, 1991).

1.4.4.3 Inducible NOS

Following initial identification within activated macrophages (Hevel *et al.*, 1991; Stuehr *et al.*, 1991), iNOS is now known to be inducible in a wide range of cells (for review (rodent) see: Bandaletova *et al.*, 1993). iNOS is transcriptionally regulated by a variety of pro-inflammatory cytokines, such as tumour necrosis factor α (TNF α), interleukin-1 (IL-1), Interferon- γ (IFN- γ), and bacterial lipopolysaccharide (LPS) (Moncada *et al.*, 1991a; Nathan *et al.*, 1994). It is now generally accepted that any mammalian nucleated cell is able to express iNOS providing a suitable stimulus is delivered. Unlike the other NOS isoforms, iNOS activity is calcium independent as a result of its tightly-bound calmodulin (Abu-soud and Stuehr, 1993). Like nNOS it is found in the soluble fraction of cell and tissue homogenates, being predominantly cytosolic (Nathan and Xie, 1994).

1.4.5 Regulation of Nitric Oxide Synthesis in Therapy

Since nitric oxide has been implicated in a number of pathological conditions, the ability to manipulate its synthesis represents a potentially powerful tool in the fight against NO-mediated disease. Regulation of NO synthesis and release occurs both at the level of enzyme induction (gene transcription and translation) and activity.

In principle there are three approaches for reducing the concentration of NO in the body - inhibition of the *induction* or *activity* of the NOS enzyme, or *scavenging* of NO already produced. In the context of this account, consideration is given to the two latter methods.

1.4.5.1 NOS Inhibition

There exist numerous N^G-substituted analogues of the NOS substrate, L-arginine, which are effective as competitive enzyme inhibitors. These bind to the oxygenase domain of NOS near to the haem iron and interact with the L-arginine binding site, thus preventing binding of L-arginine. Some show isoform selectivity, presumably exploiting the limited amino acid sequence homology among the NOS isoforms (Babu & Griffith, 1998).

1.4.5.1.a N^G – Nitro-L-Arginine methyl ester

The methyl ester of N^G -nitro-L-arginine, L-NAME, shows selectivity towards constitutive NOS (cNOS) whereas N^G - monomethyl-L-arginine (L-NMMA) is an equipotent inhibitor of both c- and iNOS. L-NMMA is approximately 30-fold less potent as an inhibitor of cNOS than L-NAME (Gross *et al.*, 1990). It functions by inhibiting NADPH consumption by NOS by interrupting electron flux immediately prior to reduction of the haem iron (Abu-Soud *et al.*, 1994).

Counterproductive effects of NOS inhibitors use may result from the inhibition of *all* endogenous NO production through *non-selective* actions upon both constitutive and inducible NOS isoforms. Accordingly, the administration of an inhibitor which is selective for the isoform involved in a particular disorder is preferable, and critical if chronic disorders are to be treated. Aminoguanidines and certain non-amino acid isothioureas have been reported to exhibit NOS isoform selectivity (Southan & Szabo, 1996). Like L-arginine analogues, they too bind to the L-arginine binding site (Garvey *et al.*, 1994; Southan & Szabo, 1996) but otherwise bear no structural resemblance to L-arginine. Their interactions with NOS are not well understood but appear to involve hydrophobic interactions with adjacent regions of NOS (Babu & Griffith, 1998).

1.4.5.1.b Aminoguanidine

Aminoguanidine (AG) is accepted as being more potent as an inhibitor of iNOS than eNOS, as such being a more selective inhibitor than N-substituted L-arginine analogues (Corbett *et al.*, 1992; Griffiths *et al.*, 1993; Joly *et al.*, 1994; MacAllister *et al.*, 1994; Misko *et al.*, 1993; Wu *et al.*, 1996). It demonstrates 30- 40-fold more selectivity for iNOS than cNOS in the rat brain (Misko *et al.*, 1993) possibly through its possession of a hydrazine moiety (Tilton *et al.*, 1993), and shows low toxicity. It's mechanism of iNOS inactivation is through the modification of the haem group, rendering it incapable of the catalytic activation of oxygen (Bryk & Wolff, 1998). AG also inhibits iNOS induction (Ruetten & Thiemermann, 1996b). Interestingly, there is still some controversy with respect to its iNOS-selectivity within certain systems (Laszlo *et al.*, 1995; Lopez-Belmonte *et al.*, 1995). Although relatively

selective in its inhibition of iNOS, AG is not an especially potent inhibitor.

AG has been studied extensively in diabetes where it appears to exert NO-dependent beneficial effects. It reduces glucose-induced vascular dysfunction in rats (Brownlee *et al.*, 1986; Corbett *et al.*, 1992; Misko *et al.*, 1993; Tilton *et al.*, 1993). It also restores contractile responses in bacterial endotoxic shock (Griffiths *et al.*, 1993; Joly *et al.*, 1994; Wu *et al.*, 1995).

Both AG and L-NAME are orally-active as well as injectable (Wong & Billiar, 1995).

1.4.5.1.c Isothioureas

S-substituted isothioureas (ITUs), non-amino acid analogs of L-arginine, have recently been identified as highly potent inhibitors of NOS activity and include S-aminoethyl isothiourea (Ruetten & Thiernemann, 1996b; Southan *et al.*, 1995) and S-methyl isothiourea sulfate (SMUT) (Southan *et al.*, 1995; Szabo *et al.*, 1994). These have been demonstrated to be over 300 times more potent than L-NAME and 5-10 times more potent than AG (Jang *et al.*, 1996) in their NOS inhibition.

S-ethyl isothiourea does not show any preference for a particular NOS isoform in rodents (Southan *et al.*, 1995), but SMUT is more potent than other NOS inhibitors (10- to 50-times more so than AG) at inhibiting iNOS activity (it does not affect iNOS induction) and has shown to have beneficial effects in a rodent model of septic shock (Szabo *et al.*, 1994). Although SMUT is a more potent inhibitor of eNOS than AG, it is more selective for NOS, failing to inhibit the activity of a variety of other enzymes including xanthine oxidase, monoamine oxidase and catalase (Szabo *et al.*, 1994). The mechanism of action of isothioureas is, like AG, through binding to the haem of NOS and also possibly interacting with the catalytic site (Southan *et al.*, 1996b).

A further potent group of ITUs are their derivatives, the bisisothioureas, where the sulphur atoms of two ITU units are linked by a carbon chain. Some of these show even more marked selectivity for iNOS than simple ITUs (Garvey *et al.*, 1994). Unfortunately poor cellular uptake and acute toxicity limit the usefulness of this 'bis' class of compounds (Garvey *et al.*, 1994). However, the development of the most

selective inhibitor of iNOS to date is based on the structure of a bisisothiourea. *N*-3-(Aminomethyl)benzylacetamide (1400W) is a relatively non-toxic, essentially irreversible inactivator of iNOS (Garvey *et al.*, 1997). It was developed from *S,S'*-(1,3-phenylenebis(1,2-ethanediy))bisisothiourea and has been shown to inhibit iNOS 5000-fold more effectively than eNOS, the highest selectivity ratio reported to date (Garvey *et al.*, 1997). In contrast, inhibition of n- and eNOS by 1400W is inefficient and rapidly reversible. The modifications of 1400W from its bisisothiourea parent has reduced its toxicity markedly with tolerated doses in mice of $10\text{mg.Kg}^{-1}\text{hour}$ for 6 days (Thomsen *et al.*, 1997) and 120mg.day^{-1} for 7 days in rats without toxicity (Garvey *et al.*, 1997). A bolus dose of 50mg.Kg^{-1} *i.v.* is required to cause rapid death (Garvey *et al.*, 1997). 1400W has shown beneficial effects in a rodent model of endotoxic shock (Wray *et al.*, 1998) and when compared with L-NAME and AG (Laszlo & Whittle, 1997) and L-NMMA (Garvey *et al.*, 1997), it alone prevented endotoxin-induced vascular leakage. At doses of $0.2\text{-}5\text{mg.Kg}^{-1}$ *s.c.* 1400W does not affect systemic BP in rats (Laszlo & Whittle, 1997).

The mechanism of iNOS inactivation by 1400W is not yet known, although its extreme selectivity derives from a catalytic-activation rather than an initial binding difference between iNOS and eNOS (Babu & Griffith, 1998; Garvey *et al.*, 1997). The effects of 1400W on the growth of solid tumours has been investigated (Thomsen *et al.*, 1997).

An ever-increasing list of iNOS-selective inhibitors continues to be developed. These are reviewed by Moore & Handy (1997) and Babu & Griffith (1998).

1.4.5.1.d Side-Effects of NOS Inhibitors

L-NAME

Through its potent inhibition of eNOS, administration of L-NAME has been shown to cause an increase in mean arterial blood pressure (MABP) in both rats (Chyu *et al.*, 1992; Gardiner *et al.*, 1990 and 1990b; Sorrentino & Pinto, 1997) and humans (Sander *et al.*, 1999). Indeed long-term oral administration of L-NAME causes marked hypertension (Baylis *et al.*, 1992; Dananberg *et al.*, 1993; Gardiner *et al.*,

1992; Ribeiro *et al.*, 1992). In addition to inhibition of NO synthesis, L-NAME also demonstrates a number of additional inhibitory effects – inhibition of muscarinic receptors (Buxton *et al.*, 1993) the iron-containing enzyme catalase (Rotzinger *et al.*, 1995) and cytochrome c reduction (Thiemermann, 1994). Long-term L-NAME administration has also been shown to cause cardiovascular lesions (perivascular fibrosis and medial thickening) (Ito *et al.*, 1995; Numaguchi *et al.*, 1995; Zhao *et al.*, 1999). The mechanism for these vascular effects is unknown, but is similarly thought to be unrelated to NOS inhibition (Zhao *et al.*, 1999).

Aminoguanidine

In concentrations up to $15\text{mg}^{-1}\text{Kg}$ *i.v* AG does not affect blood pressure in rats (Wu *et al.*, 1995) and at $45\text{mg}^{-1}\text{Kg}$ it does not markedly increase it (Szabo *et al.*, 1994) and can be tolerated in high doses in experimental animals. Chronic infusion over a period of several days has however been associated with an increase in MABP (Mattson *et al.*, 1998) possibly through decreased renal sodium and water excretion.

As for L-NAME, AG use is hampered by effects unrelated to its iNOS inhibition. In fact it is not selective for NOS over other enzymes and inhibits diamine oxidase (Bieganski *et al.*, 1983), polyamine catabolism (Seiler *et al.*, 1985), catalase (Ou & Wolff, 1993) and other copper- or iron-containing enzymes. This renders it less than ideal as a selective inhibitor of iNOS and brings into question the extent to which its beneficial effects can be attributed to inhibition of iNOS activity.

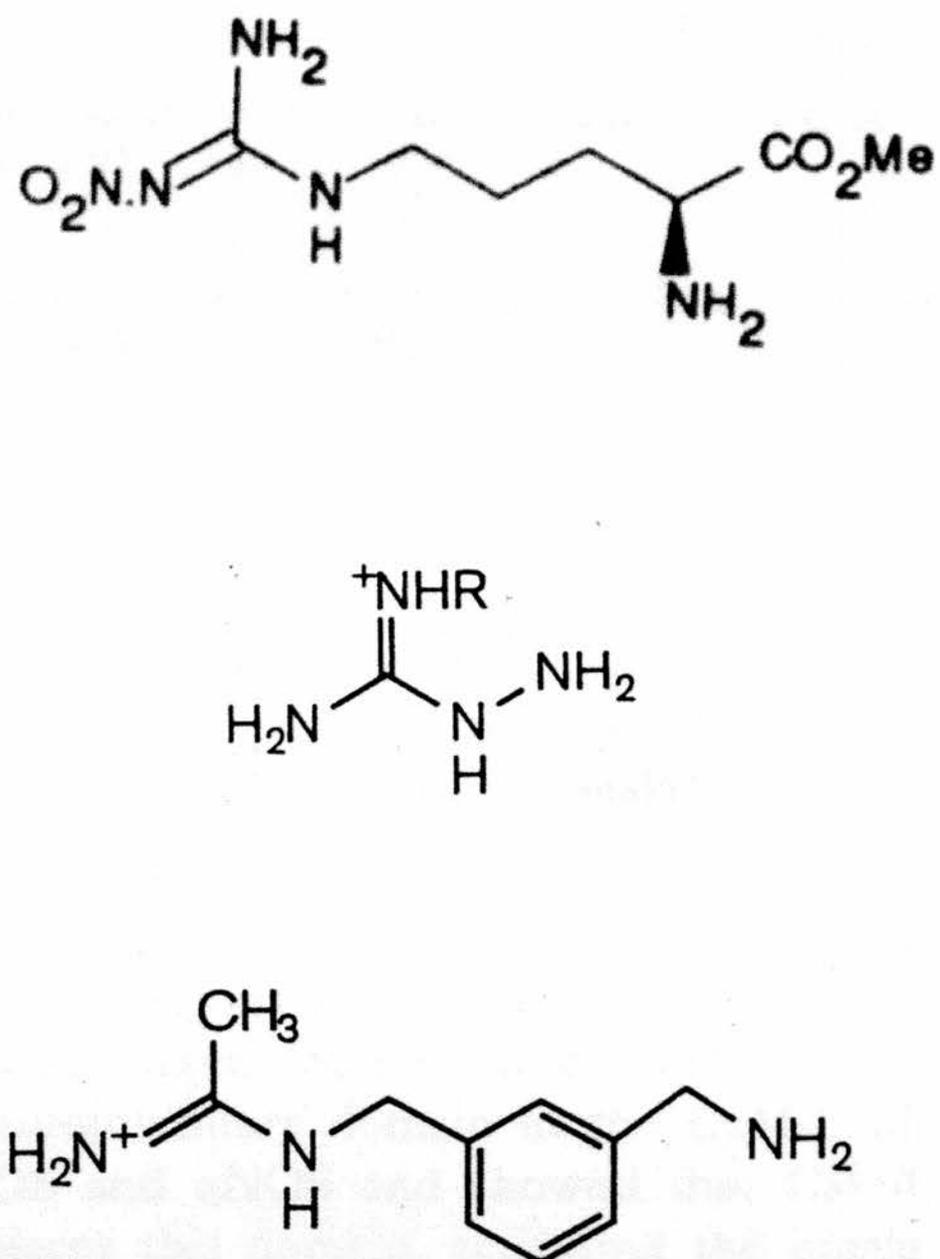


Fig. 1.3 Structures of NOS Inhibitors used in this Study
(top - L-NAME; middle - AG; bottom - 1400W)

1.4.5.2 NO Scavenging

Although inhibition of NOS is an effective therapeutic target, there are haemodynamic problems associated with the use of NOS inhibitors (see section 1.4.5.1.d). NO scavenging represents an alternative strategy, removing excess NO rather than preventing its synthesis. The selectivity of scavengers for NO is based, not on specificity for a particular enzyme but on compartmental localisation. The rate of NO scavenging depends on both the [NO] and [scavenger]. Scavenging will therefore be promoted where [NO] is elevated in contrast to NOS inhibitors which are independent of [NO] and inhibit NO synthesis equally in regions of high and low NO production. In principle therefore, scavengers have the potential to reduce excess levels of NO while having a minimal effect on essential basal NO production. Also, unlike NOS inhibitors, their actions are rapidly reversible if required and they do not have the potential to generate unforeseen metabolic consequences through interference with the L-arginine/urea-ornithine cycle.

A number of both organic and inorganic molecules have been utilised as NO scavengers and have demonstrated not only effective NO removal, but beneficial haemodynamic effects over NOS inhibitors (Bone *et al.*, 1998; Yoshida *et al.*, 1994). One such organic scavenger, 2-phenyl-4,4,5,5-tetramethylimidazole-1-*oxyl*-3-oxide, (PTIO), has proven effective at inhibiting NO-dependent vascular relaxation *ex vivo*, decreasing solid tumour vascular permeability (see section 1.11.2.2) and reversing the acute hypotension of septic shock (Maeda *et al.*, 1994 and 1994b; Yoshida *et al.*, 1994). In contrast, one study has concluded PTIO to be ineffective at reducing blood flow in tumour tissue (Tozer *et al.*, 1997). This has been attributed to an insufficient concentration of active drug.

Metal complexes, by exploiting the properties of NO as a ligand for metals, can be used as NO scavengers, reacting readily with NO to form metal nitrosyl complexes. Ruthenium forms more nitrosyl complexes than any other metal (Bottomley, 1978) and the Ru-NO bond is generally very stable and consequently the nitrosyl moiety is not easily displaced (Abrams, 1996; Davies *et al.*, 1997). Ruthenium (III) will react rapidly with NO to form six-coordinate Ru (II) mononitrosyl complexes. Chelation of the metal ion with a suitable ligand can be employed to confer water solubility, facilitating rapid *in vivo* clearance and low toxicity. The polyamioncarboxylates such

as ethylenediaminetetraacetic acid (EDTA) satisfy these requirements as ligands. Two ruthenium (III)-EDTA complexes were utilised in this study, potassium chloro[hydrogen(ethylenedinitrilo)tetraacetato]ruthenate (referred to as AMD6221) and its aqua derivative which forms from the reaction of this chloro complex with NO in aqueous solution, aqua[hydrogen(ethylenedinitrilo)tetraacetato] ruthenium (referred to as AMD6245) (see fig. 1.4). Each complex has one free coordination site available for NO binding.

These ruthenium complexes have been examined for their ability to scavenge NO in both *in vitro* and *in vivo* biological systems (Fricker *et al.*, 1997; Fricker, 1999) and have proved highly effective. Ruthenium complexes have also recently demonstrated favourable haemodynamic effects over NOS inhibitors in rats (Beirith *et al.*, 1999).

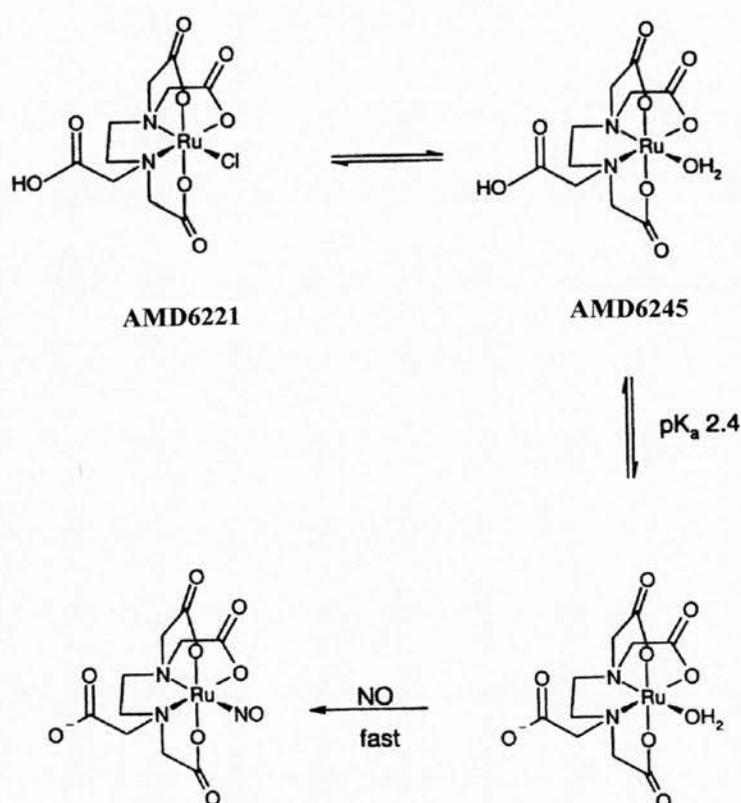


Fig. 1.4 Equilibrium diagram of the reaction between the ruthenium-EDTA complexes AMD6221 and AMD6245 and NO in aqueous solution

1.5 Physiological and Pathophysiological Roles of Nitric Oxide

1.5.1 The Cardiovascular system

1.5.1.1 Physiology

NO is an important regulator of basal vascular tone in the arterial circulation, causing relaxation of vascular smooth muscle (VSM) and consequently vasodilation (Ignarro *et al.*, 1987; Vallance *et al.*, 1989). Accordingly, an increase in vessel tone results from the *in vitro* and *in vivo* administration of specific inhibitors of NOS (Moncada *et al.*, 1991b). Thus NO affects blood perfusion and oxygen delivery by contributing to the regulation of vascular resistance.

Endothelial cells (ECs) are the source of a number of factors which bring about vasorelaxation or vasoconstriction either spontaneously (i.e. basal release) or in response to stimulation (FSS or endothelium-dependent agonists). NO is just one such endothelium-derived relaxing factor. The mechanical shear stress exerted on the luminal surface of endothelial cells, as a result of blood flow, is considered to be the principle factor responsible for the release of NO and consequently the maintenance of *basal* (unstimulated) vessel tone (Busse *et al.*, 1994; Koller *et al.*, 1993) (see 1.15).

In addition to basal release of NO, *active* vascular tone results from chemical stimulation of the endothelium by vasodilators which enhance NO release, including acetylcholine, ATP, ADP, substance P, bradykinin, serotonin, norepinephrine and platelet-activating factor (PAF) (Vane *et al.*, 1990). These compounds lead to a rapid increase in free $[Ca^{2+}]_i$. This, coupled with covalent modifications to NOS, cause its activation. Maintained production of NO following the (transient) Ca^{2+} peak involves changes in intracellular pH, whereby receptor-dependent agonists such as bradykinin activate the Na^+/H^+ exchanger and effect alkalinization which is associated with maintained NOS activity (Fleming *et al.*, 1994).

The functional consequences of NO production within the endothelium are due to the stimulation of guanylate cyclase (GC) and enhanced cGMP formation. The mechanism of vasorelaxation is thought to be twofold: (1) In smooth muscle cells, cGMP activates cGMP-dependent protein kinase producing phosphorylation and

activation of Ca^{2+} -ATPase (Lincoln & Cornwall, 1993; Schmidt *et al.*, 1993). The resulting reduction in available Ca^{2+} for myosin light-chain kinase activation (Kai *et al.*, 1987; Kobayashi *et al.*, 1985; Lincoln & Cornwall, 1993; Schmidt *et al.*, 1993) causes muscle relaxation. (2) cGMP activates calcium-dependent potassium channels (K^+_{Ca}), inducing hyperpolarisation in VSM cells (Archer *et al.*, 1994; Bolotina *et al.*, 1994). Membrane hyperpolarisation inactivates voltage-gated Ca^{2+} channels, again promoting relaxation (Luckoff & Busse, 1990).

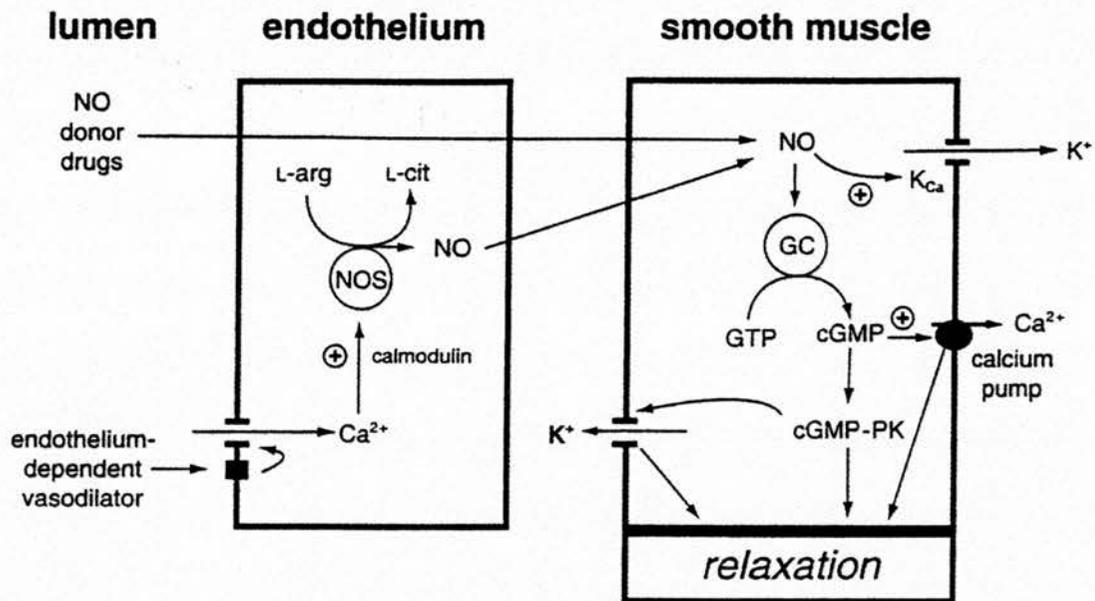


Fig. 1.5 *The Mechanism of Endothelial-Dependent Relaxation of Vascular Smooth Muscle* (Reproduced from Murad, 1996).

1.5.1.2 Vascular Disease

The endothelium plays an important role in maintaining the underlying VSM in a quiescent state. The endothelial denudation which occurs during balloon angioplasty leads to migration of underlying VSM into the vessel intima and intimal hyperplasia. Endogenous NO appears to modulate this hyperplasia with increased expression of iNOS by VSM following balloon injury (Hansson *et al.*, 1994). The resulting local release of NO suppresses VSM proliferation in an attempt to protect from thrombosis and restenosis.

Impaired synthesis or depletion of NO within the vasculature is involved in a number of circulatory pathologies. Hypertension is associated with impaired NO synthesis (Panza *et al.*, 1990) and eNOS knockout mice have been shown to be hypertensive (Huang *et al.*, 1995). Excessive NO production leads to systemic hypotension (Kilbourn *et al.*, 1991), forming the basis for bacterial septic shock (Thiemermann, 1994 and 1997). Hypercholesterolemia (Creager *et al.*, 1990) and atherosclerosis (Cohen, 1995) are associated with a reduction in vasorelaxation and an enhancement of vasoconstriction largely due to a reduction in NO activity. It is thought the oxidised low-density lipoprotein (LDL) component of cholesterol may chemically combine with and inactivate NO (Chin *et al.*, 1992).

Diabetes mellitus is associated with a reduced response of VSM cells to endothelial NO (Abiru *et al.*, 1990).

1.5.2 The Platelet

Within damaged blood vessels at sites of VEC loss, the aggregation of platelets and their adhesion to the vessel wall prevents excessive bleeding. NO inhibits both aggregation and adhesive processes (Radomski *et al.*, 1987a) through effects on cell surface adhesion molecules. It therefore acts to protect against thrombosis, counteracting the effects of the prothrombotic substances of the clotting cascade and restoring bloodflow. Although an independent L-arginine : NO pathway exists within platelets (Radomski *et al.*, 1990), the NO utilised is mostly derived from iNOS expression which is triggered within the lesion by the arterial damage (Yan *et al.*, 1996).

1.5.3 The Immune System

The L-arginine : NO pathway is an important element of the macrophage non-specific immune response against tumour cells, invading microorganisms, fungi and parasites (Nathan and Hibbs, 1991). Macrophages exposed to certain cytokines including interleukin-1 (IL-1), interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) or to bacterial endotoxin are induced to express iNOS and therefore generate cytotoxic amounts of NO (Hibbs *et al.*, 1987). NO is also generated by iNOS in human neutrophils (Moncada, 1992) although the biological significance of

this remains to be elucidated. Unregulated NO synthesis is involved in autoimmune disease (Moncada & Higgs, 1993), immune rejection of allografted organs (Langrehr *et al.*, 1992), graft-versus-host disease (Langrehr *et al.*, 1992) and sepsis (Thiemermann, 1994).

NO can mediate anti-inflammatory functions such as the inhibition of neutrophil adhesion to vascular endothelium (Kubes *et al.*, 1991). The NO is produced following iNOS expression within the endothelium (Binion *et al.*, 1998). Conversely however, elevated levels of NO can cause inflammation and are associated with a number of chronic inflammatory states, including arthritis and osteoarthritis (Farrell *et al.*, 1992), inflammatory nephritis (Weinberg *et al.*, 1994) and ulcerative colitis (Middleton *et al.*, 1993). The origin of NO is unclear but it may derive from blood vessels, neutrophils and macrophages (Moncada & Higgs, 1993).

1.5.4 The Nervous System

1.5.4.1 The Central Nervous System (CNS)

NOS is widely distributed within the brain (Knowles *et al.*, 1989) and the resultant NO has both neuroprotective and neurodestructive effects within the CNS (Lipton *et al.*, 1993). A neurotransmitter function for NO in 'nitrinergic' neurones was first demonstrated by its release following activation of glutamate N-methyl-D-aspartate (NMDA) receptors (Garthwaite *et al.*, 1988).

A messenger role for NO in the synaptic plasticity associated with memory establishment (long-term potentiation, LTP) has also been shown (Kendrick *et al.*, 1997; Wilson *et al.*, 1997). Neuronal NO may also be important in regulating the cerebral circulation, coupling increased local blood flow to neural activity (Gally *et al.*, 1990).

Elevated production of NO contributes to the CNS degeneration associated with a variety of pathologies, including Parkinson's disease (Snyder, 1996) and Alzheimer's disease (Vodovotz *et al.*, 1996). During cerebral ischaemia (stroke) NOS expression produces large amounts of NO which results in tissue damage (Iadecola, 1997).

1.5.4.2 The Peripheral Nervous System (PNS)

NO has a transmitter role in non-adrenergic non-cholinergic (NANC) neurones in the PNS (Gibson *et al.*, 1990). It plays a part in controlling autonomic functions including gastrointestinal peristalsis (Bredt *et al.*, 1990) and relaxation of the corpus cavernosum smooth muscle to allow penile erection (Ignarro *et al.*, 1990).

1.5.5 Cytotoxicity of NO

NO has a number of actions which are mediated independently of the guanylate cyclase/cGMP system. These are predominantly cytotoxic in their effect and may result from a number of processes. Many effects result from the ability of NO to interact with the Fe-S centre of a variety of enzymes, and thus modulate their activity (Feldman *et al.*, 1993; Nathan, 1992). NO produced by activated macrophages has cytostatic and cytotoxic effects on tumour cells as a result of its inhibition of complexes I and II of the electron transport chain (NADH:ubiquinone oxireductase and NADH:succinate oxireductase) and cis-aconitase of the tricarboxylic acid cycle, thereby resulting in the inhibition of cellular respiration (Stuehr and Nathan, 1989).

In an oxidative situation, NO may nitrosate important thiols, thus disrupting key metabolic processes. It can cause post-translational modifications in proteins (ADP-ribosylation) by its activation of ADP-ribosyltransferase (Brune *et al.*, 1994). This has been implicated in its neurotoxic actions (Zhang *et al.*, 1994). By S-nitrosylating the glycolytic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Dimmeler *et al.*, 1992; Kamoshima *et al.*, 1997; Molina y Vedia *et al.*, 1992; Zhang *et al.*, 1994) and causing an excessive activation of poly (ADP-ribose) polymerase (PARP) (Kamoshima *et al.*, 1997), NO inhibits energy-dependent DNA repair mechanisms, causing cell death.

High local levels of NO can impair DNA synthesis (Maragos *et al.*, 1993) and consequently cell division, by inhibiting ribonucleotide reductase (RNR) (Kwon *et al.*, 1991; Lepoivre *et al.*, 1991). NO is therefore thought to be involved in tumour-induced immunosuppression by preventing the proliferation of immune cells (Lejeune *et al.*, 1994). NO is also able to cause single strand breaks in DNA directly through the inhibition of DNA ligase activity (Graziewicz *et al.*, 1996).

NO is also potentially toxic through its induction of both apoptosis (following prolonged exposure to low doses) and necrosis (following acute exposure to high doses) (Nicotera *et al.*, 1995). This is thought to result from either altered mitochondrial function or by S-nitrosylation of the enzymes which mediate apoptosis (caspases and tissue transglutaminase, tTG) (Melino *et al.*, 1997).

PART B

THE SOLID TUMOUR

1.6 Introduction

There has been little improvement in survival prospects in relation to the four most common solid tumours in humans (lung, colorectal, breast and prostate) in recent years (Parker *et al.*, 1997). Ten million new cases of cancer will be diagnosed worldwide in the year 2000 (Boyle, 1997) and 40% of people in the UK will develop cancer during their lifetime (CRC press release, Jan 2000). As a consequence, cancer remains a major cause of death in the United States and in many industrialised countries (Beardsley, 1994). Since more than 85% of these malignancies are solid tumours (Jain, 1996) there exists an urgent need for more effective solid tumour therapy.

1.7 Tumour Architecture

1.7.1 Malignant cells and Stroma

Solid tumours are composed of three distinct but interdependent compartments: the malignant cancer cells, the abundant interstitial stroma, which they induce and in which they are dispersed, and the vasculature (Dvorak, 1997). The major components of tumour stroma are interstitial fluid containing plasma exudate and fibrinogen/fibrin; structural proteins such as collagens and individual cells of connective tissue (e.g. fibroblasts) and immune (e.g. macrophage) origin (Yeo & Dvorak, 1995).

In order for a systemic or orally-administered drug to be effective it must therefore pass through the vascular space, across the microvascular wall, through the interstitial space and across the cell membrane of the cancer cells. Despite an array of effective traditional chemotherapeutic and novel state-of-the-art 'molecular' anti-cancer agents, many of the short comings of current treatments result from their

inability to overcome these ‘barriers’ and reach the malignant cells in quantities sufficient to be effective (Jain, 1994). The vascular compartment represents a lifeline for the acquisition of nutrients, exchange of gases and waste disposal. Targeting the vasculature rather than tumour cells themselves therefore represents a potentially-valuable therapeutic means of anti-cancer therapy.

1.7.2 Tumour Vasculature

The vascularisation of a growing tumour involves two different vessel populations - pre-existing normal host vessels which are incorporated into the tumour mass, and microvessels which arise as neovasculature within the growing tumour, through the process of angiogenesis (Day, 1964; Folkman, 1995; Jain, 1988). Anti-tumour therapy may be based on the concept of occluding the blood supply within a tumour, anti-vascular therapy (Denekamp *et al.*, 1983), preventing the formation of new vasculature, the anti-angiogenic approach (Folkman, 1971), or a combination of the two.

Although tumour tissue can show a high vascular density, this does not usually indicate a high nutritive flow (Jain, 1987a). The development of new vasculature within tumours is hasty and consequently the resultant vessels are tortuous, elongated and dilated. Vessels fuse randomly into peculiar branching patterns, developing shunts (including arterio-venous shunts), loops, bi- and trifurcations and blind ending sprouts (Jain, 1987a; Less *et al.*, 1991). Vessel wall structure is frequently abnormal with the EC lining often missing or incomplete and characterised by simple immature cell contacts. Different cell types, including tumour cells, may even be involved in the formation of the vessel wall (Konerding *et al.*, 1989). Basement membrane (BM) and VSM/pericytes are also frequently absent (Bertossi *et al.*, 1997; Konerding *et al.*, 1989; Vaupel *et al.*, 1989).

The rapidly growing tumour cells have a high metabolic demand which cannot be met by the inadequate, chaotic blood supply. Metabolism of carbon produces H^+ ions. The high metabolic rate and lactic acid production, combined with insufficient drainage leads to an accumulation of H^+ ions and the pH drops to acidic levels (Kallinowski *et al.*, 1989). Insufficient perfusion also results in low oxygen tension

and acutely hypoxic areas develop, within which cells die giving rise to chronically-hypoxic, necrotic regions (Ahlstrom *et al.*, 1988). A characteristic pattern of spatial and temporal heterogeneity in tissue blood flow exists. Since the fraction of necrotic and semi-necrotic tissue increases with size, the average tumour perfusion rate decreases with the age of the tumour. Gazit *et al.* (1995) analysed the vascular patterns of normal tissue and compared the results with four different tumour lines in immunodeficient mice. Their findings clearly demonstrate the abnormal characteristics of the vasculature within tumour tissue.

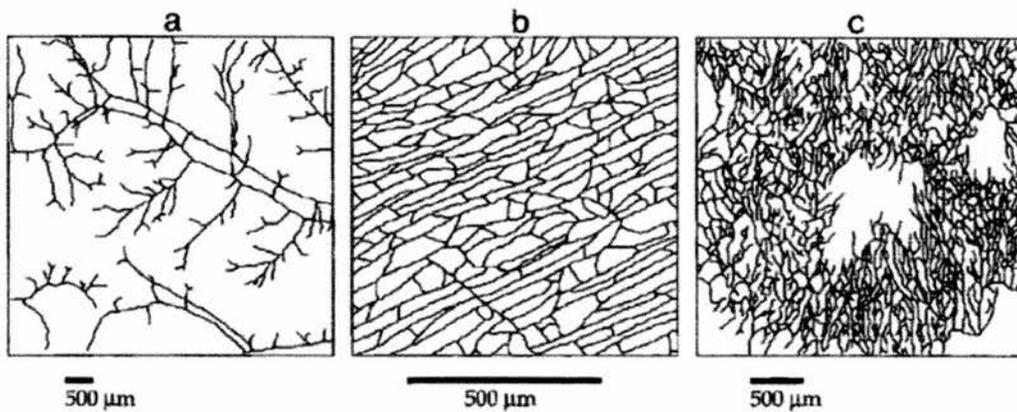


Fig. 1.6 *Skeletonised vascular patterns for normal subcutaneous arteries and veins (a) normal subcutaneous capillaries (b), and adenocarcinoma (c)*

Vessels in healthy tissue (a) seem to develop in an orderly fashion leading to optimal transport of nutrients and blood gases. Capillaries in normal tissue (b) form a regular space-filling array which leaves all points in the tissue within diffusion distance of oxygen and nutrients. In contrast tumour vessels (c) appear randomly distributed within the tumour tissue, resulting in an architecture which is dominated by great heterogeneity and irregular spacing, leaving some tissue too distant from the nearest vessel to avoid hypoxia.

The aberrant tumour vasculature responds to vasoactive agents in an unpredictable way (Andrade *et al.*, 1992b) and is frequently unresponsive to physiological and pharmacological stimuli (Tozer *et al.*, 1996; Vaupel *et al.*, 1989). The success of anti-vascular therapy is therefore limited. However, anti-angiogenic agents, by targeting the angiogenic process rather than the structurally-abnormal, heterogeneously-distributed vasculature, represent a more effective anti-tumour strategy.

1.8 NO and the Solid Tumour

The precise role(s) of NO in the biology of solid cancers remain unclear and somewhat controversial. The involvement of NO has been studied in a number of cancers, and both growth-inhibitory and growth-promoting roles have been proposed. These opposing effects appear to be related, in part, to the levels of NO produced (Chinje & Stratford, 1997; Hajri *et al.*, 1998; Jenkins *et al.*, 1995). NO in low concentrations can stimulate cellular proliferation and protect against peroxide-mediated toxicity (Wink *et al.*, 1995) and apoptosis (Dimmeler *et al.*, 1997), whilst at higher levels it is known to have cytostatic/cytotoxic effects, causing DNA damage, inducing apoptosis and causing immunosuppression of lymphocytes (Cui *et al.*, 1994; Ioannidis & de Groot, 1993; Lejeune *et al.*, 1994; Maragos *et al.*, 1993; Nicotera *et al.*, 1995) (for review see: Wink *et al.*, 1995). Clearly, NO will be expected to affect tumour growth in a dose-dependent manner. Additionally, the tumour type (including genetic status, see section 1.8.3.4), stage of growth, localisation of NOS activity and *in vivo* or *in vitro* setting will be expected to influence the effect of NO on tumour growth. The pro- and anti-tumour effects of NO are summarised in tables 1.2 and 1.3.

1.8.1 NOS Expression

Both inducible and constitutive NOS have been identified within solid tumours and their expression is frequently correlated with tumour grade (see table 1.2). Some investigators have detected only the inducible NOS isoform, including cancers of the liver (Ahn *et al.*, 1999), prostate (Klotz *et al.*, 1998) and experimental rat tumours (Doi *et al.*, 1996). Conversely, endothelial NOS has been detected in human cervical and CNS tumours (Cobbs *et al.*, 1995; Thomsen *et al.*, 1994) in which iNOS was below detectable levels. Most studies, however, have identified expression of both i- and eNOS isoforms, in human cancers of the colon (Radomski *et al.*, 1991), bladder (Jansson *et al.*, 1998), Kaposi's sarcoma (Weninger *et al.*, 1998), breast (Thomsen *et al.*, 1995) and head and neck cancer (Gallo *et al.*, 1998; Prazma *et al.*, 1995). Experimental rodent tumours have also demonstrated an upregulation of both isoforms (Fukumura *et al.*, 1997; Thomsen *et al.*, 1998; Whittle *et al.*, 1996).

1.8.2 NOS Localisation

NOS isoforms have been detected across a variety of cell types within tumours.

1.8.2.1 Inducible NOS

Expression of iNOS confined exclusively to the tumour endothelium (Ahn *et al.*, 1999; Ambs *et al.*, 1998b; Buttery *et al.*, 1993), leukocytes (Weninger *et al.*, 1998) and tumour/stromal cells (Gallo *et al.*, 1998; Klotz *et al.*, 1998; Onier *et al.*, 1999; Shimizu *et al.*, 1998; Tschugguel *et al.*, 1999) has been reported. An absence of iNOS expression in tumour macrophages (Klotz *et al.*, 1998) and in both macrophages and ECs (Kundu *et al.*, 1998; Onier *et al.*, 1999; Xie *et al.*, 1995a) has been noted by some researchers.

1.8.2.2 Endothelial NOS

Expression of eNOS confined exclusively to the tumour endothelium (Jansson *et al.*, 1998b; Klotz *et al.*, 1998; Tschugguel *et al.*, 1999; Weninger *et al.*, 1998;) and tumour cells (Thomsen *et al.*, 1994) has been reported. Concurrent EC and tumour localisation has been observed by Cobbs *et al.* (1995). An absence of eNOS has also been documented within some tumours (Buttery *et al.*, 1993; Onier *et al.*, 1999).

Clearly both the NOS isoform expressed and its specific localisation is tumour-dependent and the vascular response to NOS inhibition is therefore likely to be heterogeneous across tumours. Most of the cellular components of the tumour mass (tumour cells, ECs, immune cell infiltrate) have demonstrated the capacity to generate NO.

NOS expression also appears to be tumour stage-specific and the time-course has been examined by Buttery *et al.* (1993) in two experimental murine tumours. They noted a lack of expression during the earlier stages of tumour development (first 7 days of growth) until 12-14 days post-implantation when immunostaining for iNOS became evident.

1.8.3 Roles of NO in the Solid Tumour

1.8.3.1 Macrophages

Macrophages, through their production of NO, demonstrate both positive and negative influences on tumour growth (for a review see: Polverini, 1997). Their opposing actions may relate, in part, to the pathway through which L-arginine is metabolised (Mills *et al.*, 1992) (see table 1.5).

1.8.3.1.a Cytostasis/Cytotoxicity

More than a century ago, Fehleisen (1882) showed that resistance to cancer could be enhanced in a non-specific way by bacterial products. This phenomenon has since been linked to macrophage activation and the induction of NOS (Moncada, 1992). Macrophages, when activated by cytokines from sensitised lymphocytes, demonstrate cytostatic or cytotoxic effects on tumour cells (Stuehr & Nathan, 1989; Weinberg *et al.*, 1978) via a release of NO (Hibbs, 1991; Kwon *et al.*, 1990). The tumouricidal mechanisms include an inhibitory effect on mitochondrial respiration and DNA synthesis and peroxynitrite-induced protein and DNA damage (as discussed in section 1.5.5) (Lepoivre *et al.*, 1990).

1.8.3.1.b Angiogenesis

In addition to their cytotoxic immune function, macrophages are also involved in the stimulation of tumour angiogenesis (Leibovich *et al.*, 1994; Polverini & Leibovich, 1984). Their angiogenic potential is linked to both the L-arginine:NO pathway (Leibovich *et al.*, 1994) and their production of vascular endothelial growth factor (VEGF) (Xiong *et al.*, 1998). They produce in excess of 20 pro-angiogenic molecules (Sunderkotter *et al.*, 1991) and are able to liberate additional angiogenic substances from the ECM.

1.8.3.2 Regulation of Tumour Bloodflow

Unlike hastily-developed neovasculature, tumour-supply vessels recruited from the host possess normal wall structure (Hirst *et al.*, 1991) and are therefore reactive targets for anti-vascular tumour therapy. Relative to 'normal' vasculature, these

supply vessels exist in a permanent state of exaggerated, almost maximal dilation (for a review see Peterson, 1991) (see also section 1.9.4.1.a).

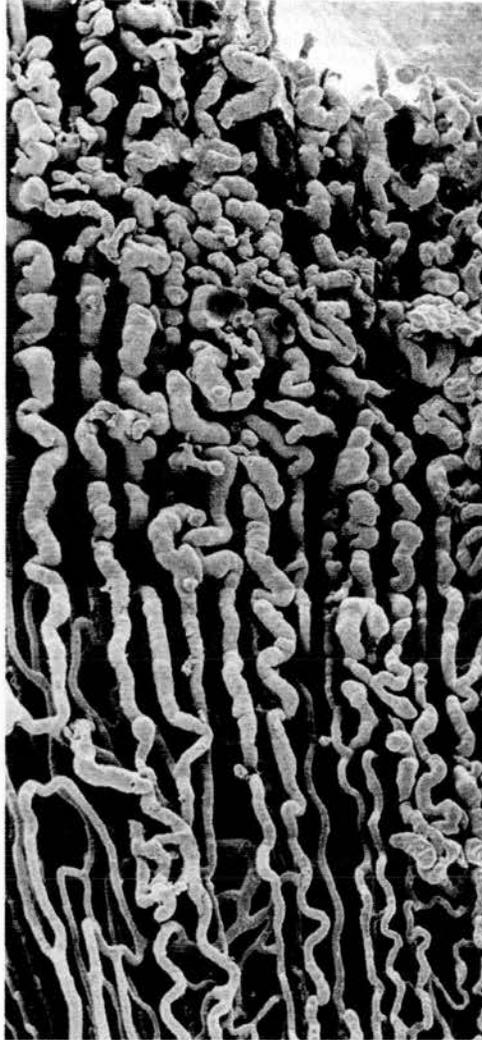


Plate 1.1 *Dilation of host vessels at the tumour base*

(Reproduced from Grunt *et al.*, 1986 with the kind permission of A. Lametschwandtner, University of Salzburg).

This vasodilation can be observed within each vessel which approaches the tumour (Grunt *et al.*, 1986; Gullino & Grantham, 1961) and is a result of the increased expression of NOS (Kennovin *et al.*, 1994a) and subsequently enhanced production of NO.

Therapy exploiting this distinctive property of tumour-supply vessels, selectively reducing blood flow within them, was first demonstrated by Andrade *et al.* (1992) using L-NAME and L-NMMA in mice. Since then, a number of investigators have shown that L-NAME is able to reduce tumour vessel diameter and bloodflow (Fukumura *et al.*, 1997; Gallo *et al.*, 1998; Meyer *et al.*, 1995; Swaroop *et al.*, 1998; Tozer *et al.*, 1997; Whittle *et al.*, 1996). The vasoconstriction resulting from chronic, oral administration of L-NAME has been shown to produce marked inhibition of *in vivo* solid tumour growth in the rat (Kennovin *et al.*, 1994a).

There is evidence that enhanced NO production within tumour-supply and intra-tumour vessels results from the expression of *inducible* NOS (Buttery *et al.*, 1993; Doi *et al.*, 1996; Rosbe *et al.*, 1995). Some studies have therefore utilised iNOS selective inhibitors in order to retain the vasoconstrictive effect but to avoid systemic hypertension resulting from concomitant eNOS inhibition. AG has been observed to cause vasoconstriction of isolated *ex vivo* tumour-supply vessels. Moreover, the chronic, oral administration of AG has produced tumour growth-retarding effects in subcutaneous rat tumours (Bisland, 1996), although the effect was less marked than that seen with L-NAME.

Thomsen *et al.* (1997) examined the effects of 1400W on the growth of two murine tumours and one human tumour xenograft in mice. The growth rate of a murine mammary adenocarcinoma was reduced by a continuous 6 day infusion of 10-12mg⁻¹Kg⁻¹hr 1400W. The growth rate of a human adenocarcinoma DLD-1 xenograft, engineered to express iNOS constitutively (see table 1.2), was reduced by a continuous 13 day infusion of 6mg⁻¹kg⁻¹hr 1400W. The growth of a murine colon adenocarcinoma was however, unaffected. This observed lack of effect was postulated to be due to high intratumour arginine concentrations, producing competition between L- arginine and 1400W for the iNOS binding site.

Adequate tumour blood flow is necessary to supply oxygen and nutrients and to maintain a suitable pH (Peterson, 1991). Clearly the reduction in tumour perfusion resulting from vessel constriction with NOS inhibitors plays an important role in the retardation of tumour growth rate.

1.8.3.3 Tumour Angiogenesis

NO plays a role in the regulation of tumour angiogenesis. This is given consideration in section 1.12.

1.8.3.4 NO and Cancer Genetics

The tumour suppressor protein *p53* plays a role in inhibiting cell proliferation, and over half of all human cancers are associated with a loss of its function (Levine *et al.*, 1991). A correlation exists between NOS activity and *p53*, wherein NO induces mutation in the *p53* gene by causing DNA damage (Fujimoto *et al.*, 1998). An accumulation of mutant *p53* has been observed in cells exposed to NO (Forrester *et al.*, 1996). Conversely the expression of mutant *p53* down-regulates the expression of NOS (Forrester *et al.*, 1996). This implies the existence of a negative feedback loop in which NO-induced DNA damage results in mutant *p53* accumulation and *p53*-mediated repression of iNOS gene expression (Ambs *et al.*, 1997).

Wild-type *p53* inhibits tumour angiogenesis by enhancing the effect of an angiogenesis inhibitor, thrombospondin -1 (Dameron *et al.*, 1994). NO inhibits the growth of tumour cells containing wild-type *p53*, but accelerates that of mutant *p53*-containing cells. It achieves this through the induction of VEGF (Ambs *et al.*, 1998; Kieser *et al.*, 1994). (see section 1.12.2). Clearly the effect of NO on tumour growth may, in addition to other factors, depend on the *p53* status. (For a review see: Chiarugi *et al.*, 1998).

Table 1.2 Positive Effects of NO on Solid Tumour Growth

PRO-TUMOUR EFFECTS	REFERENCE
The anti-tumour drug curcumin inhibits iNOS induction in activated macrophages.	Brouet <i>et al.</i> , 1995.
The induction, by tumour cells, of a low level of NO production by spleen macrophages inhibits T-cell proliferation and promotes tumour growth.	Alleva <i>et al.</i> , 1994; Lejeune <i>et al.</i> , 1994.
Transfection of iNOS into a human adenocarcinoma cell line (DLD-1) leads to continuous NO generation and increases tumour growth and metastasis <i>in vivo</i> .	Jenkins <i>et al.</i> , 1995.
Continuous 1400W infusion reduced the growth of two murine tumours (EMT-6; Colon 38) and a human tumour xenograph (DLD-1) in mice.	Thomsen <i>et al.</i> , 1997.
Chronic oral administration of L-NAME retards solid tumour growth in rats.	Kenovin <i>et al.</i> , 1994a.
Chronic oral administration of AG retards solid tumour growth in rats.	Bisland, 1996.
Chemotherapeutic drugs (4'-epi-doxorubicin and mitomycin C) strongly inhibit iNOS induction.	Inagaki <i>et al.</i> , 1999; Sakai <i>et al.</i> , 1996.
Human oral carcinoma cells transfected with eNOS and inoculated into mice grow larger tumours than control-transfected cells.	Liu <i>et al.</i> , 1998b.
NO production in breast cancer cells (EMT-6) <i>in vitro</i> inhibits cell growth. L-NAME reduces <i>in vivo</i> tumour size in mice.	Edwards <i>et al.</i> , 1996.
iNOS expression in colon cancer contributes to tumour development/progression.	Ambs <i>et al.</i> , 1998b.
Excessive production of NO is implicated in the rapid growth of a rat solid tumour.	Doi <i>et al.</i> , 1996
NOS isoforms are expressed in a number of human cancers including breast, colon, liver, prostate, bladder, cervical, the CNS, head and neck tumours, Kaposi's sarcoma and skin carcinoma. Activity is often positively correlated with tumour grade, with the extent of NOS expression increasing with tumour development.	Ahn <i>et al.</i> , 1999; Cobbs <i>et al.</i> , 1995; Gallo <i>et al.</i> , 1998; Jansson <i>et al.</i> , 1998b; Klotz <i>et al.</i> , 1998; Kojima <i>et al.</i> , 1999; Prazma <i>et al.</i> , 1995; Thomsen <i>et al.</i> , 1994, 1995, 1998; Villiotou & Deliconstantinos, 1995; Weninger <i>et al.</i> , 1998;

Table 1.3 Negative Effects of NO on Solid Tumour Growth

ANTI-TUMOUR EFFECTS	REFERENCE
The cytotoxic anti-tumour drug adriamycin has been shown to stimulate NO production. Aminoguanidine blocks adriamycin's inhibitory effect on tumours.	Lind <i>et al.</i> , 1997.
Relaxin, a peptide hormone, inhibits growth of breast adenocarcinoma cells. It increases iNOS expression, potentiating NO production.	Bani <i>et al.</i> , 1995.
Anti-tumour Bacillus Calmette-Guerin (BCG) induces NOS, increasing NO production. L-NAME abolishes the tumouricidal effect.	Farias-Eisner <i>et al.</i> , 1994; Jansson <i>et al.</i> , 1998b.
Lipid A treatment induces colon tumour regression. Through induction of cytokines it increases iNOS expression and NO production within tumours.	Onier <i>et al.</i> , 1999.
Tumour rejection in tumour-preimmunised mice is associated with a local upregulation of NOS.	Mills <i>et al.</i> , 1992.
L-NMMA increases the growth of squamous cell carcinoma in mice.	Yim <i>et al.</i> , 1993.
The expression of iNOS is associated with slow growth and tumour regression in melanoma cells transfected with the iNOS gene and also in non-transfected bystander cells.	Xie <i>et al.</i> , 1995b; 1996; 1997b.
The anti-tumour effects of IL-10 and IL-12 in mice is associated with increased production of NO in the tumour.	Kundu <i>et al.</i> , 1998; Tsung <i>et al.</i> , 1997.
Anti-tumour agent taxol (and its analogues) induce iNOS in murine macrophages <i>in vitro</i> .	Kirikae <i>et al.</i> , 1996.
The growth of tumour cells co-cultured with activated macrophages (which produce a high [NO]) is inhibited, and cytotoxicity greatly increased.	Stuehr & Nathan, 1989.
NO-generating compounds inhibit tumour cell proliferation.	Chenais <i>et al.</i> , 1993.
The growth rate of eNOS-transfected cells <i>in vitro</i> is lower than that of controls.	Liu <i>et al.</i> , 1998.
<i>In vivo</i> , both an NO donor and endogenous induction of NO by endotoxin significantly reduced tumour growth in rats.	Hajri <i>et al.</i> , 1998.
IFN β -transfected tumour cells stimulate significant iNOS expression in macrophages. The NO produced correlates with antitumour activity.	Xie <i>et al.</i> , 1997a.
INOS-transfected adenocarcinoma cells (DLD-1) grew more slowly <i>in vitro</i> than control cells.	Jenkins <i>et al.</i> , 1995.
The <i>in vitro</i> growth of some tumour cell lines is greatly inhibited by SNAP.	Takeshita <i>et al.</i> , 1997.
The anti-tumour effects of IL-1 α and IFN γ result from the production of NO.	Chang <i>et al.</i> , 1996.
eNOS activity and subsequent NO levels are lower in malignant renal cell carcinoma than controls. NO levels are suppressed further with advancing tumour growth.	Jansson <i>et al.</i> , 1998.

1.9 Angiogenesis

1.9.1 History

Hertig (1935) first coined the term 'angiogenesis' to describe the physiological development of blood vessels in the placenta. The importance of neovascularisation in tumour development was first demonstrated by Algire *et al.* (1945). They observed subcutaneous tumour growth in transparent chambers in mice and showed that before vascularisation occurred, growth was slow and linear, but became rapid, nearly exponential once angiogenesis had been initiated. This was followed by a series of observations made during the 1960s and 1970s which further implicated vascularisation as a vital stage in the growth of a tumour. Folkman *et al.* (1966) observed that tumours grown in the absence of blood vessel proliferation were limited in size ($\sim 1\text{-}2\text{mm}^3$) but expanded rapidly after vascularisation. Similarly, Gimbrone *et al.* (1972) observed that avascular tumours suspended in the aqueous fluid of the eye remained small ($< 1\text{mm}^3$) but once implanted contiguous to the proliferating iris vessels, were able to induce neovascularisation and to enlarge up to 16,000 times their original volume within two weeks.

Since these early beginnings, a wealth of evidence has been uncovered which unequivocally demonstrates that tumour growth is wholly dependent on the induction of angiogenesis (for reviews see: Bicknell, 1997; Folkman, 1990) and as a result, an entirely new approach to the therapy of solid tumours has been spawned.

1.9.2 The 'Switch' to an Angiogenic Phenotype

During the pre-vascular stage of tumour growth, simple diffusion is sufficient to allow the exchange of nutrients, oxygen and waste products to the tumour cells. Avascular tumours can persist *in vivo* for many years (*in situ* cancer). Transition from a pre-vascular to a vascular stage occurs when diffusion of substances across the outer surface of the tumour is no longer adequate. The 'angiogenic switch' is thrown, and exponential growth takes place.

With the exception of physiological angiogenesis and wound healing, normal ECs *in vivo* are essentially quiescent, with an estimated 'turnover' time in the range of 1000 days or more in adult mammals. In contrast, continuous labelling studies have shown

that tumour ECs can grow with a turnover rate of only 4-5 days (Denekamp & Hobson, 1982). Tumour angiogenesis is in fact under multiple positive and negative regulatory controls. Specific pro-angiogenic molecules initiate the angiogenic process and specific inhibitory molecules halt it (see tables 1.4 and 1.5). The 'switch' to endothelial activation within an angiogenic tumour is determined by a *balance* between positive and negative regulators of microvessel growth (Hanahan & Folkman, 1996; Liotta *et al.*, 1991) and the cross-talk between the tumour and vascular compartments is extensive, involving numerous growth factors and inhibitors interacting in an extremely complex and diverse network (Bussolino *et al.*, 1996; Rak *et al.*, 1996). In activated (angiogenic) endothelium, positive regulators predominate, whereas endothelial quiescence is due to the dominance of negative regulators.

Table 1.4 *Pro-Angiogenic factors*

ANGIOGENIC FACTOR	REFERENCE
<i>Polypeptides</i>	
a FGF	Abraham <i>et al.</i> , 1986.
b FGF	Esch <i>et al.</i> , 1988.
Angiogenin	Fett <i>et al.</i> , 1985.
Epidermal growth factor (EGF)	Yates <i>et al.</i> , 1991.
TGF α	Folkman & Klagsbrun, 1987.
TGF β	Roberts <i>et al.</i> , 1986.
Platelet derived growth factor (PDGF)	Risau <i>et al.</i> , 1992.
VEGF	Senger <i>et al.</i> , 1983.
Platelet activating factor (PAF)	Andrade <i>et al.</i> , 1992c.
TNF α	Leibovich <i>et al.</i> , 1987.
IL-1 α	Bicknell & Harris, 1997.
IL-8	Koch <i>et al.</i> , 1992.
IL-3	Dentelli <i>et al.</i> , 1999.
<i>Peptides</i>	
Ceruloplasmin	Folkman & Klagsbrun, 1987.
Angiotensin II	Fernandez <i>et al.</i> , 1985.
Substance P	Fan <i>et al.</i> , 1993.
Plasminogen activator (PA)	Berman <i>et al.</i> , 1982.
<i>Lipids</i>	
Prostaglandins E1 and E2	Benezra, 1978.
Ercamide	Wakamatsu <i>et al.</i> , 1990.
<i>Others</i>	
Heparin	Folkman & Klagsbrun, 1987.
Hyaluronic acid fragments	West & Kumar, 1989; 1989b.
Nicotinamide	Morris <i>et al.</i> , 1991.

Table 1.5 *Angiogenesis Inhibitors*

ANGIOGENESIS INHIBITOR	REFERENCE
<i>Tumour Suppressor Genes</i>	
P53	Dameron <i>et al.</i> , 1994,
Retinoblastoma (RB)	Antelman <i>et al.</i> , 1995.
Von Hippel-Lindau (VHL)	Chen <i>et al.</i> , 1995.
<i>Enzyme Inhibitors</i>	
Placental Ribonuclease Inhibitor	Shapiro & Valee, 1987.
Prostaglandin Synthesis Inhibitor	Peterson, 1986.
Laminin peptides	Grant <i>et al.</i> , 1989.
<i>Modulators of Collagen Synthesis</i>	
Proline analogues	Ingber & Folkman, 1988
α,α -dipyridyl	Ingber & Folkman, 1988.
β -aminoproponitrile	Ingber & Folkman, 1988.
GPA 1734	Maragoudakis <i>et al.</i> , 1988.
<i>Proteins</i>	
Angiostatin	O'Reilly <i>et al.</i> , 1994.
Endostatin	O'Reilly <i>et al.</i> , 1997.
16KDa aminoterminal prolactin fragment	Clapp <i>et al.</i> , 1993.
Thrombospondin	Rastinejad <i>et al.</i> , 1989.
<i>Soluble Mediators</i>	
Interferon α	Sidky & Borden, 1987.
Heparinase	Sasisekharan <i>et al.</i> , 1994.
<i>Others</i>	
High mass Hyaluron	West & Kumar, 1989;1989b
Medroxyprogesterone	Gross <i>et al.</i> , 1981.
Angiostatic steroids	Folkman <i>et al.</i> , 1988.
Protamine	Taylor & Folkman, 1982.
Retinoids	Arensman & Stolar, 1979.
Platelet Factor IV	Maione <i>et al.</i> , 1990.
Herbimycin A	Yamashita <i>et al.</i> , 1989.
D-penicillamine	Matsubara <i>et al.</i> , 1989.
Methotrexate	Hirata <i>et al.</i> , 1989.

The most extensively studied pro-angiogenic factors produced by tumour cells are acidic and basic fibroblast growth factor (aFGF, bFGF) and VEGF (Thommen *et al.*, 1997). These affect EC behaviour directly, stimulating migration, proliferation and/or tubule formation (see section 1.9.4.1). They also function indirectly by recruiting macrophages and mast cells (Polverini & Leibovich, 1984). Growth factors (e.g. bFGF) are sequestered in bio-active forms in the ECM (Vlodavsky *et al.*, 1987) and BM (Folkman *et al.*, 1988) and can be mobilised by collagenases or heparinases

secreted by tumour cells, macrophages or heparin-rich mast cells (Folkman *et al.*, 1988). Peripheral blood and tumour-infiltrating lymphocytes also express VEGF (Freeman *et al.*, 1995), indicating that they may be cellular effectors of angiogenesis, capable of exporting VEGF into the extracellular space.

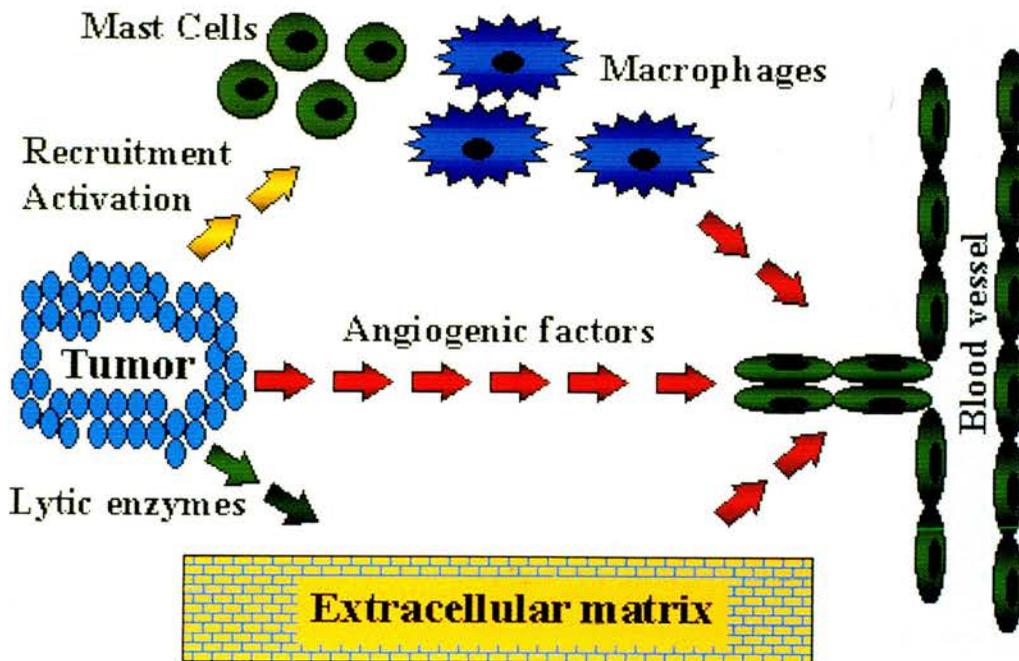


Fig. 1.7 *Mechanisms Involved in the Switch to an Angiogenic Phenotype in a Tumour.* (Reproduced from: <http://www.med.unibs.it/~airc/>)

Tumours recruit macrophages which provide proangiogenic factors (such as NO) and mast cells (source of heparin). Pro-angiogenic molecules are also exported out of tumour cells themselves. The tumour also provides proteolytic enzymes which serve to mobilise further growth factors from the ECM. Angiogenic molecules stimulate ECs of the parent vessel to produce collagenases and plasminogen activator to degrade the BM of the parent vessel. VEGF increases the permeability of the capillary bed, leading to leakage of fibrin into the extracellular space. ECs lining the host vessels are stimulated to divide and migrate to form the bud of a new vessel.

1.9.3 Tumour-Conditioned Cell Culture Medium

In an attempt to recreate the angiogenic environment of a tumour *in vitro*, the use of cell culture medium in which tumour cells have been grown, 'tumour-conditioned medium' (TCM) has been utilised by a number of investigators. A rapid growth of ECs (Folkman *et al.*, 1979; Folkman & Haudenschild, 1980) and the formation, by single ECs, of rings (Folkman & Haudenschild, 1980) have been observed in TCM.

Tumour-derived factors within conditioned medium also stimulate the rapid migration of capillary ECs *in vitro* (Zetter, 1980). The first isolation of an angiogenic factor from TCM was by Klagsbrun *et al.* in 1976. This has subsequently been confirmed as VEGF (Nakagawa *et al.*, 1992).

1.9.4 Anti-Angiogenic Therapy

The proposal that negative regulators of blood vessel growth must exist endogenously was based on i) the cessation of physiological angiogenesis (e.g. in the endometrium during the menstrual cycle and following wound healing) and ii) the observation that dormant metastases often appear following the surgical removal of a primary tumour in cancer patients. Although numerous angiogenesis inhibitors have subsequently been detected, most attention has focused on the two most powerful, angiostatin and endostatin.

Angiostatin is a 38 KDa internal fragment within the sequence of plasminogen and has been shown to inhibit EC proliferation *in vitro* and angiogenesis and metastatic growth *in vivo* (O'Reilly *et al.*, 1996). Administration of angiostatin to mice has produced not only a potent growth inhibitory effect of both murine (O'Reilly *et al.*, 1994; Wu *et al.*, 1997) and human (O'Reilly *et al.*, 1996) carcinomas, but also a regression of tumours to microscopic foci for as long as administration is continued.

Three years after the discovery of angiostatin, endostatin, a 20 KDa fragment of collagen XVIII was discovered (O'Reilly *et al.*, 1997). Its administration also induced regression of tumours to microscopic lesions for as long as it was administered (Boehm *et al.*, 1997). It was observed, however, that following a number of drug cycles, tumours remained responsive to the drug. Tumours allowed to regrow between treatments could be shrunk again and again. Endostatin treatment eventually induced a state of dormancy in which tumours failed to re-grow even in the absence of therapy.

There are four stages in the angiogenic process which may be altered by anti-angiogenic substances. Firstly the inhibition of the release and/or neutralisation of angiogenic factors, secondly, inhibition of EC proliferation or thirdly, migration and

finally inhibition of the synthesis of vessel BM. Although the underlying mechanism of action of angiostatin and endostatin treatment remains unclear, both have been shown to induce apoptosis of ECs (Dhanabal *et al.*, 1999; Lucas *et al.*, 1998).

Clinically, anti-angiogenic strategies are showing great promise (Barinagi, 1997; Folkman, 1996; Pepper, 1997). These agents have two major advantages over existing anti-cancer drugs. Drug resistance plagues 30% of patients undergoing chemotherapy (Young, 1989) and is instrumental in its high failure rate. By targeting 'normal' ECs, rather than genetically-unstable, rapidly mutating tumour cells, angiogenesis inhibitors do not appear to induce drug resistance (Boehm *et al.*, 1997). Secondly, since anti-angiogenic drugs are derived from endogenous proteins and target only proliferating ECs, toxicity appears to be low (Barinagi, 1997; Folkman, 1995 and 1995b) and the distressing symptoms which characterise standard chemotherapy are avoided (Folkman, 1995).

About 20 anti-angiogenic agents are currently being tested in human trials (see: <http://pharmacology.miningco.com/library/weekly/bl980512.htm>). Angiostatin™ is currently in pre-clinical investigation and Endostatin™ entered phase I clinical trials (safety) in September 1999 (<http://www.EntreMed.com>).

For reviews of anti-angiogenic therapy see: Folkman (1996); Gastl *et al.* (1997); Harris, (1997); Ono *et al.* (1996).

1.9.5 Mechanisms of Angiogenesis

Angiogenesis is a complex process involving the intricate interplay between vascular and non-vascular cells, soluble pro- and anti-angiogenic factors and ECM components.

1.9.5.1 Capillary Sprouting

Capillary sprouting is the end result of a sequence of five overlapping events:

- a) *Vasodilation* b) *degradation of the EC BM and surrounding ECM* c) *EC migration* d) *EC proliferation* e) *capillary tube formation and maturation*

(for review see: Paku & Paweletz, 1991; Risau, 1997). These are considered in turn below.

1.9.5.1.a Vasodilation

As discussed in section 1.8.3.2, vasodilation has been observed in the vessels supplying/surrounding a solid tumour (Grunt *et al.*, 1986; Gullino & Grantham, 1961; Kennovin *et al.*, 1993, 1994b; Paku & Paweletz, 1991) and it is due, at least in part, to the expression of NOS (Bisland, 1996; Kennovin *et al.*, 1994a). Intratumoural vessels have been observed in a similarly dilated state (Eddy & Casarett, 1973; Liotta *et al.*, 1974; Vogel, 1965; Yamaura & Sato, 1974) and this dilation of 'parent' vessels is an important and much overlooked early event in angiogenesis. It instigates a cascade of events which are instrumental in the process of neovascularisation.

Resultant mechanical (i.e. tension-dependent) interactions between ECs and the ECM serve to regulate capillary development (Ingber & Folkman, 1987). Vasodilation may stretch the EC lining of the vessel, rendering ECs more responsive to angiogenic growth factors (Acevedo *et al.*, 1993; Ingber, 1990). Disruption of the BM through mechanical distortion may itself enable sprouting to occur. Experiments in tissue culture have demonstrated that mechanical forces, such as fluid shear stress (FSS) (Ando *et al.*, 1987) cyclic strain (Iba *et al.*, 1991) or stretch (Ingber & Folkman, 1987), can induce EC proliferation by modifying either the apical (glycocalyx) or basal (basement membrane, ECM) part of the EC or by effecting prostaglandin/NO release. Accordingly, early experiments demonstrated the greatest capillary growth at the bends of pre-existing vessels (Thoma, 1911).

The increase in bloodflow resulting from the widened vessel lumen of the dilated parent vessel may also play a part a critical role in new vessel sprouting (Hudlicka, 1998). The role of bloodflow in capillary growth was first demonstrated by Clark in 1918 using tadpole tails. Capillaries with a high velocity of flow sprouted to form new vessels, while those with sluggish flow gradually disappeared. Accordingly, the

long-term administration of vasodilators has been shown to induce capillary growth (Dawson & Hudlicka, 1990; Ziada *et al.*, 1984).

Growth factors including VEGF (Hariawala *et al.*, 1996; Horowitz *et al.*, 1997; Ku *et al.*, 1993; Malavaud *et al.*, 1997; Yang *et al.*, 1996) and bFGF (Brown *et al.*, 1996; Cuevas *et al.*, 1991; Hudlicka *et al.*, 1989; Wu *et al.*, 1996b) are themselves vasodilators and may therefore, in addition to their direct mitogenic effects, contribute indirectly to capillary growth by increasing blood flow.

It appears therefore that the initiation of vessel sprouting from the dilated parent vessel is induced both by mechanical factors, relating to the vessel distension itself, and the subsequent increase in bloodflow.

1.9.5.1.b Degradation of EC Basement Membrane and Surrounding ECM

Tumour and ECs secrete two major families of proteolytic enzymes which are responsible for extracellular proteolysis in angiogenesis, the serine proteases, in particular the plasminogen activator (PA)/plasmin system (Vassalli *et al.*, 1991) and the matrix metalloproteinases (MMPs) (e.g. collagenase) (Basbaum & Werb, 1996; Fischer *et al.*, 1994). Plasmin is generated from inactive plasminogen by the actions of urokinase and tissue-type (u-PA, t-PA) plasminogen activator. It is able to degrade most matrix components either directly or through the activation of other latent enzymes (Pepper & Montesano, 1990). VEGF is responsible for regulating the EC-expression of proteolytic enzymes (see section 1.11.2.3) and the increased FSS resulting from vasodilation stimulates further production (Brown *et al.*, 1995).

Co-production of protease inhibitors helps to restrict proteolytic activity to the immediate pericellular environment (Pepper *et al.*, 1996b). The activity of u-PA and t-PA can be inhibited by specific PA inhibitors, namely plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2). Tumour cells simultaneously secrete both proteases and their inhibitors and the balance between them precisely regulates the level of extracellular proteolysis and thus promotes or suppresses angiogenesis (Pepper & Montesano, 1990; Pepper *et al.*, 1991).

The composition of the tumour ECM is an important factor in angiogenesis. The increased permeability displayed by tumour vessels (see section 1.11.2.2) facilitates

the extravasation of fibrinogen. Extracellular conversion to fibrin forms a three-dimensional gel matrix which is chemotactic and provides for a provisional stroma (Dvorak *et al.*, 1995; Yeo & Dvorak, 1995). Collagen synthesis is also a prerequisite for angiogenesis (Rooney *et al.*, 1997).

1.9.5.1.c Endothelial Cell Migration

Resting ECs are guarded by pericytes which help to maintain the endothelium in a quiescent, state (Antonelli-Orlidge *et al.*, 1989) and also inhibit EC movement (Sato & Rifkin, 1989). These effects must be overcome before angiogenesis can proceed (see section 1.14.2.2 for the involvement of pericytes in angiogenesis). A loose ECM is generated following proteolysis and ECs elongate and migrate into and through the spaces. They align with one another forming cords connected by intercellular junctions. Fibroblasts are also induced to migrate inwards where they synthesise and secrete the interstitial components (matrix proteins, proteoglycans and glycosaminoglycans) that comprise mature stroma (Brown *et al.*, 1993c; Dvorak *et al.*, 1987).

1.9.5.1.d Endothelial Cell Proliferation

Behind the leading front of migrating ECs, others in the cord actively divide in response to growth factors, providing new ECs for the elongating vessel sprout. The sprout gradually develops a lumen (canalization) between the migrating ECs.

1.9.5.1.e Tubulogenesis

Anastomosis of capillary sprouts at their tips results in the formation of capillary loops in which blood flow is soon established. ECs resume their tubular morphology. Pericytes migrate to the sites of capillary loops and become associated with the capillary and a BM is laid down around the new vessel. Remodelling, regression and rearrangement of newly formed capillaries occurs as part of vessel differentiation and maturation.

1.9.5.2 Intussusception

In addition to endothelial 'sprouting', new vessel growth and remodelling in tumours also occurs by intussusceptive microvascular growth (IMG) (Nagy *et al.*, 1995; Patan

et al., 1996b). Intussusception involves the insertion of interstitial tissue columns into the lumen of vasodilated vessels. These tissue pillars appear to arise within infoldings of the vessel wall (Patan *et al.*, 1996a and 1996b) and their subsequent growth divides the vessel into two segments. Flow appears to model the tissue pillars and FSS seems to influence their formation (Patan *et al.*, 1996b). It has even been suggested that the intermittent bloodflow which characterises the circulation of tumours may be the result of the architectural changes caused by IMG (Patan *et al.*, 1996b).

1.9.6 Genetics and Angiogenesis

Recent work has focussed on the genetic switches regulating angiogenesis (Hanahan & Folkman, 1996) although the detailed genetic changes and their effects on the process still remain to be identified. Angiogenic cells may evolve from non-angiogenic cells as a result of accumulating genetic changes, including the activation of oncogenes (e.g. *ras*-induced synthesis and secretion of GFs), inhibition of tumour suppressor genes or existence of mutant forms of mitogenic signalling pathways and cell-cycle control switches (Arbiser *et al.*, 1997; Folkman, 1995; Sager, 1989; Weinberg, 1989). These can lead to malignant transformation and neovascularisation.

1.9.7 Assessment of Angiogenesis

The concept of quantifying angiogenesis was introduced by Weidner *et al.* (1991) who first demonstrated a correlation between breast cancer angiogenesis and the propensity of the tumour to invade and metastasise. Subsequently, numerous studies involving a large variety of tumour types have reinforced the positive association between tumour angiogenesis and the risk of metastasis, tumour recurrence or death (Weidner *et al.*, 1995 and 1995b). Most studies assessing angiogenesis have employed a method based on the pioneering work of Weidner *et al.* (1991). This involves tumour biopsy and histological sectioning, followed by the identification of the *most vascular* areas of a tumour slice, vascular 'hot spots', using immunohistochemical markers specific for elements of the vasculature. Microvessels are then counted within these hot spots and the extent of angiogenesis scored. Microvessel density (MVD) has proved to be an effective gauge of angiogenesis and

consequently a powerful prognostic tool in many human tumour types (for review see: Engels *et al.*, 1997).

1.9.7.1 Vascular Markers

A number of different EC markers are available to highlight tumour blood vessels. Clearly the choice of marker is critical if staining is to be both highly specific for the vasculature and able to detect all vessels present. Many available antibodies suffer from drawbacks, such as a restricted distribution associated with only a subtype of endothelium, lack of specificity for endothelium, reaction with lymphatic endothelium or an overall lack of sensitivity (see table 1.6). Early studies tended towards the use of tinctorial stains (Protopapa *et al.*, 1993) or polyclonal anti-von Willibrand factor (factor VIIIrAg) (for a review see: Engels *et al.*, 1997). However the use of highly-specific, sensitive monoclonal antibodies has led to an improvement in complete and reproducible staining.

Most studies have utilised *endothelial* antigens in vascular detection although other components of the vasculature (pericytes, basal lamina components) have also been tested.

Advantages and disadvantages of available vascular markers are summarised in Table 1.6.

CELL MARKER	ANTIGEN	ADVANTAGES	REFERENCES	DISADVANTAGES	REFERENCES
Endothelial					
Anti-von Willebrand Factor (vWF) / Factor VIII related antigen (FVIIIrAg)	Weibel-palade bodies	Specific stain for ECs.	Offersen <i>et al.</i> , 1998; Weidner <i>et al.</i> , 1991.	WP bodies scarce/absent in some capillary ECs. Therefore absent / heterogeneous staining observed, especially in tumour tissue. Stains a proportion of lymphatic ECs. Also stains lymphocytes and may cross-react with tumour cells. Low sensitivity.	Engels <i>et al.</i> , 1997; Fox <i>et al.</i> , 1995; Giatromanolaki <i>et al.</i> , 1997; Horak <i>et al.</i> , 1992; Mietinen <i>et al.</i> , 1994; Ruiter <i>et al.</i> , 1989; Schlingemann <i>et al.</i> , 1991; Vermeulen <i>et al.</i> , 1996.
Anti-PECAM-1/CD31	Junctional CAM	Stains both macro- and microvasculature widely. Highly specific and sensitive. Failure to stain lymphatic ECs. Equally effective EC staining in tumour and normal tissue. Most superior marker in paraffin sections and excellent on frozen sections.	Charpin <i>et al.</i> , 1995; Engels <i>et al.</i> , 1997; Fox <i>et al.</i> , 1995; Giatromanolaki <i>et al.</i> , 1997; Hoerl & Goldblum, 1997; Horak <i>et al.</i> , 1992; Mietinen <i>et al.</i> , 1994; Schlingemann <i>et al.</i> , 1991; Vermeulen <i>et al.</i> , 1996.	One study has claimed it failed to stain vasculature in part of a section. Stains inflammatory infiltrate.	Offersen <i>et al.</i> , 1998; Schlingemann <i>et al.</i> , 1991; Weidner, 1995.
Anti-CD34	Membrane CAM	Sensitive marker. Equally effective EC staining in tumour and normal tissue.	Engels <i>et al.</i> , 1997; Schlingemann <i>et al.</i> , 1990; Vermeulen <i>et al.</i> , 1996.	Absent / heterogeneous staining in tumour tissue. Expressed by haematopoietic progenitor cells. Lack of EC specificity. Stains lymphatic ECs.	Fox <i>et al.</i> , 1995; Heimburg <i>et al.</i> , 1997; Hoerl & Goldblum, 1997; Mietinen <i>et al.</i> , 1994; Vermeulen <i>et al.</i> , 1996.
PAL-E	Not known. Associated with vesicles	Widespread and specific staining. Equally effective EC staining in tumour and normal tissue. Failure to stain lymphatic ECs.	Jones <i>et al.</i> , 1986; Ruiter <i>et al.</i> , 1989; Schlingemann <i>et al.</i> , 1985 and 1991; Vermeulen <i>et al.</i> , 1996.	Not expressed by all ECs.	Ruiter <i>et al.</i> , 1989.

Table 1.6 Markers used in the identification of the vasculature

CELL MARKER	ANTIGEN	ADVANTAGES	REFERENCES	DISADVANTAGES	REFERENCES
Endothelial (cont)					
Ulex europaeus agglutinin-1 (UEA-1)	Cell surface Lectin	Equally effective staining in tumour and normal tissue.	Vermeulen <i>et al.</i> , 1996.	Lacks sensitivity and specificity. Often stains non-EC, including tumour cells. Stains lymphatic ECs.	Jaffe <i>et al.</i> , 1973; Ruiter <i>et al.</i> , 1989; Vermeulen <i>et al.</i> , 1996.
EN4	Not known. Localised to EC junctions	Reactive with ECs in tumours.	Jones <i>et al.</i> , 1986.	Some areas left unstained. Some non-EC cells stained, including tumour cells. Stains lymphatic endothelium.	Jones <i>et al.</i> , 1986; Vermeulen <i>et al.</i> , 1996.
BMA 120 / BW 200	EC-specific antigen	Broad, specific staining. Equally effective staining in tumour and normal tissue.	Alles & Bosslet, 1986; Schlingemann <i>et al.</i> , 1991; Vermeulen <i>et al.</i> , 1996.	Occasionally stains tumour cells.	Vermeulen <i>et al.</i> , 1996.
Pericyte					
High-MW melanoma-associated antigen	Proteoglycan	Stained vasculature broadly and strongly.	Schlingemann <i>et al.</i> , 1991.		
Alpha smooth muscle actin	Cytoskeleton			Failure to stain large areas of vasculature.	Schlingemann <i>et al.</i> , 1991.
Basement membrane					
Collagen type IV / Laminin	Basement membrane-specific components			Staining not restricted to vasculature. Some tumour cells stained. Low sensitivity.	Engels <i>et al.</i> , 1997; Fox <i>et al.</i> , 1995; Schlingemann <i>et al.</i> , 1991.

Table 1.6 (continued)

1.9.7.2 Method of Microvessel Density Assessment – Chalkley Point Counting

A number of methods of assessing MVD have been established (for reviews see: Vermeulen *et al.*, 1996; Weidner, 1995). These include the manual counting of individual microvessels within hot spots (Weidner *et al.*, 1991) and computer image analysis (Fox *et al.*, 1995).

The method used in the present study is ‘Chalkley point counting’, first described by Chalkley (1943) for quantifying cells and nuclei, and subsequently applied by Fox *et al.* (1995) for quantifying vasculature.

The method comprises vascular staining of a paraffin-embedded/frozen tumour section followed by scanning at low magnification (X10-100) to identify the microvascular hot spots. The rationale for selecting areas of highest MVD for quantification is that they are likely to be biologically important, being the areas of most intense neovascularisation. Other studies have examined random fields (Protopapa *et al.*, 1993) or average vascularity (Srivastava *et al.*, 1988) but most have assessed hot spots (Weidner *et al.*, 1991). The number of hot spots counted per tumour section varies from one to five, but, in line with the original criteria of Weidner *et al.* (1991), is generally three. The tumour field area currently assessed ranges from 0.12 – 1.00 mm². Clearly too small an area will always give a high vascular index and too large an area will ‘dilute’ out the hot spot.

Having identified the requisite number of hotspots at low power, a Chalkley 25-point eyepiece graticule (Chalkley, 1943) is inserted into the microscope ocular. In order to visualise individual stained microvessels each hot spot is then viewed at high power (X250). The graticule is orientated so that the maximum number of points are on or within areas of highlighted vessels. The overlying points are counted.

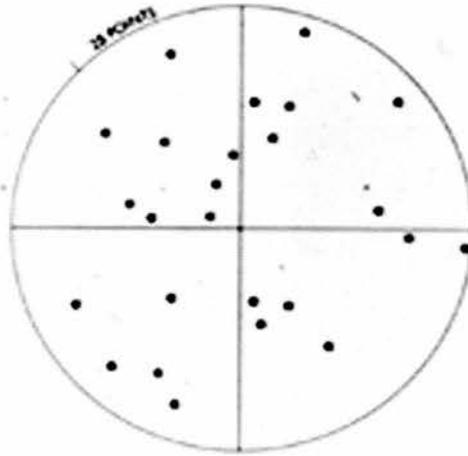


Plate 1.2 *The Chalkley 25-point eyepiece graticule “...(provides) a random distribution of points throughout the tissue. (By) summing up the number of points lying in the various components, the ratios of these sums will also approach the ratios of the volume fractions occupied by the respective components....” (Chalkley, 1943).*

The degree of subjectivity related to hot spot selection is deemed acceptable (Fox *et al.*, 1995). Most investigators have concluded that individual tumours tend to have low intratumour variation in MVD, demonstrating the same microvascular architecture throughout. Accordingly quantifying vessels in a single tissue section from a tumour is generally accepted as characterising the whole tumour specimen (Offersen *et al.*, 1998; Schlingemann *et al.*, 1991; Vermeulen *et al.*, 1997; Weidner *et al.*, 1991).

Chalkley point counting is rapid and reproducible. It reduces the laborious nature and varying inter-observer interpretation of counting individual microvessels, as required by the Weidner *et al.* (1991) method, and it eliminates costly, time-consuming methods of computer image analysis. An excellent correlation of vascular grade and Chalkley count has been observed (Fox *et al.*, 1995) suggesting that the method can be used to quantify tumour angiogenesis effectively. An international consensus on angiogenesis quantification recommended Chalkley point counting as the method of choice (Vermeulen *et al.*, 1996).

1.10 Metastasis

The formation of tumours at secondary sites by metastasis is a complex multistage process (for a review see: Liotta, 1992). Tumour cells detach from the primary tumour mass, intravasate into the lymphatics or vasculature and are carried in the general circulation until they lodge in a capillary bed. They must then extravasate through the blood vessel wall and through the ECM to invade the surrounding tissues and begin to proliferate (Fidler, 1990). The metastasised secondary tumour must then induce angiogenesis in order to grow. A tumour cell undergoing metastasis must secrete proteolytic enzymes to allow its passage through the ECM. Numerous adhesion molecules have been identified which mediate the adherence of tumour cells to the endothelium (for a review see: Albelda, 1994; Tang & Honn, 1994).

NO has been shown both to reduce (Dong *et al.*, 1994; Tschugguel *et al.*, 1999; Xie *et al.*, 1996, 1997a; Yamamoto *et al.*, 1994) and to enhance (Edwards *et al.*, 1996; Yudoh *et al.*, 1997) the process of metastasis. Accordingly L-NAME has been shown both to increase (Yamamoto *et al.*, 1994) and to reduce (Edwards *et al.*, 1996) lung metastasis in mice. Inhibitory actions of L-NAME may derive from a vasoconstrictive effect (Yamamoto *et al.*, 1998), the inhibition of platelet-tumour cell aggregation (Mehta *et al.*, 1987) or a reduction in the adhesion of tumour cells to the endothelium (Kong *et al.*, 1996; Kubes *et al.*, 1993; Vidal *et al.*, 1992). Blood turbulence influences tumour cell arrest in capillary beds. Clearly the role of NO in metastasis is not clear at the present time.

1.11 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), was unwittingly discovered by Gullino in 1975, when he noted that the protein and water concentration of tumour stroma was significantly greater than that of normal tissue stroma. This indicated that tumour vessels are hyperpermeable. In

1983, VEGF was first described and purified from tumour ascites fluid (Senger *et al.*, 1983).

Although many angiogenic factors have been defined, only VEGF meets all the criteria required for the definition of a vasculogenic or angiogenic factor (Connolly *et al.*, 1989; Murohara *et al.*, 1998; Pepper *et al.*, 1996; Risau, 1996). It is therefore considered to be the principle regulator of angiogenesis (Klagsbrun & Soker, 1993).

1.11.1 General Properties

VEGF is a family of four dimeric, vasoactive polypeptides which are alternatively spliced from a single gene (Bacic *et al.*, 1995; Tischer *et al.*, 1991). The two shortest forms, VEGF₁₂₁, VEGF₁₆₅, are secreted proteins and readily diffusible. The two longer forms, VEGF₁₈₉, VEGF₂₀₆, are bound to heparin-containing proteoglycans on the cell surface or BM (Houck *et al.*, 1992). VEGF₁₆₅ is the most abundant form (Ferrara & Davis-Smyth, 1997) and has the most potent mitogenic activity *in vivo* (Keyt *et al.*, 1996). It is a 46 kDa protein which is secreted by tumour cells and specifically affects VECs (Dvorak *et al.*, 1995; Dvorak, 1997; Ferrara *et al.*, 1991; Jakeman *et al.*, 1992).

1.11.2 VEGF Functions

VEGF performs a number of both direct and indirect actions within the vasculature during physiological and pathophysiological angiogenesis.

1.11.2.1 Vascularisation

VEGF is involved in the development of new vasculature, acting upon VECs to cause them to assume an elongated shape, migrate, divide and assemble into tubular structures (Gospodarowicz *et al.*, 1989; Koch *et al.*, 1994; Leung *et al.*, 1989; Nakagawa *et al.*, 1992; Senger *et al.*, 1983). As such, it is found in many tissues during both primary (foetal) vasculogenesis when ECs differentiate from mesodermal precursors (Breier *et al.*, 1992; Jakeman *et al.*, 1993) and adult physiological angiogenesis (Brown *et al.*, 1992; Harper & Klagsbrun, 1999; Kamat *et al.*, 1995). Disruption of the genes for VEGF (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996) and

its receptors (Fong *et al.*, 1995; Shalaby *et al.*, 1995) results in embryonic lethality, highlighting the importance of VEGF in the process of vascularisation.

VEGF is also associated with a number of pathologies which involve persistent unregulated angiogenesis, such as rheumatoid arthritis (Koch *et al.*, 1994), psoriasis (Detmar *et al.*, 1994), ischaemic ocular diseases (Aiello *et al.*, 1995) and the delayed hypersensitivity (DH) immune response (Brown *et al.*, 1995c). Of particular relevance to this research is the positive correlation between VEGF expression and angiogenesis in malignant tumours (Brown *et al.*, 1995b; Cornali *et al.*, 1996; Flore *et al.*, 1998; Guidi *et al.*, 1995). VEGF and its receptors have been found to be substantially over-expressed in the majority of human cancers thus far studied (Brown *et al.*, 1993; 1995b; Dvorak, 1997; Guidi *et al.*, 1996; Hatva *et al.*, 1995; Pietsch *et al.*, 1997; Plate *et al.*, 1992; Senger *et al.*, 1983) and anti-VEGF antibodies reduce tumour growth by interfering with tumour angiogenesis (Asano *et al.*, 1995; Borgstrom *et al.*, 1996 and 1998; Kim *et al.*, 1992; Kondo *et al.*, 1993; Presta *et al.*, 1997).

1.11.2.2 *Vascular Permeability*

VEGF expression occurs in several normal adult tissues in the absence of angiogenesis, particularly kidney, lung and adrenal gland (Berse *et al.*, 1992). Within these tissues it is assumed to be playing its other major role, that of regulating vascular permeability (Brown *et al.*, 1997). VEGF increases vessel permeability either by inducing endothelial fenestrations (Roberts & Palade, 1995) or by up-regulating the function of vesiculo-vacuolar organelles (VVOs). VVOs comprise a series of membrane-bound, interconnecting vesicles and vacuoles which cluster together in endothelial cell cytoplasm to form organelle-like structures, (Dvorak *et al.*, 1996; Feng *et al.*, 1996). They span the entire cytoplasm of ECs, and communicate with each other and with the EC plasmalemma by stomata (Dvorak *et al.*, 1996). VEGF up-regulates VVO function at the level of stomatal diaphragms (Dvorak *et al.*, 1996).

The result of VEGF production within tumours therefore, is to render their vasculature hyperpermeable (Dvorak *et al.*, 1988; Potgens *et al.*, 1996). As reviewed in section 1.9.5.1.b, this property is critical for the onset of angiogenesis.

1.11.2.3 Gene Expression

VEGF alters the pattern of expression of several angiogenesis-related genes in ECs. It up-regulates the expression of PA and PAI (Pepper *et al.*, 1991) and the urokinase receptor (Mandriota *et al.*, 1995). It also induces the expression of interstitial collagenase (Unemori *et al.*, 1992), and of pro-coagulant tissue factor (Clauss *et al.*, 1990), thereby increasing the activity of clotting factors and the formation of fibrin from fibrinogen.

1.11.2.4 Vascular Morphology

VEGF also functions in maintaining vessel integrity. Low levels are present in normal plasma (Yamamoto *et al.*, 1996) and appear to prevent EC replicative senescence (Watanabe *et al.*, 1997). VEGF therefore appears to maintain the latent capacity of ECs for replication. Similarly, VEGF expression has been detected around microvessels where ECs are normally quiescent (Ment *et al.*, 1997) and its receptor mRNA is expressed in both proliferating and quiescent ECs (Ferrara & Davis-Smyth, 1997). It appears therefore that VEGF may act as an *in vivo* vascular survival factor.

1.11.3 VEGF Receptors

Two high-affinity VEGF receptors have been identified on VECs, the Flt-1 receptor (or fms-like tyrosine kinase) (VEGF-R1) (de Vries *et al.*, 1992) and the KDR/Flk-1 receptor (kinase insert domain-containing receptor / fetal liver kinase-1) (VEGF-R2) (Terman *et al.*, 1992). These are transmembrane receptor tyrosine kinases (RTKs) with seven IgG-like domains (Vaisman *et al.*, 1990). Both are strikingly over-expressed in tumour microvasculature (Brown *et al.*, 1993, 1993b;1995b; Dvorak, 1997; Hatva *et al.*, 1995; Plate *et al.*, 1992,1993, 1994). Although originally thought to be confined exclusively to VECs (Jakeman *et al.*, 1992; Olander *et al.*, 1991; Peters *et al.*, 1993), and still frequently misquoted as such, VEGF receptors have

been identified on a number of non-endothelial cell types (Brown *et al.*, 1997b), suggesting that VEGF may have important, previously unsuspected roles.

Non-EC expression of receptors has included a number of transformed cells. Expression of novel VEGF receptors of various molecular weights has been described on a number of different tumour types (Gitay-Goren *et al.*, 1993; Omura *et al.*, 1997; Soker *et al.*, 1996). Expression of KDR/Flk-1 has been detected on tumour cells from a number of different tumour types (Boocock *et al.*, 1995; Brown *et al.*, 1996b). The role played by VEGF on tumour cells expressing KDR receptors remains to be elucidated. Expression implies that VEGF may have a direct effect on tumour cells and although it fails to induce mitogenesis in these cells (Boocock *et al.*, 1995), it may be involved in invasion rather than growth. It is possible that aberrant expression of VEGF receptors on tumour cells is one of the phenotypic changes that occurs during malignant transformation (Gitay-Goren *et al.*, 1993).

VEGF induces upregulation of its own receptors (Brogi *et al.*, 1996; Kim *et al.*, 1993; Plate *et al.*, 1993 and 1994; Wilting *et al.*, 1996) and is unable to function until receptor expression occurs (Llan *et al.*, 1998). Of the two subtypes, KDR/Flk-1 appears to be the major transducer of the VEGF signal in ECs (Waltenberger *et al.*, 1994). It mediates VEGF-induced enhancement of vascular permeability (Murohara *et al.*, 1998), EC proliferation (Malavaud *et al.*, 1997) and migration/chemotaxis (Millauer *et al.*, 1993). KDR/Flk-1 is expressed at a much higher density on ECs than Flt-1 (Vaisman *et al.*, 1990) and the extent of neovascularisation correlates directly with its expression (Takahashi *et al.*, 1995). One study has concluded that Flt-1 expression is restricted to *lymphatic* ECs (Wilting *et al.*, 1996) and that mutant forms of VEGF which preferentially bind to Flt-1 do not stimulate EC proliferation, raising questions regarding the role of Flt-1 in angiogenesis (Keyt *et al.*, 1996). Anti-KDR/Flk-1 antibodies are effective at blocking tumour growth (Fong *et al.*, 1999; Lin *et al.*, 1998; Millauer *et al.*, 1994 and 1996; Skobe *et al.*, 1996; Strawn *et al.*, 1996; Witte *et al.*, 1998) and agonists specific for KDR/Flk-1 are sufficient, in themselves, to elicit angiogenesis (Ortega *et al.*, 1997). It is the activation of

KDR/Flk-1 which leads to the upregulation of NOS in response to VEGF (Kroll & Waltenberger, 1998).

1.11.4 VEGF Signal Transduction

Stimulation of VECs with VEGF results from its binding to the extracellular domain of a receptor, inducing receptor dimerisation. The tyrosine kinase in the cytoplasmic domain is activated and the receptors become autophosphorylated on tyrosine residues. Phosphotyrosines serve as binding sites for intracellular signalling molecules. Investigations of downstream phosphorylation targets for VEGF receptor activation have yielded varying results. The cascade has included phospholipase C- γ (PLC- γ) (producing inositol trisphosphate, IP₃ and increasing intracellular [Ca²⁺]_i), p120 guanine nucleotide exchange factor (GEF), the mitogen-activated protein kinases (MAPK) ERK-1 and ERK-2, focal adhesion kinase (FAK) and paxillin (Abedi & Zachari, 1997; D'Angelo *et al.*, 1995; Seetharam *et al.*, 1995; Shawver *et al.*, 1997; Xia *et al.*, 1996; Zachary, 1997). (For a review of VEGF signalling pathways see: Llan *et al.* (1998) or Zachary, (1997)).

The effects of VEGF on actin cytoskeletal reorganisation and cell migration may result from stimulation of FAK phosphorylation (Abedi & Zachari, 1997). VEGF signalling has been shown to cause an increase in [Ca²⁺]_i (Bates & Curry, 1997; Brock *et al.*, 1991) and also, at high concentrations, to augment NO production (Ku *et al.*, 1993; van der Zee *et al.*, 1997) (see section 1.12.2). The mobilisation of Ca²⁺ from intracellular stores in response to IP₃ production has the potential to modulate the effect of Ca-calmodulin regulated enzymes such as eNOS (Ahmed *et al.*, 1997; Ku *et al.*, 1993). Activation of the MAPK cascade in ECs has also been shown to require NO production (Parenti *et al.*, 1998), further implicating NO in the response to VEGF.

1.11.5 Regulation of VEGF

Although constitutively expressed by many tumour cells and transformed cell lines, VEGF expression is subject to regulation by a number of mechanisms (for review see Brown *et al.*, 1997).

Oxygen concentrations play a regulatory role in VEGF expression, with hypoxia up-regulating the expression of VEGF (Kourembanas *et al.*, 1997; Minchenko *et al.*, 1995; Mukhopadhyay *et al.*, 1995; Namiki *et al.*, 1995; Tuder *et al.*, 1995; Tufro-McReddie *et al.*, 1997) and both KDR/Flk-1 (Tufro-McReddie *et al.*, 1997; Waltenberger *et al.*, 1996) and Flt-1 (Tuder *et al.*, 1995; Tufro-McReddie *et al.*, 1997) receptors. This increased expression is mediated by an upregulation of the transcription factor hypoxia-inducible factor-1 (HIF-1) (Semenza, 1996). An upregulation of VEGF expression has been demonstrated in hypoxic zones of solid tumours (Brown *et al.*, 1993; Hlatky *et al.*, 1994; Plate *et al.*, 1992; Shweiki *et al.*, 1992), presumably in an attempt to alleviate necrosis by stimulating new vessel formation.

The expression of both i- and eNOS is similarly up-regulated by hypoxia (Hampl *et al.*, 1995; Lecras *et al.*, 1996) and there are a number of reports suggesting that NO is itself capable of both suppressing (Liu *et al.*, 1998; Tsurumi *et al.*, 1997) and enhancing (Ambs *et al.*, 1998; Chin *et al.*, 1997; Frank *et al.*, 1999) the induction of VEGF expression (see section 1.12.2).

Oncogenes also regulate VEGF expression. Cells transfected with mutant *ras* oncogenes express increased levels of VEGF mRNA and protein (Grugel *et al.*, 1995; Rak *et al.*, 1995, 1995b) and disruption of mutant *K-ras* in carcinoma cells leads to decreased VEGF expression (Rak *et al.*, 1995, 1995b). As discussed in section 1.8.3.4, mutant forms of *p53* also increase expression of VEGF mRNA (Kieser *et al.*, 1994).

1.12 Nitric Oxide and Angiogenesis

As discussed in section 1.8.3, NO clearly influences the growth of solid tumours. There is a considerable body of evidence implicating the L-arginine: NO pathway in determining the angiogenic potential of tumours. However despite the apparent association between NO and angiogenesis, conflicting data on the effects and possible function(s) of NO exists and its involvement therefore merits further consideration.

1.12.1 Pro- and Anti-angiogenic Actions of NO

The evidence demonstrating both positive and negative effects of NO in angiogenesis is documented in tables 1.7 and 1.8 overleaf.

PRO-ANGIOGENIC EFFECT OF NO	REFERENCE
Rabbits received a corneal implant of either a Substance P analogue or prostaglandin E1 and an adjacent implant of an NO donor. Angiogenesis promoted by Sub P/PGE1 was potentiated by the presence of the NO donor. Systemic L-NAME at 0.5g/litre was provided for 17 days (from 7 days prior to surgery) <i>ad libitum</i> in drinking water. L-NAME greatly reduced Sub P/PGE-induced angiogenesis (0.1g/litre did not reduce the angiogenic response). <i>In vitro</i> exposure of ECs to NO donors increased proliferation and migration. This was also abolished by NOS inhibitors. Exposure of cells to Sub P activated eNOS. NO production from vasoactive agents such as Sub P/PGE1 mediates their <i>in vitro</i> and <i>in vivo</i> angiogenic response.	Ziche <i>et al.</i> , 1994.
Rabbits received a corneal implant of A-431 tumour fragments. Systemic L-NAME at 1g/litre was provided: (1) from 7 days prior to implantation for a total of 19 days; and (2) once a consistent vascular network had been established (from day 26 post-implantation) for 7 days. In regime (1) no angiogenesis was produced by the implants until 8 days after treatment interruption when vascularisation started and progressed to match that of controls. In regime (2) vascular diameter was reduced by 50% within 24 hours of treatment, and over the following days the vasculature regressed and tumour size decreased to 50% that of controls. Tumour total NOS (i and e), iNOS and cGMP activities increased with increased tumour vascularisation. Angiogenesis in A-431 cells <i>in vitro</i> was blocked by a NOS inhibitor.	Gallo <i>et al.</i> , 1998.
Mice received subcutaneous injections of a mammary adenocarcinoma. Systemic L-NAME at 0.5g/litre was provided for 26 days from the day of implantation. L-NAME significantly reduced tumour growth from day 17 of administration. Growth inhibition was shown to result from a reduction in the angiogenic response.	Jasnis <i>et al.</i> , 1997.
Subcutaneous murine tumours grown from iNOS-transfected carcinoma cells (Calu-6) contained significantly more small blood vessels than control tumours lacking iNOS. Control tumours contained large necrotic areas indicative of limited angiogenesis. 1% AG <i>ad libitum</i> in the drinking water significantly reduced tumour growth.	Ambs <i>et al.</i> , 1998.
Subcutaneous murine tumours grown from iNOS-transfected adenocarcinoma cells (DLD-1) grew faster and were markedly more vascularised and invasive than control tumours.	Jenkins <i>et al.</i> , 1995.
L-NAME inhibits <i>in vitro</i> EC sprouting and migration and surface expression of integrin $\alpha\beta 3$.	Murohara <i>et al.</i> , 1999.
Spontaneous angiogenesis following experimental limb ischemia is severely impaired in mice lacking the gene for eNOS.	Murohara <i>et al.</i> , 1998b.
Oral L-arginine significantly enhanced angiogenesis in experimental limb ischemia in rabbits. L-NNA at 3.3mg/Kg/d added to the drinking water of portal hypertensive rats significantly inhibited the angiogenic response in the rat mesenteric window assay.	Murohara <i>et al.</i> , 1998b. Sumanovski <i>et al.</i> , 1999.

Table 1.7 Pro-angiogenic effects of NO

Murine mammary adenocarcinoma cells (C3-L5) constitutively express high levels of e- but not iNOS protein <i>in vivo</i> and <i>in vitro</i> and are highly invasive. NOS inhibitors reduce their invasiveness <i>in vitro</i> . <i>In vitro</i> NO production and invasiveness is paralleled by a down-regulation of TIMP2 and TIMP3. Induction of iNOS and extra NO also lead to upregulation of MMP-2. Suggests that NO promotes tumour cell invasiveness by altering the balance between expression of MMP-2 and its inhibitors TIMP2 and TIMP3.	Orucevic <i>et al.</i> , 1999.
An NO donor induces bFGF expression which promotes proliferation and up-regulates uPA activity in ECs <i>in vitro</i> . A NOS inhibitor blocks these effects. This represents a mechanism of NO-mediated matrix degradation during angiogenesis. NO sustains EC proliferation by inducing endogenous bFGF expression. NOS inhibition, by reducing bFGF, induces apoptosis, therefore ultimately suppressing tumour bloodflow.	Ziche <i>et al.</i> , 1997b.
IL-2-induced NO promotes degradation of articular cartilage by stimulation of MMP-2 and MMP-9 in cultured rabbit chondrocytes. These effects are blocked by L-NMMA. bFGF released from the degraded ECM stimulates EC proliferation. NO may therefore mediate angiogenesis associated with arthritis.	Tamura <i>et al.</i> , 1996.
NO induced by IL-1 β , TNF α and LPS stimulates stromelysin and collagenase activity in cartilage. NOS inhibitors inhibited, and SNAP further induced this enzyme activity.	Murrell <i>et al.</i> , 1995;
VEGF but not TGF β induced an increase in cGMP levels and eNOS in ECs <i>in vitro</i> . VEGF-stimulated EC proliferation and tubulogenesis were blocked by L-NAME and the removal of Ca ²⁺ .	Papapetropoulos <i>et al.</i> , 1997.
L-NAME/L-NMMA inhibit TGF β -induced EC tubulogenesis in a 3D collagen gel <i>in vitro</i> . An NO donor or cGMP analogue restore tubule formation.	Papapetropoulos <i>et al.</i> , 1997b.
NOS inhibitors reduce angiogenesis, inhibiting healing in gastric ulcers.	Konturek <i>et al.</i> , 1993.
bFGF up-regulates eNOS production in ECs <i>in vitro</i> .	Kostyk <i>et al.</i> , 1995.
bFGF induces tubulogenesis and enhanced eNOS expression and NO production in ECs on a fibrin matrix. NOS inhibitors reduced and SNAP increased tubulogenesis.	Babaei <i>et al.</i> , 1998.
<i>In vitro</i> Endothelin (ET)-induced angiogenesis requires functional eNOS, is mediated by the release of NO and is blocked by L-NAME and an eNOS anti-sense construct. NO is considered necessary for EC podokinesis and ET for direction of movement.	Goligorsky <i>et al.</i> , 1999.
Mice were injected subcutaneously with matrigel, to form a solid gel plug into which vessel growth could be observed. Systemic L-NAME at 0.5mg/ml was provided <i>ad libitum</i> in the drinking water. The angiogenic response induced in the plug by the inclusion of PAF or TNF, but not bFGF, was blocked by the L-NAME regime. L-NAME also inhibited the <i>in vitro</i> migration of ECs in response to PAF and TNF, but not bFGF.	Montrucchio <i>et al.</i> , 1997.
An iNOS-dependent mechanism is involved in the angiogenesis of granuloma pyogenicum.	Shimizu <i>et al.</i> , 1998.
An L-arginine:NOS-dependent mechanism is required for the production of angiogenic activity by macrophages. NOS inhibitors prevent the expression of the this angiogenic phenotype.	Leibovich <i>et al.</i> , 1994.

Table 1.7 (continued)

ANTI-ANGIOGENIC EFFECTS OF NO	REFERENCE
An NO donor and L-arginine inhibited, and L-NMMA increased angiogenesis in the <i>in vivo</i> chick chorioallantoic membrane (CAM) angiogenesis assay.	Pipili Synetos <i>et al.</i> , 1993.
An NO donor inhibited angiogenesis in both the <i>in vivo</i> CAM and the <i>in vitro</i> EC tubulogenesis assay. NOS inhibitors and dexamethasone (inhibitor of iNOS induction) stimulated angiogenesis in the CAM suggesting both e- and iNOS contribute to NO-mediated angiogenesis inhibition.	Pipili-Synetos <i>et al.</i> , 1994.
NO donors inhibit EC proliferation <i>in vitro</i> . Inhibitory effects are blocked by a GC inhibitor.	RayChaudhury <i>et al.</i> , 1996; Yang <i>et al.</i> , 1994.
L-NAME (0.5g/l) in the drinking water of rats enhanced the angiogenic response produced by bFGF and IL-1 in the rat mesenteric window assay, but had no effect on that produced by VEGF. Suggests that different pathways mediate these angiogenic responses.	Norrby, 1998.
NO is involved in the anti-angiogenic effect of irradiation in the CAM. X-rays are anti-angiogenic through their activation of NOS. L-NAME reversed the irradiation-induced anti-angiogenic effect in the CAM. The authors hypothesise that radiation-induced NO interacts with superoxide radicals, producing peroxynitrite which causes stromal, endothelial and other tissue injury, including inhibition of angiogenesis.	Hatjikondi <i>et al.</i> , 1996.
NO-releasing compounds (ISMN, ISDN) inhibited angiogenesis in the CAM and significantly reduced Lewis lung carcinoma (LLC) growth and metastasis in mice. They had had no effect on bFGF-stimulated ECs or LLC cells <i>in vitro</i> suggesting the the anti-angiogenic mechanism is independent of EC growth.	Pipili-Synetos <i>et al.</i> , 1995.
IL-2-mediated angiogenesis inhibition in the CAM is abolished by L-NAME. Suggests that observed anti-tumour effects of IL-2 are therefore mediated through the inhibition of angiogenesis, and that the effect is mediated by NO.	Sakkoula <i>et al.</i> , 1997.
Elevated levels of glucose inhibit the proliferation of ECs (Graier <i>et al.</i> , 1995; Lorenzi <i>et al.</i> , 1986). AG prevents glucose-induced inhibition of neovascularisation in a rat model.	Teixeira & Andrade, 1999.
NO prevents the dissociation of the transcription factor (TF) NFκB from its inhibitory factor, IκBα, prevents TF activation. NFκB initiates transcription of genes encoding for certain cell adhesion molecules and monocyte chemoattractant protein (MCP-1). NO therefore inhibits cellular adhesion and migration through its interaction with the NFκB/IκBα complex.	Collard <i>et al.</i> , 1999.
NO donors inhibited EC DNA synthesis and L-NAME stimulated it. A regulatory switch from an angiogenic to a non-angiogenic phenotype in ECs involves a change in L-arginine metabolism from polyamines to NO (see Joshi <i>et al.</i> , 1997 and Mills <i>et al.</i> , 1992 below).	Joshi <i>et al.</i> , 1999.
L-arginine metabolism can proceed through two distinct pathways - NOS catalysis, producing NO, or arginase/ornithine decarboxylase catalysis, producing polyamines. Polyamines (Pas) increase EC proliferation (Takigawa <i>et al.</i> , 1990) and inhibit NO synthesis (Szabo <i>et al.</i> , 1994b). Their role in angiogenesis is proposed to be in direct opposition to that of NO. During early stages of malignancy, PA synthesis promotes EC proliferation and NO is maintained at low levels. During the vascular stage of growth, NO synthesis is elevated, reducing EC proliferation and maintaining a vasodilator tone in and around the tumour. The author proposes that angiogenesis is regulated through a switch in the pathway of L-arginine metabolism to produce either NO or PAs.	Joshi <i>et al.</i> , 1997; Mills <i>et al.</i> , 1992.

Table 1.8 Anti-Angiogenic Effects of NO

1.12.2 A Nitric Oxide - VEGF Association

Direct associations between NO and VEGF have been documented for each stage of the angiogenic process - vasodilation, increased vascular permeability, EC proliferation and migration. There is experimental evidence to suggest that the angiogenic effects of VEGF are mediated by increased NO synthesis. Conversely however, NO has itself been observed both to induce and suppress VEGF expression. Clearly the relationship between the two mediators is complex and somewhat conflicting. However, based on the pivotal role played by VEGF in angiogenesis, the association between it and NO is clearly an important consideration when investigating the involvement of NO in the process. The current evidence linking the actions of VEGF and NO is provided in table 1.9 overleaf.

Table 1.9 NO - VEGF Association

NO –VEGF RELATIONSHIP	REFERENCE
VEGF signal transduction pathways increase $[Ca^{2+}]_i$, having the potential to modulate calcium-sensitive enzymes such as eNOS. (see section 1.11.3). This would generate short-term stimulation of endothelial NO generation.	Ahmed <i>et al.</i> , 1997; Ku <i>et al.</i> , 1993.
Exposure of confluent ECs or aortic rings to VEGF significantly increases eNOS mRNA and protein levels and the basal production of cGMP.	Bouloumie <i>et al.</i> , 1999.
NOS inhibitors abolish VEGF-induced proliferation of cultured ECs.	Morbidelli <i>et al.</i> , 1996.
NOS inhibitors abolish VEGF-induced tubulogenesis in 3D collagen gels.	Papapetropoulos <i>et al.</i> , 1997.
VEGF up-regulates eNOS mRNA, protein and NO production in human ECs.	Hood <i>et al.</i> , 1998.
VEGF induces expression of eNOS and iNOS protein in ECs.	Kroll & Waltenberger, 1998.
NO donors can suppress the induction of VEGF by interfering with the binding of the transcription factor AP-1 to the VEGF promoter (non-transformed cells).	Liu <i>et al.</i> , 1998; Tsurimi <i>et al.</i> , 1997.
NO donors induce the expression of VEGF mRNA and protein in tumour cells and L-NMMA or guanylate cyclase inhibitors inhibit VEGF production.	Ambs <i>et al.</i> , 1997; Chin <i>et al.</i> , 1997.
AG prevents the expression of VEGF in galactosemic rats.	Frank <i>et al.</i> , 1997.
L-NAME and AG inhibit the VEGF-induced increase in vascular permeability in mouse skin. This suggests that NO produced from iNOS is involved in the process.	Fujii <i>et al.</i> , 1997.
GSNO can induce the expression of VEGF in rat mesangial cells.	Frank <i>et al.</i> , 1999.
VEGF protein concentration is higher in cell extracts of iNOS-expressing tumour cells than in control cells. L-NMA reduces VEGF secretion.	Ambs <i>et al.</i> , 1998.
VEGF induces NO release from cultured ECs and L-NAME abolishes VEGF-mediated reduction in smooth muscle cell proliferation.	Laitinen <i>et al.</i> , 1997.
<i>In vitro</i> VEGF-stimulated EC migration requires functional NOS and is abolished in the presence of L-NAME or antisense oligonucleotides targeting eNOS. NO appears to attenuate EC adhesion to several matrix proteins at focal adhesions. The authors propose that NO induces a switch from a stationary to a locomoting EC phenotype, producing scalar motion ('podokinesis') and VEGF provides guidance cues that impart a vectorial component to the movement.	Noiri <i>et al.</i> , 1998.
VEGF increases NO release from cultured ECs. NOS inhibitors reduce the stimulatory effect of VEGF.	Ahmed <i>et al.</i> , 1997; Hariawala <i>et al.</i> , 1996; Van der Zee <i>et al.</i> , 1997.
L-NMMA and L-NAME prevent VEGF-induced hypotension <i>in vitro</i> and <i>in vivo</i> .	Horowitz <i>et al.</i> , 1997; Ku <i>et al.</i> , 1993; Malavaud <i>et al.</i> , 1997; Yang <i>et al.</i> , 1996.
VEGF stimulated cGMP levels and <i>in vitro</i> angiogenesis in ECs. It increased cNOS ~ 2 fold and iNOS by 36%. The effects were blocked by L-NMMA or a GC inhibitor.	Ziche <i>et al.</i> , 1997.
L-NAME and an NO scavenger reduce the VEGF-enhanced permeability of tumour vessels.	Maeda <i>et al.</i> , 1994; Murohara <i>et al.</i> , 1998.
L-NMMA and a GC inhibitor abolish the VEGF-induced increase in vascular permeability in isolated vessels.	Wu <i>et al.</i> , 1996.
NO-producing vasodilators reduce VEGF expression and L-NAME increases transcription of mRNA for both VEGF and its receptors.	Tuder <i>et al.</i> , 1995.
The angiogenic activity of macrophages is, in part, mediated by VEGF and is blocked by L-NAME and AG.	Xiong <i>et al.</i> , 1998.

1.12.3 NO and Angiogenesis - Conclusions

Clearly the involvement of NO in tumour angiogenesis is complex and contentious. In summary, a number of factors may offer some insight into the possible causes of apparent confliction:

- The role of NO in the growth of solid tumours is clearly a multi-faceted one. It functions in both growth-inhibitory and growth-supporting capacities, determined at least in part by its concentration. It could be supposed, therefore, that the effect of NO on angiogenesis may be a concentration-dependent one.
- In light of the association between NO, VEGF, *p53* and oncogenes, the genetic status of a tumour would be expected to influence the involvement of NO. Accordingly, both tumour type and normal versus transformed phenotype would have the potential to generate conflicting results.
- *In vitro* actions of NO in the process may bear no relation to *in vivo* events.
- Differing results obtained with different *in vivo* models/assays of angiogenesis raise the possibility that the involvement of NO may be species specific and/or tissue-specific (fully-differentiated adult or embryonic). The presence or absence of transformed tumour versus normal tissue within the assay could also influence the process, as could the vascular status of the tissue i.e. natively-vascularised or avascular (e.g. cornea, CAM).
- More than one mechanism/pathway may be involved in the regulation of angiogenesis. For example, differing pro-angiogenic cytokines, bFGF and VEGF appear to induce angiogenesis by different mechanisms (Brooks *et al.*, 1994; Friedlander *et al.*, 1985) and only that induced by VEGF involves NO (Murohara *et al.*, 1998; Ziche *et al.*, 1997). At least two distinct angiogenic pathways are therefore said to exist. Thus although the NO pathway integrates several chemical and physical modulators of the angiogenic process, not all angiogenic factors depend on this signalling cascade and the involvement of NO appears to be growth stimuli-dependent.

A summary of the involvement of NO within the circulation of a solid tumour is provided in figure 1.8 overleaf.

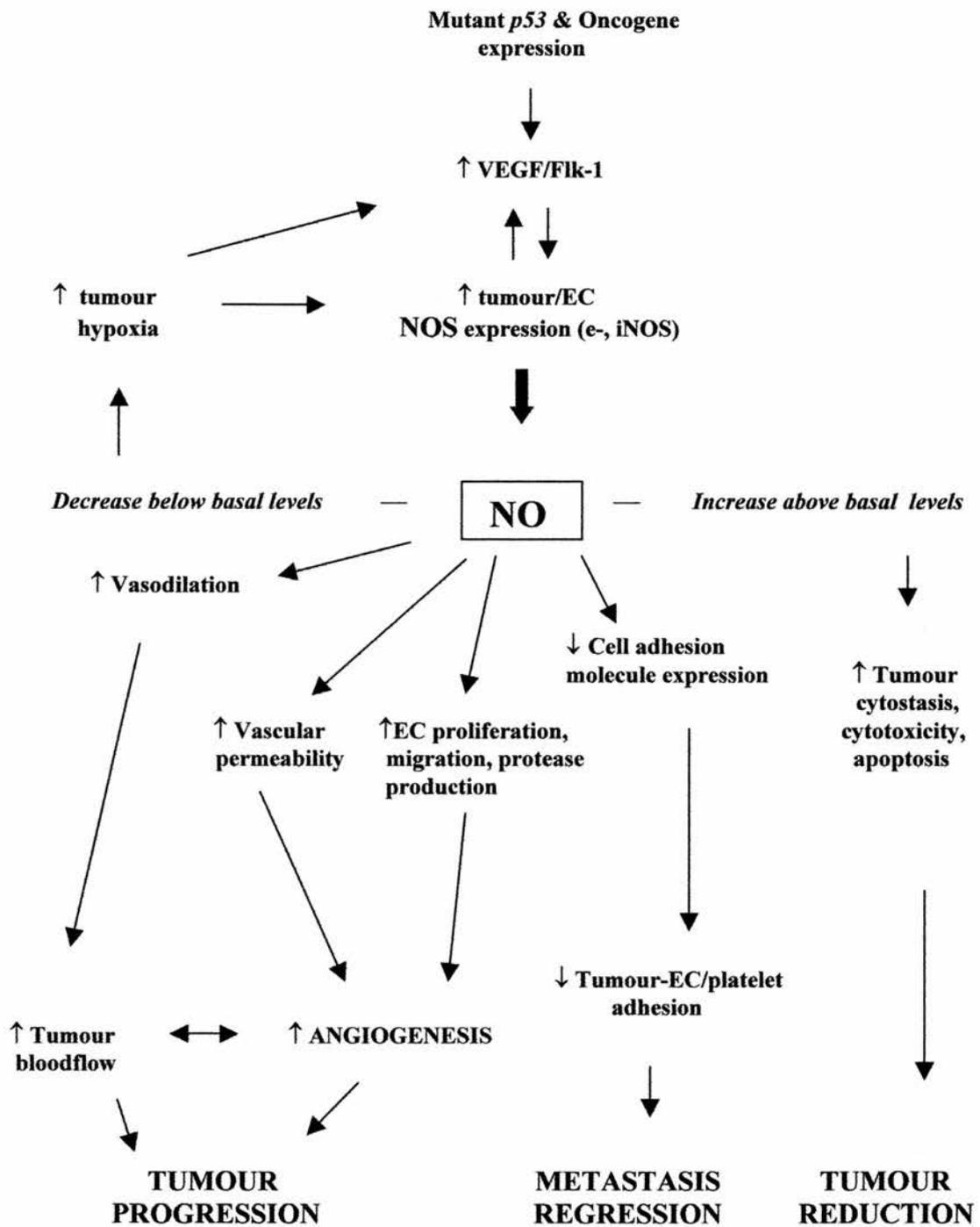


Fig. 1.8 Schematic of the role of NO in tumour microcirculation

PART C

THE VASCULAR ENDOTHELIUM

1.13 The Vascular Endothelial Cell

1.13.1 Introduction

Vascular endothelial cells (VECs) are a diverse population of flattened polygonal squamous epithelial cells which form a continuous monolayer over the luminal surface of the vasculature. They are bounded abuminally by a basement membrane (BM) (basal lamina) which, in arteries contacts the elastic, muscular and connective tissue tunics of the vessel wall. Within the microvasculature, ECs are closely associated with pericytes (see section 1.14). Inter-endothelial junctions vary within different parts of the vasculature. The majority of blood vessels consist of continuous endothelium, in which the ECs form an uninterrupted lining. Endothelium in visceral capillaries however, is fenestrated, containing numerous large pores, and in certain organs such as the bone marrow, liver and spleen, the endothelium is discontinuous. ECs grow in an obligate monolayer both *in vivo* and *in vitro* and thus manifest characteristic contact inhibition. *In vivo* they demonstrate polarity, with each cell exhibiting a 'sidedness' with regard to different functions. This is not obvious with cells in culture. Both luminal and abluminal surfaces have a carbohydrate-rich molecular coat, the glycocalyx (Fan & Dale, 1994). This is comprised of plasma proteins and glycosaminoglycans (GAGs), oligosaccharide moieties of cell membrane glycoproteins and glycolipids as well as sialoconjugates (Mitchell, 1983). The glycocalyx is involved in cell adhesion, stabilisation of receptors, regulation of extravasation and the detection of FSS.

The vascular endothelium is strategically-located at the blood-tissue interface and is an extremely active, multi-functional tissue, a full discussion of which is beyond the scope of this report (for a review see: Fan & Dale, 1994). It expresses a wide variety of receptors and cell adhesion molecules and it has an extensive repertoire of interactions with other cells (including the recruitment of immune cells) and humoral

factors. By its elaboration of a diverse array of paracrine substances, it exerts major control over blood vessel function, including the previously discussed maintenance of vascular tone and haemostasis.

1.13.2 Endothelial Heterogeneity

The microcirculation (arterioles, capillaries, venules) is the part of the vascular system concerned with blood-tissue exchange. The vast majority of ECs in the body are in the microvasculature, most being in the capillaries. Unlike large vessels which occur as isolated anatomical entities, the microvasculature appears as an integral part of the tissue it supplies.

Since the techniques for long-term culture of microvascular ECs have only recently been firmly established, most *in vitro* investigations have been carried out on ECs derived from the macrovascular portion of the circulation (arteries, veins). However, it is now apparent that extensive morphological and physiological heterogeneity exists between macro- and microvascular endothelia (Beekhuizen & van Furth, 1994; Geiger *et al.*, 1997; Kumar *et al.*, 1987; Schnitzer *et al.*, 1994; Swerlick *et al.*, 1992) and also between microvascular endothelium from different tissue locations (Belloni & Nicolson, 1988; Belloni & Tressler, 1990; Dodge *et al.*, 1989). This diversity results from both the innate characteristics of different EC populations and also from their biological adaptation to local conditions within different areas of the vascular tree. Distinctions include the expression of cell surface proteins which affect EC interactions with leukocytes and tumour cells (Belloni & Tressler, 1990), migratory activity (Beekhuizen & van Furth, 1994; Klagsbrun *et al.*, 1976; Zetter, 1980) and the potential for NO production (Geiger *et al.*, 1997), an important consideration for intertumour EC studies.

When studying the role of the endothelium in a given pathology it is therefore imperative to work with an appropriate endothelial isolate.

1.13.2.1 The Microvasculature

In rapidly-growing, transplantable experimental tumours the blood vessels are of a capillary structure (Huseby *et al.*, 1975). Therefore in order to mimic an intertumour

capillary endothelium most closely, the human dermal microvascular endothelial cell (HDMEC) was used for all static *in vitro* research presented here.

The culture of dermal microvascular ECs was first attempted in the rabbit by collecting cells from the central ear artery (Davison *et al.*, 1980). Later ECs from the microvessels of newborn foreskin (Davison *et al.*, 1980b) and adult human dermis were successfully cultivated (Davison *et al.*, 1983).

1.13.2.2 The Macrovasculature

In vivo, it is large-vessel ECs which are primarily exposed to mechanical forces (Mitchell, 1983). Therefore, human coronary artery endothelial cells (HCAEC) were utilised for *in vitro* fluid shearing experiments. However, in order to extrapolate FSS-induced effects to the tumour microenvironment, experiments were also performed using HDMEC.

1.14 The Pericyte

1.14.1 Introduction

Mature capillaries and venules are comprised of two cell types – VECs and pericytes. Pericytes are the perivascular supporting cells of the microvasculature and were first described in 1873 by Rouget as ‘vascular companion cells’. *In vivo*, pericytes are located within the BM of capillaries and venules, having an intimate structural relationship with the underlying ECs. The two cell types communicate through both gap junctions and the release of paracrine factors (Hirschi & D’Amore, 1997). Pericytes are stellate cells with distinctive bulging cell bodies, cytoplasmic branching and rough endoplasmic reticulum (Tilton *et al.*, 1979). Their cellular processes form an incomplete layer which envelops the abluminal surface of the endothelium – *peri* ‘around’, *cyte* ‘cell’.

Considerable variation in pericyte morphology, frequency and distribution occurs across different regions of the microvasculature (Frank *et al.*, 1987; Rhodin, 1968; Zimmerman, 1923) possibly suggesting distinct functions in different tissues. In the

retinal microvascular bed, for example, there is a relatively high number of pericytes per unit length of microvessel (Tilton *et al.*, 1985) with a pericytes:EC ratio of 1, whereas ECs outnumber pericytes by 10 to 1 in other microvascular beds.

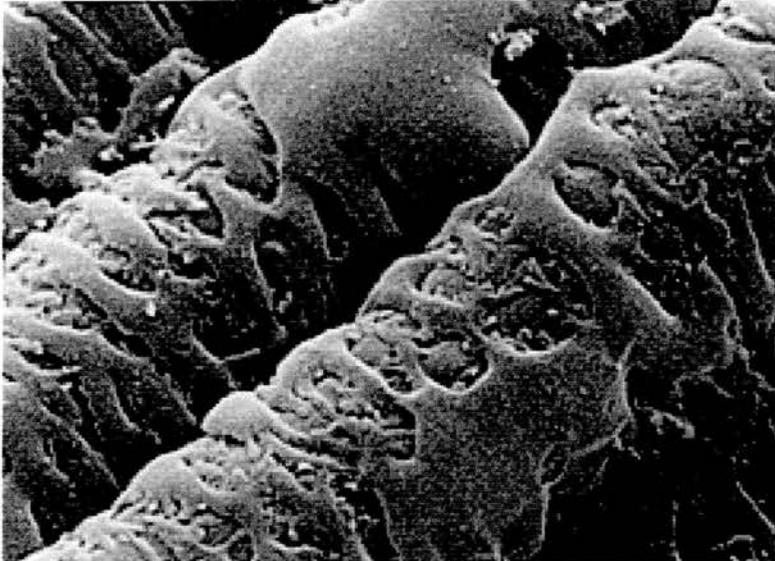


Plate 1.3 *EM of Pericytes investing Capillaries*
(From Mammalian Histology, Dept. of Biological Sciences, University of Delaware – <http://www.co.udel.edu/biology/wags/histopage/empage/ebv/ebv/htm>).

1.14.2 Pericyte Function

Several important roles have been postulated for the pericyte in the microvasculature (for a review see: Chakravarthy & Gardiner, 1999). Their major role is a contractile function for the VSM-devoid capillary wall (Clark & Clark, 1925) although they also provide structural support and regulate capillary permeability (De Oliveira, 1966). Pericytes also appear to play a role in angiogenesis (Nehls *et al.*, 1992; Schultze & Kerr, 1981). It has been suggested that they may have phagocytic ability (Andrews *et al.*, 1971; Schlondorff, 1987).

1.14.2.1 Contractile Function

The evidence for pericytes possessing a contractile function is strong. Their abundant cell processes extend both longitudinally and circumferentially around the vessel wall (Tilton *et al.*, 1985) (see plate 1.3) and they contain a cytoskeleton with bundles of

actin (Herman & D'Amore, 1985) and myosin (Joyce *et al.*, 1985). *In vitro* experiments have shown that pericytes are capable of acting like contractile cells (Kelley *et al.*, 1987). By exerting tension on the substratum on which they were grown, they cause it to wrinkle and contract.

Pericyte contraction and relaxation is likely to cause major alterations in capillary lumen and hence alter local blood flow. Pericytes express guanylate cyclase and have been shown to relax in response to NO donors (Haefliger *et al.*, 1994, 1997; Kelley *et al.*, 1987). Coupled with a documented expression of NOS by retinal capillaries (Chakravarthy *et al.*, 1995) there is therefore potential for the operation of the L-arginine: NO pathway in an equivalent way to VSM in the macrovasculature.

1.14.2.2 Pericytes and Angiogenesis

Of particular interest to the present study is the role of pericytes in regulating EC proliferation and differentiation, processes fundamental to angiogenesis (for review see: Hirschi & D'Amore, 1997). The function(s) of pericytes in angiogenesis are contentious and a summary of experimental observations to date is provided in Table 1.10 overleaf.

Table 1.10 Summary of the Involvement of Pericytes in Angiogenesis.

OBSERVATION	REFERENCE
<i>In vivo</i>	
In the initial phases of capillary sprouting during wound healing, pericytes are largely absent from the growing capillaries. At the later stages, when capillary growth ceases, pericytes become visible within the capillary basal lamina.	Crocker <i>et al.</i> , 1970.
In retinopathy, new vessels grow from capillaries without pericytes or where pericyte degeneration has occurred. There is a decline in EC proliferation and capillaries stop growing when re-invested with pericytes.	De Oliveira, 1966.
Capillaries with the highest pericyte coverage have the lowest EC turnover rate.	Frank <i>et al.</i> , 1987.
The incorporation of pericytes into the basal lamina of newly-formed capillaries may be responsible for inhibiting EC proliferation. Pericytes may perform a dual role, (i) preventing EC proliferation at the proximal end of the sprout, and (ii) providing a scaffold for migration of EC at the distal end of the sprout.	Rhodin & Fujita, 1989.
Reduction in contacts between ECs and pericytes, and a withdrawal of pericyte processes correlates with an increase in capillary growth in rat cardiac and skeletal muscle.	Eggington <i>et al.</i> , 1996.
During angiogenesis induced by artificial implantation of bone marrow, the number of contact surfaces between pericytes and EC is noticeably lower than in normal control blood vessels.	Diaz-Flores <i>et al.</i> , 1992.
In comparison between normal and peri-tumoural human brain microcapillaries, peri-tumoural microcapillary pericytes demonstrate reduced contacts with ECs.	Bertossi <i>et al.</i> , 1997.
Background retinopathy is associated with a loss of retinal microvascular pericytes.	Speiser <i>et al.</i> , 1968.
The authors suggest that a build up of aldose reductase in diabetic retinal capillaries causes pericyte degeneration and consequently proliferation of ECs.	Robinson <i>et al.</i> , 1989.
<i>In vitro</i>	
Capillary microvessel fragments, with and without pericytes were grown in culture. Those without pericytes sprouted ECs which migrated, divided and produced a confluent monolayer. Segments with pericytes did not form an EC monolayer.	Frank <i>et al.</i> , 1981.
Authors developed a novel co-culture system in which ECs and pericytes could be grown together with and without direct contact. Pericytes inhibited the growth of ECs in a contact dependent manner.	Antonelli-Orlidge & D'Amore, 1987.
Retinal pericytes inhibit the growth of EC when grown together in co-culture.	Yamagashi <i>et al.</i> , 1993.

The exact nature of pericyte incorporation into newly-formed capillaries is not well understood. According to the most popular view, pericytes become involved in microvessel growth after the EC loops have been formed (Ausprunk & Folkman, 1977; Paku & Pawleta, 1991). They therefore contribute to the maturation of pre-existing capillaries (maintaining the endothelium in a quiescent, non-proliferating state) rather than to the sprouting process itself. Accordingly, young capillary sprouts are largely devoid of pericytes (Rhodin & Fujita, 1989). However, it has also been suggested that pericytes participate in angiogenesis from earlier stages. During initial phases of angiogenesis, pericytes have been seen to undergo sudden, brief and intense activity (Diaz-Flores *et al.*, 1992). They undergo hypertrophy with shortened processes and there is a decrease in contact with the endothelium, and an increase in DNA synthesis in both themselves and ECs (Diaz-Flores *et al.*, 1994). Pericytes have also been observed at the advancing tips of EC sprouts acting as a scaffold along which ECs migrate during sprouting (Diaz-Flores *et al.*, 1992; Nehls *et al.*, 1992). Other authors similarly suggest an early involvement of pericytes in capillary sprouting (Crocker *et al.*, 1970; Nehls *et al.*, 1992) although they do not agree on the exact time of occurrence.

Clearly the function of pericyte involvement in angiogenesis remains relatively unclear, with conflicting reports on the degree and timing of pericyte investment. What is clear however, is that pericytes do exert control over EC growth (Crocker *et al.*, 1970; Antonelli-Orlidge & D'Amore., 1987) and their absence can be correlated with the onset of EC proliferation. Their inhibitory effect has been attributed to activation of the latent form of TGF β (Kanzaki *et al.*, 1997; Antonelli-Orlidge & D'Amore, 1987 and 1989b; Roberts & Sporn, 1989; Sato & Rifkin, 1989). TGF β in a biologically-active form, is a potent inhibitor of EC growth (Antonelli-Orlidge *et al.*, 1989b).

1.14.3 Pericytes in the Tumour Vasculature

From the limited amount of information available, it appears that pericyte distribution, morphology and/or contact with ECs in tumour vasculature is frequently altered from that of normal vessels. Accordingly, the roles played by pericytes in the

normal microvasculature – regulation of EC proliferation, vascular permeability, vessel diameter and tone – are the very control mechanisms which are compromised in the tumour vascular system (Vaupel, 1989). In tumour capillaries an absence / significant reduction in pericyte investment has been observed (Bertossi *et al.*, 1997; Bicknell & Harris, 1992; Suzuki & Yoshida, 1998). Irregular pericytes (Suzuki & Yoshida, 1998) and abnormalities in the contact between ECs and pericytes (Caruso *et al.*, 1996) have also been detected. Schlingemann *et al.* (1990) noted both that staining for pericytes in tumour microcirculation was variable and also that, in opposition to most findings, pericytes were more numerous in vessels undergoing angiogenesis than in quiescent vessels.

1.14.4 Pericyte-Conditioned Cell Culture Medium

Pericytes may be cultured *in vitro* and their growth medium harvested and applied to other cells. The effect of such ‘pericyte-conditioned medium’ (PCM) on the growth of ECs has been examined by a number of investigators. In contradiction to their generally accepted, contact-dependent growth inhibitory effect (see table 1.10), pericytes have been shown to release an angiogenic factor into their culture medium (Murata *et al.*, 1994). Accordingly, PCM stimulates EC growth and angiogenesis *in vitro* (Sato *et al.*, 1987; Watanabe *et al.*, 1997b; Wong *et al.*, 1997). The PCM-increase in EC proliferation demonstrated by Watanabe *et al.* (1997b) was significantly inhibited by a neutralising antibody to bFGF, suggesting that the angiogenic molecule within PCM is a bFGF-like molecule. It appears that pericytes function in both growth-promoting and inhibiting capacities for ECs, and these differential actions may depend upon paracrine versus cell contact mechanisms, respectively (Antonelli-Orlidge & D’Amore, 1987).

1.15 Fluid Shear Stress (FSS)

1.15.1 Introduction

Endothelial cells are subjected to two mechanical stresses from the flow of blood in the systemic circulation, (1) shear stress (parallel to the vessel wall) generated by streaming of blood, and (2) blood pressure (wall distension) (perpendicular to the wall) resulting from blood pulsations caused by the heart pumping. Within this account consideration is given to the first parameter, fluid shear stress (FSS).

1.15.2 Flow

1.15.2.1 Characteristics of Flow

Blood flow within the vasculature can be considered *laminar* or *turbulent* (oscillatory). Laminar flow is defined as flow through a container with unvarying internal geometry, where uniform velocity gradients develop such that fluid velocity is zero at the stationary vessel wall and rises with increasing distance from it. The flow can be viewed as a series of molecular layers (laminae) slipping past each other with increasing velocity as the centre of the container is approached (Ballermann *et al.*, 1998).

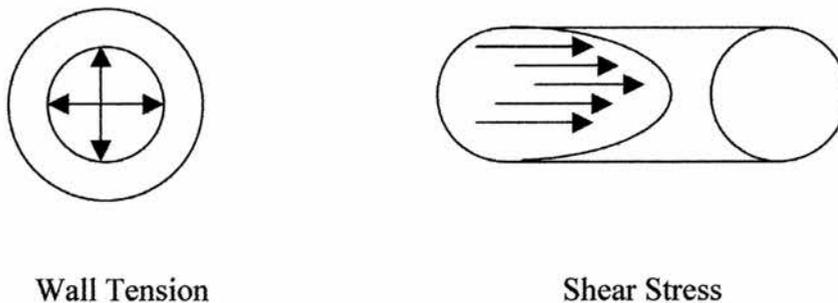


Fig.1.9 Mechanical stresses of blood flow

In a uniform, rigid cylinder, shear stress (τ) at the vessel wall can be derived from Poiseuille's law as: $\tau = 4Q \times \eta / \Pi \times r^3$ where Q is the fluid flow rate, and η is the fluid viscosity. If mean flow rate is constant, then the greater the resistance to flow (either because of high fluid viscosity or small vessel diameter), the greater the shear stress.

Biological systems depart from defined conditions however, as blood is non-Newtonian i.e. its viscosity tends to decrease with increasing velocity, and vessels are non-uniform, variably-distensible containers (Milnor, 1974). At very low blood velocities, aggregation of cellular elements tends to raise blood viscosity markedly, while its viscosity at high velocities is only about fourfold greater than that of water (Chabanel & Chien, 1990). The determination of FSS within the experimental system used here is considered in more depth in chapter 5.

Fluid shear stress can be either *acute* or *chronic*. Acute FSS refers to conditions in which ECs not previously exposed to FSS are suddenly exposed to this force with a time frame measured in seconds to hours (Davies *et al.*, 1992). Chronic FSS refers to conditions in which ECs are cultured for several days to weeks under the influence of shear stress, with or without superimposed acute alterations in the level of shear stress.

Although blood flow tends to be laminar in vessels $> 0.5\text{mm}$ in diameter, it departs somewhat from this behaviour in very small vessels. Oscillatory (turbulent) flow is characterised by a low mean component and a considerable amount of flow in the reverse direction. Manifestations of dysfunctional endothelium can be readily observed in areas of the vascular tree, such as branch points, which experience low mean shear stress and flow reversal (Ku *et al.*, 1995).

1.15.2.2 Flow and Endothelial Cell Morphology

High unidirectional (but not oscillatory) shear stress (either *in situ* or in culture) induces a characteristic elongation and alignment of ECs in the direction of flow (Dewey *et al.*, 1981; Flaherty *et al.*, 1972). This is accompanied by a flow-induced rearrangement of the actin cytoskeleton into bundles of stress fibres (Franke *et al.*, 1984). It serves to streamline the cell, decreasing resistance and lowering shear stress (Barbee *et al.*, 1995).

1.15.2.3 Physiological Fluid Shear Stress

Physiological levels of FSS vary widely across different areas of the vasculature and estimates of wall shear stress at various locations in the circulation have been made (Jones *et al.*, 1997). Mean shear stress is lowest in the large veins where it is often $< 1 \text{ dyn.cm}^{-2}$. It tends to be highest in small arterioles, where it can reach 60-80 dyn.cm^{-2} . 20-40 dyn.cm^{-2} represents an approximation of arterial FSS in regions of uniform geometry (Dewey, 1979) but vessel curvature and configuration can dramatically affect wall shear with levels ranging from $< 1 \text{ dyn.cm}^{-2}$ to $> 600 \text{ dyn.cm}^{-2}$ within the same vessel (Zarins *et al.*, 1983).

1.15.2.4 Mechanotransduction

The transduction of mechanical FSS into chemical signals is not fully understood (for a review see: Davies, 1995). Of the major mechanisms, shear stress-induced cytoskeletal disturbances and/or rearrangement appear to play an essential role (Davies & Barbee, 1994). The EC cytoskeleton - microtubules, intermediate filaments and actin fibres - transverses the cell, ending at the surface in characteristic adhesion complexes. The cytoskeleton is maintained under tension even in the absence of cell stimulation. Tensegrity permits mechanical forces (such as FSS) to be directly transmitted from the cell surface, across the physically interconnecting filaments to the nucleus (Ingber *et al.*, 1997).

Mechanosensitive ion channels (e.g. stretch-activated Ca^{2+} channel) also play an important role in sensing FSS (Davies, 1995). Shear stress induces a biphasic increase in EC $[\text{Ca}^{2+}]_i$, characterised by a rapid initial peak followed by a steady plateau phase (Falcone *et al.*, 1993; Shen *et al.*, 1992). The initial transient component reflects IP_3 -mediated Ca^{2+} release from intracellular stores, whereas the second phase is characterised by a more prolonged Ca^{2+} influx achieved through opening of $\text{K}_{\text{Ca}^{2+}}$ channels, K^+ efflux and membrane hyperpolarisation (Luckhoff & Busse, 1990).

The EC glycocalyx (Davies & Barbee, 1994), plasmalemma fluidity (Knudsen & Frangos, 1997), receptor tyrosine kinases and integrins (Chen *et al.*, 1999) have also been implicated as potential mechanosensing candidates.

A common promoter element has been identified within a number of flow-responsive genes, the shear stress response element (SSRE). This interacts with shear stress-induced transcription factors (Resnick *et al.*, 1993) and appears to represent an important mechanism of FSS-induced alterations in gene expression.

1.15.3 Flow in the Tumour Vasculature

The abnormal architecture of tumour vasculature (as reviewed in section 1.7.2) will be expected to generate a number of distinct flow properties and consequently FSS characteristics within a tumour. Although highly complex, a number of generalisations can be postulated:

- i) Flow and FSS within tumours will lack uniformity:
 - a) In normal tissue, blood velocities correlate strongly with branching order and diameter of the vessels (Popel, 1987). In contrast tumours show no such correlation (Leunig *et al.*, 1992; Yuan *et al.*, 1994).
 - b) Elevated interstitial fluid pressure within the centre of solid tumours contrasts with a zone of almost normal pressure around the periphery (Boucher & Jain, 1992). Vascular occlusion within the core will therefore be expected to produce relatively elevated levels of FSS in this region by comparison.
- ii) On average, flow and FSS within tumours will be lower than that of normal tissue:
 - a) Tumour capillary diameter is greater than that in most normal tissues (Less *et al.*, 1991). (Because vessel distension increases vessel diameter, its effect on shear can be significant as shear is inversely proportional to the third power of the vessel radius).
 - b) Tumour vasculature is malformed and tortuous.
- iii) As tumour cell proliferation continues, flow will be gradually reduced in the core of tumours due to 'solid' pressure. FSS will concomitantly increase with increasing tumour age.
- iv) As a result of non-uniform vessel branching patterns, loops, shunts, bi- and trifurcations present within tumours (Less *et al.*, 1991) flow will be relatively turbulent (oscillatory).

v) Heterogeneous bloodflow leads to stagnation and the consequent opening and closing of regions of the vasculature. When blood flow is reinitiated in a vessel after temporary occlusion, *acute* FSS will be generated.

1.15.4 Fluid Shear Stress and Tumour Growth

The inclusion of FSS studies within the research presented here is appropriate for a number of reasons:

- i) The disparate flow/FSS condition in tumours (see above).
- ii) FSS affects tumour-EC adhesion and therefore the potential for metastasis. Several investigators have found that the *in vitro* adherence of tumour cells to the endothelium fails under low physiological ($> 1.5 \text{ dyn.cm}^{-2}$) or even dramatically-reduced shear stresses (Giavazzi *et al.*, 1993; Kong *et al.*, 1996). However, a short interruption in perfusion (blood flow) increases tumour cell adherence (Kong *et al.*, 1996). Thus, circulating tumour cells which are normally prevented from establishing adhesive interactions by the flow of blood, may settle onto the EC surface during periods of no-flow within tumours.
- iii) The extent of blood flow correlates with angiogenesis by sprouting (see section 1.9.5.1.a).
- iv) Through its generation of NO, flow mediates vascular permeability (Yuan *et al.*, 1992).
- v) Fluid shear stress (up to 18 dyn.cm^{-2}) increases EC expression and secretion of t-PA, therefore having the potential to enhance fibrinolytic activity (Diamond *et al.*, 1989 and 1990) and hence angiogenesis (see section 1.9.5.1.b).
- vi) FSS can stimulate the proliferation and migration of ECs (Ando *et al.*, 1987; Masuda & Fujiwara, 1993) in the direction of flow.
- vii) Flow appears to model intussusceptive tissue pillars and FSS seems to influence their formation (Patan *et al.*, 1996b) (see section 1.9.5.2).

1.15.5 Proteins under study

1.15.5.1 Endothelial NOS

a) Expressional Regulation

Within ECs, eNOS has been assigned to several membrane locations, the Golgi apparatus (O'Brien *et al.*, 1995), plasma membrane (Hecker *et al.*, 1994) and caveolae (Shaul *et al.*, 1996), invaginations of the plasma membrane which function to bind and organise a variety of signalling molecules (Lisanti *et al.*, 1994). Its membrane anchoring results from the covalent modifications of myristoylation (attachment of the fatty acid myristate) (Sessa *et al.*, 1993) and palmitoylation (attachment of the fatty acid palmitate) (Robinson *et al.*, 1995).

Steady laminar shear stress elevates eNOS mRNA in both a time and dose-dependent manner and the induction is dynamic, reversing after the cessation of flow (Malek *et al.*, 1999). Circumferential stretch also induces eNOS expression, but compared with shear is considerably less important as a stimulus (Ziegler *et al.*, 1998). The ability of shear stress to enhance eNOS activity may be the consequence of an increase in $[Ca^{2+}]_i$, although a direct effect on membrane-bound eNOS itself may also occur. The close association between caveolae and the cytoskeleton may render eNOS more susceptible to activation by physical stimuli (Hecker *et al.*, 1994).

A comparison of steady laminar, pulsatile laminar and turbulent shear stresses in the range of 2-16 dyn.cm^{-2} has revealed that steady and pulsatile laminar flow up-regulate eNOS mRNA and increase NO release, whereas turbulent flow does neither (Noris *et al.*, 1995). Similarly, oscillatory shear stress has been shown to induce a lower expression of eNOS mRNA compared with unidirectional shear (Ziegler *et al.*, 1998). A purely oscillatory flow can fail to increase $[Ca^{2+}]_i$ (Helmlinger *et al.*, 1995) which may explain its failure in inducing eNOS activation.

Varying time and force-dependent effects have been reported to induce eNOS expression *in vitro*. Very low shear stress (0.08dyn.cm^{-2}) in bovine aortic ECs (BAE) fails to generate increased mRNA expression, although a 6-fold induction occurs when force is increased to 0.3dyn.cm^{-2} (Ziegler *et al.*, 1998). Similarly, high FSS

(15dyn.cm⁻²) in BAEs and human aortic ECs (HAEC) stimulates a 2-3 fold increase in eNOS mRNA (Uematsu *et al.*, 1995). Of interest, high FSS (25dyn.cm⁻²) in BAE and human umbilical vein ECs (HUVEC) concomitantly enhance eNOS protein levels, although lower FSS (4 dyn.cm⁻²) does not (Ranjan *et al.*, 1995).

In saphenous vein ECs, high FSS (30dyn.cm⁻²) and zero FSS (static control) have been reported to induce much lower levels of eNOS expression than low (2 dyn.cm⁻²) FSS. Intermediate FSS (6dyn.cm⁻²) stimulates markedly increased expression (Zhu *et al.*, 1997). It appears that the particular EC type studied may affect eNOS induction by shear.

Reported shearing time required to induce eNOS expression similarly varies between studies. Most investigators conclude that longer shearing times (>24 hr) are required for significant eNOS induction (Ziegler *et al.*, 1998) although others have reported mRNA and protein expression as early as 3 hours after exposure (Ranjan *et al.*, 1995; Uematsu *et al.*, 1995).

Following activation, eNOS is dissociated from the membrane fraction (as a consequence of depalmitoylation) and translocates to the cytosol. It is phosphorylated in response to FSS (Garcia-Cardena *et al.*, 1996). Regulation of the enzymatic activity/cellular location is clearly under several control mechanisms including Ca²⁺, phosphorylation and post-translational modifications of myristoylation, palmitoylation. (For a review of eNOS activation/signal transduction see: Fleming & Busse, 1999).

The eNOS gene promoter contains several SSREs (Lowenstein *et al.*, 1992; Marsden *et al.*, 1992), and binding sequences for the transcription factors AP-1 and NFκB (Zhang *et al.*, 1995).

b) NO Release

Shear stress is the principle regulator of NO release within the vasculature (Lamontagne *et al.*, 1992; Rubanyi *et al.*, 1986). In cell culture studies of the acute EC response to the onset of flow, there is a dramatic rise in NO release which increases by several orders of magnitude over the range <1 dyn.cm⁻² to 10dyn.cm⁻²

(Taylor *et al.*, 1991). This is in keeping with the generally observed enhancement of eNOS activation with increasing shear force. In line with the shear-induced elevation of $[Ca^{2+}]_i$, a biphasic increase in NO release occurs in response to FSS, an initial peak followed by a plateau. These phases are differentially-sensitive to extracellular Ca^{2+} in that the initial peak is abolished by the removal of Ca^{2+} while the sustained phase remains. It has been suggested both that the initial Ca^{2+} -dependent phase is an artefact of *in vitro* conditions rather than a real physiological response (Ayajika *et al.*, 1996) or that it results from phosphorylational activation of eNOS (Corson *et al.*, 1996).

1.15.5.2 PECAM-1 / CD31

Although utilised solely as a vascular marker for the *in vivo* angiogenesis studies undertaken in this research, platelet endothelial cell adhesion molecule 1 (PECAM-1) / CD31 activity was examined under conditions of FSS as it functions as an important protein in angiogenesis in its own right (for a review see: Newman, 1997). PECAM-1/CD31 is a 130KDa member of the transmembrane immunoglobulin (IgG) supergene family of cell adhesion molecules (Newman *et al.*, 1990) and occurs in six, alternatively-spliced isoforms (Kirschbaum *et al.*, 1994). It is constitutively-expressed in large amounts on both capillary and large vessel ECs and it mediates both homophilic and heterophilic cell-cell interactions (Albelda *et al.*, 1990) and also serves a signalling function (DeLisser *et al.*, 1994). In ECs during angiogenesis (Berger *et al.*, 1993) and *in vitro* seeding (Ayalon *et al.*, 1994), CD31 becomes redistributed from a circumferential cell membrane pattern to expression confined to intercellular junctions (Shen *et al.*, 1995; Wong & Dorovini, 1996). In addition to ECs, CD31 is expressed on platelets, monocytes, neutrophils and a subset of lymphocytes (DeLisser *et al.*, 1994). It appears to have a number of *in vivo* physiological roles. It is crucial for the transmigration of neutrophils through the vessel wall in inflammation (Muller *et al.*, 1993) and it may be involved in tumour metastasis (Tang & Honn, 1994). Increasing evidence implicates PECAM-1 in angiogenesis (Berger *et al.*, 1993; DeLisser *et al.*, 1997). It is critical for blood vessel formation (Baldwin *et al.*, 1994; DeLisser *et al.*, 1994) and EC tubulogenesis *in vitro* involves a 3- to 4-fold increase in PECAM-1 gene expression (Llan *et al.*, 1998).

Accordingly anti-PECAM-1 antibodies inhibit both *in vitro* tubulogenesis (DeLisser *et al.*, 1997; Matsumura *et al.*, 1997; Sheibani *et al.*, 1997) and angiogenesis *in vivo* (DeLisser *et al.*, 1997). CD31 has also been implicated in migration (Kim *et al.*, 1998; Schimmenti *et al.*, 1992) and permeability (Ferrero *et al.*, 1995). The angiogenic integrin $\alpha v\beta 3$ is a ligand for CD31 (Buckley *et al.*, 1996). The angiogenesis inhibitor thrombospondin completely suppresses CD31 expression (Sheibani *et al.*, 1997).

Interendothelial junctions contribute to the adhesiveness of the endothelium, helping it to resist mechanical forces. Of the three types of intercellular adhesion molecules (PECAM-1, cadherins, integrins), PECAM-1 is sufficient for maintaining monolayer cohesiveness under resting conditions, but not under flow, when cadherins are essential for sustaining integrity (Schnittler *et al.*, 1997). Unlike cadherins, PECAM-1 expression is independent of calcium (Schnittler *et al.*, 1997). It is tyrosine phosphorylated in response to FSS (Fujiwara *et al.*, 1998; Osawa *et al.*, 1997) and may be involved in mechanosensing/signal transduction. The PECAM-1 gene contains both a SSRE and an NF κ B binding site (Almendro *et al.*, 1996).

1.15.5.3 KDR/Flk-1

Little information is available on KDR/Flk-1 and shear stress. A striking expression of VEGF binding sites occurs along the EC lining of the heart valves, the aorta and the pulmonary artery of rats (Jakeman *et al.*, 1992), structures constantly subjected to blood under high flow and therefore high FSS. A force of 12dyn/cm^2 has recently been shown to induce a rapid and transient tyrosine phosphorylation of Flk-1 in BAECs (Chen *et al.*, 1999). Membrane perturbation appears to activate Flk-1 by inducing receptor clustering on the luminal surface. Tyrosine phosphorylation and receptor clustering was observed after 1 minute of FSS, reached a peak level at 5 minutes and returned to basal level at 30 minutes.

PART D

AIMS OF THE PRESENT STUDY

The investigations undertaken in the present study were founded on two major issues, (i) the observation that vasodilation of a tumour-supplying blood vessel represents the first stage of tumour angiogenesis (Folkman & Shing, 1992; Grunt *et al.*, 1986) (section 1.9.4.1.a) and (ii), the evident, yet ambiguous involvement of NO in the angiogenic process (section 1.12).

Previous work from this laboratory (Bisland, 1996; Kennovin *et al.*, 1994a) has demonstrated the efficacy of NO synthase inhibitors at retarding the growth of experimental solid tumours, possibly through anti-vascular mechanisms. The research undertaken here has expanded on these findings, investigating further the involvement of NO in tumour growth and determining its potential role in the angiogenic response. An *in vivo* animal model was used to examine the impact of two distinct NO-reducing strategies – the inhibition of NO synthesis (Chapter 2) and the ‘scavenging’ of NO (Chapter 3).

Research was also extended to endothelial cells in culture where the involvement of angiogenically-relevant proteins (NOS, PECAM-1/CD31 and the KDR/Flk-1 receptor) in two aspects of angiogenesis was considered. Based on the ‘paracrine’ regulation of tumour angiogenesis (section 1.9.2) and the involvement of pericytes in the process (section 1.14.2.2), the association of these proteins with the pro-angiogenic activity of both tumour cells and pericytes was investigated (Chapter 4). Finally, based on the potential of blood flow/fluid shear stress to modulate tumour angiogenesis (section 1.15.4), the impact of ‘fluid shearing’ on protein expression was examined (Chapter 5).

Chapter Two

NOS Inhibitors and the Solid Tumour

2.1 Introduction

Previous work from this laboratory has demonstrated that chronic oral administration of NOS inhibitors retards the growth of experimental solid tumours. It has been suggested that this may result from selective occlusion of the main supply vessels, creating a reduction in tumour perfusion (Bisland, 1996; Kennovin, 1994a). The research presented here examines whether, in light of the documented role of NO in angiogenesis, the growth-retarding mechanism of systemic NOS inhibition also involves an impairment of the angiogenic response of a tumour.

To this end, experiments were performed to determine the effect of systemic NOS inhibition on:

1. Animal general well-being, by direct observation (survival, body weight, drinking rate). This provided a gauge of possible drug toxicity.
2. Haemodynamic parameters (systolic, mean and diastolic blood pressure). This provided a gauge of drug selectivity for different NOS isoforms.
3. Histology (including necrosis) of primary experimental solid tumours.
4. The localisation of eNOS, iNOS and KDR/Flk-1 within tumours, by immunohistochemistry.
5. The expression of e- and iNOS isoforms within tumours, by biochemical techniques.
6. The growth rate of tumours.
7. The angiogenic response of tumours, to determine a possible involvement of NO in the process of new vessel formation.
8. Metastatic tumour formation at the two most common secondary sites (lung, liver).

A variety of NOS inhibitors with different isoform selectivity (non-specific, iNOS-specific, highly iNOS-specific) were utilised. The impact of both time of inhibitor administration (pre- and post-tumour implantation) and a period of drug-withdrawal were also examined.

2.2 Materials and Methods

2.2.1 Methods

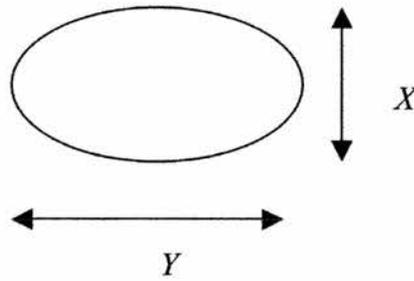
2.2.1.1 Animal tumour model

Isogenic male rats (BD-IX strain; 300-450g) were used and were age-matched to minimise possible age-related differences in both the angiogenic response (Pili, 1994; Rivard *et al.*, 1999) and vascular eNOS expression (Cernadas *et al.*, 1998).

A mixed carcinosarcoma (p22) was selected as tumour line. This was originally radiation induced and displayed aggressive, rapidly-growing characteristics. It was used for all experiments at 4th generation, close to its original phenotype to minimise genetic drift. The expansion of 3rd generation tumour, maintained as a frozen suspension, was performed by passaging in a female BD-IX. Freshly-passaged tumour was used for all experiments.

Tumour load was not permitted to exceed 10% body weight, in line with UKCCR guidelines and UK Home Office (Project licence) requirements (1997). Initial experiments were performed with multiple tumour nodules on the same animal (4 subcutaneous sites in different quadrants of the dorsal surface). However, to produce measurable (>5mm) tumours without exceeding maximum tumour load, later studies involved two tumours grown towards the front and rear of the dorsal surface. Small pieces of tissue (~1mm³) were taken from the cortical region of a freshly-passaged tumour and implanted *s/c* under anaesthesia (90 mg.Kg⁻¹ *Ketamine*; 10mg.Kg⁻¹ *Xylazine*) using a 16G trochar. This method was found to produce more symmetrical tumours than did an injection of a volume of tumour cell suspension (for protocol see appendix 8.1). *Lidocaine* spray was used as analgesia on implantation wounds immediately following surgery and *Carprofen* (10mg.Kg⁻¹ *per os* in raspberry jam) or *Buprenorphine* (0.5mg.Kg⁻¹ *s/c*) were administered to control post-operative pain for 24-48 hours following surgery.

The long (*Y*) and short (*X*) axes of each tumour were measured during growth using hand-held skin-fold calipers. Tumour volume was calculated using the hemiellipsoid formula:



$$\text{where Tumour Volume} = (X^2 \times Y) \pi / 6$$

Mean tumour volumes (mm^3) were used to construct graphs of tumour growth against time.

2.2.1.2 NOS Inhibitors

The NOS inhibitors L-NAME, AG and 1400W (see section 1.4.5.1) were administered to animals systemically in the drinking water. Drugs were freshly-dissolved in water daily. Previous research from this laboratory (Kennovin 1994a) has demonstrated that L-NAME supplied systemically to rats at concentrations from $1\text{-}6 \text{ mg.ml}^{-1}$ is effective in retarding tumour growth (dose of $100\text{-}600 \text{ mg.Kg}^{-1}\text{day}$ for animals drinking 100ml water/Kg body weight per day; approximate ingested drug dose of $30\text{-}270\text{mg.day}^{-1}$ per rat for the weight range used). Growth retardation is not significantly different over this range of doses (Kennovin *et al.*, 1994a) and toxicity can become significant at high dosage ($500\text{mg.Kg}^{-1}\text{day}$) (Onier *et al.*, 1999).

Aminoguanidine administered systemically to rats at 1mg.ml^{-1} (approximate ingested drug dose of 30mg.day^{-1} per rat) has been shown to retard tumour growth significantly (Bisland, 1996). No rat oral administration dose data is available for 1400W. All NOS inhibitors were therefore dissolved to a concentration of 1 mg.ml^{-1} , providing an approximate daily ingested dose of $30\text{-}45\text{mg}$ drug per day for rats within the specified weight range:

$$\text{Dose (mg.Kg.day}^{-1}\text{)} = \frac{(\text{water drunk (ml)/day}) \times [\text{drug}]; (1\text{mg.ml}^{-1})}{\text{Weight of rat (Kg)}}$$

To disguise the taste of drugs, all animals also received 10 mg.ml⁻¹ sucrose in their drinking water for 7 days prior to, and for the duration of drug administration. Food (standard lab chow) and water were available to animals *ad libitum*.

The ambient temperature was thermostatically-maintained at 20°C. Water consumption could then be measured by daily weighing of water bottles and taking 1g weight to equal 1ml volume.

The efficacy of each of the NOS inhibiting regimes was to be tested by measuring [NO] within animal serum harvested after drug treatment. However, the integrity of serum samples was unfortunately compromised, rendering quantification unreliable.

2.2.1.3 Measuring Blood Pressure

As reviewed in section 1.4.5.1.d, a consequence of non-selective NOS inhibition in rats is a dose-dependent increase in mean arterial blood pressure (MABP). Haemodynamic parameters were therefore measured in unrestrained, unanaesthetised animals, using the non-invasive tail cuff method.

Animals were familiarised to the procedure by a 'training' regime (daily for 3 weeks prior to commencing an experiment). This avoided undue stress. Recordings of systolic (S.b.p.), mean (M.b.p.) and diastolic (D.b.p.) arterial blood pressure were made daily over the experimental period on a representative sample of animals. Before each reading, animals were placed in a warmed environment (26-29°C) for 15 minutes after which time they were cradled under one arm and comforted. It was necessary for animals to remain completely still during the procedure as the equipment was extremely sensitive to even the smallest extraneous movement. The cuff was carefully placed on the tail ~2-3cm from the body and inflated. Handling proved to be far less stressful to the animals than the perspex animal holders. Digital traces showed the tail pulses on one channel and corresponding cuff pressures on the other (see fig. 2.1). Analysis was carried out manually to avoid false readings which often occurred as the computer detected movements of the animal causing artefacts on the trace.

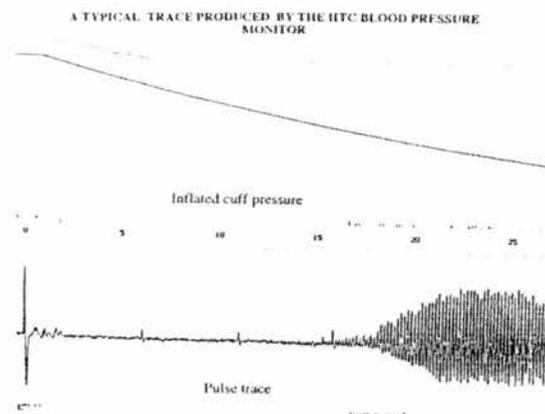


Fig. 2.1 *A trace of arterial pulse pressure obtained using the tail cuff method.*

After cuff inflation the pulse pressure returns when cuff pressure is equal to systolic b.p. (S.b.p; indicated by a tall tick mark above the cuff pressure trace baseline). The point at which the pulse amplitude is maximal represents the mean b.p. (M.b.p; small ticks above the cuff pressure trace baseline indicate updates throughout the reading cycle). Diastolic b.p. is calculated using the equation: $D.b.p. = \text{mean b.p.} - \frac{1}{2} (S.b.p. - M.b.p.)$

The heart rate was estimated from the pulse trace by measuring the time (each grid spacing = 1s) from the first of a series of sixteen small ticks to a final taller tick (i.e. seventeen ticks in all) as shown above the pulse pressure baseline (see fig. 2.1). This value was multiplied by 60 to estimate beats/min. Ticks appearing below either trace after this series of seventeen ticks indicated an interruption in the automatic reading cycle and the computer generated results were ignored.

2.2.1.4 *Tumour Harvesting*

At the time of sacrifice, mature tumours within their capsules were carefully dissected out of each animal and resected centrally into two halves. Half portions were immediately diced, placed into cryovials and snap frozen in liquid N₂ for the production of homogenates (see section 2.2.1.7). The corresponding (un-diced) portions were snap frozen in liquid nitrogen-cooled isopentane, for subsequent serial sectioning for immunohistochemistry (see section 2.2.1.6) and angiogenesis scoring (see section 2.2.1.8). One tumour per animal was dissected out and processed for histological examination (see section 2.2.1.5).

2.2.1.5 Tumour Histology

The gross structure of tumours was examined microscopically using two histological stains, haematoxylin and eosin (H+E) and Masson's trichrome. Freshly-dissected tumours were immediately placed into paraformaldehyde fixative, processed for paraffin wax-embedding, sectioned and stained (see appendix 8.2). The histology of control versus drug-treated tumours was compared in terms of tumour and stromal organisation and the extent of necrosis.

2.2.1.6 Immunohistochemistry

The localisation of eNOS, iNOS and KDR/Flk-1 within control and drug-treated tumours was examined using immunoperoxidase staining of snap-frozen, cryostat-sections (see appendix 8.3).

2.2.1.7 Biochemical Analysis

The expression of e- and iNOS proteins in control and drug-treated tumour extracts was quantified by 6% SDS-polyacrylamide gel electrophoresis and Western Blotting techniques (see appendix 8.4).

2.2.1.8 Angiogenesis Scoring - Microvessel density (MVD) determination

Quantification of angiogenesis was performed in snap-frozen tumour sections (see appendix 8.3) utilising an anti-PECAM-1/CD31 antibody and the Chalkley point counting method (see section 1.9.7.2).

Tumours for analysis were matched for rat quadrant of origin and size as closely as possible. A single tumour slice was taken to represent each tumour specimen. Three microvessel 'hot spots' were identified within each section by scanning at low magnification (X40 and X100). Chalkley counting was performed at high magnification (X250). Chalkley graticule field of view calibration was determined using a microscope stage graticule (πr^2 ; 0.126mm^2). As recommended by Weidner *et al.* (1991), any highlighted EC or cell cluster was regarded as a distinct vessel and a lumen was not required. The mean of the three Chalkley counts/tumour analysed was determined.

2.2.1.9 Secondary Tumour Spread – Lung and Liver metastasis

The metastasis of subcutaneous tumours to two of the most common secondary organ sites (lung, liver) was examined using histological stains (H+E; Masson's trichrome). A representative section of lung and liver was removed from each animal at the time of sacrifice. Sections were immediately placed into paraformaldehyde fixative and processed for subsequent paraffin wax-embedding, sectioning and histological staining (see appendix 8.2). The number of animals per drug group demonstrating one or more secondary foci was determined and expressed as a percentage of the total group.

2.2.2 Materials

2.2.2.1 Animals

Isogenic *BD-IX* rats and *p22* carcinosarcoma were obtained from the CRC Gray laboratory, Mount Vernon Hospital, Middlesex, UK.

2.2.2.2 Anaesthetics

Ketamine ('Ketaset') was purchased from Willows Francis Ltd. and *Xylazine* ('Rompun') from Bayer.

2.2.2.3 Analgesia

Lidocaine ('Xylocaine') was purchased as a spray from Astra Pharmaceuticals Ltd; *Carprofen* ('Zenecarp') was purchased from C-Vet and *Buprenorphine* ('Temgesic') was purchased from Schering.

2.2.2.4 Drugs

N^w-nitro-L-arginine methyl ester hydrochloride (L-NAME) and *Aminoguanidine hemisulfate* (AG) were purchased from Sigma Ltd ; *N-(3-(aminomethyl)benzyl)acetamide* (1400W) was synthesised by Mr Malcolm Stewart, Dept. of Chemistry, University of St Andrews.

2.2.2.5 Blood Pressure

Tail-cuff blood pressure analyser was the *Model 179 Blood Pressure Analyzer* from IITC Life Science, California, USA.

2.2.2.6 Immunological Reagents

2.2.2.6.a Primary antibodies - Primary antibodies were aliquoted and stored either frozen at -20°C or at 4°C depending on manufacturers recommendations. Optimal dilutions and incubation times for each antibody in rat paraffin/frozen sections or on nitrocellulose membrane in Western blotting were determined.

Monoclonal mouse anti-rat CD31 (PECAM-1) (Serotec). Monoclonal mouse anti-human KDR/Flk-1 (Santa Cruz Biotech). Monoclonal mouse anti-human eNOS (Transduction Labs). Monoclonal mouse anti-human iNOS (Santa Cruz Biotech). Cruz marker molecular weight standards (Santa Cruz Biotech) (23K – 132K) were used for gel electrophoresis.

2.2.2.6.b Secondary antibodies - For all immunohistochemical staining of paraffin/frozen sections, a rat pre-absorbed *labelled-streptavidin-biotin immunoperoxidase (LSAB-IP) kit (DAKO)* was employed. For Western blotting, a peroxidase-tagged *anti-mouse IgG (Santa Cruz Biotech)* secondary was used at dilutions from 1:500 – 1:2K.

2.2.2.7 MVD determination - A *Leitz Laborlux 12 microscope* was employed with a 'Chalkley point' *graticule (Graticules Ltd., Tonbridge, Kent)*.

2.3 Experimental Protocol

Two separate tumour growth studies were conducted to examine the effects of NOS inhibitor administration in rats. Taking day 0 to represent the day of tumour implantation, drug administration was commenced at two time points - appreciably before tumour implantation (day -7), to precede the angiogenic response, and during the early/mid tumour growth phase (day +10 /day +17).

Study One

Rats were separated into 4 groups of 3 animals/group. Group 1 were controls, fed water + sucrose without drugs. Groups 2, 3 and 4 received L-NAME, AG and 1400W respectively, from day +17 for 17 consecutive days, until day + 33. One rat in each group was sacrificed immediately. The remaining 2 rats underwent a period of 5 days of drug withdrawal, until day +38. This study served to compare the efficacy of different NOS inhibitors administered at the same time point. All results presented here derive from this study.

Study Two

Rats were separated into 5 groups of 5 animals/group. Group one were controls, fed water + sucrose without drugs. Group 2 received L-NAME from day -7. Group 3 received L-NAME from day +12. Group 4 received AG from day -7. Group 5 received AG from day +12. Unfortunately financial constraints prevented the further use of 1400W in this study. Drugs were continued until day +24 when one rat in each group was sacrificed. The remaining 4 rats in each group underwent a period of drug withdrawal for 7 days, until day +32, when they were sacrificed. This study served to replicate study one and to expand the experiment to consider differing administration time points. Only tumour growth data (section 2.4.7) is presented from this second study.

2.4 Results

2.4.1 Body Weight

The graphs below (Fig. 2.2 – 2.4) illustrate the changes in body weight of the animals during the course of study one (mean \pm SEM). Arrows denote the commencement and cessation of drug administration on days +17 and +33 respectively.

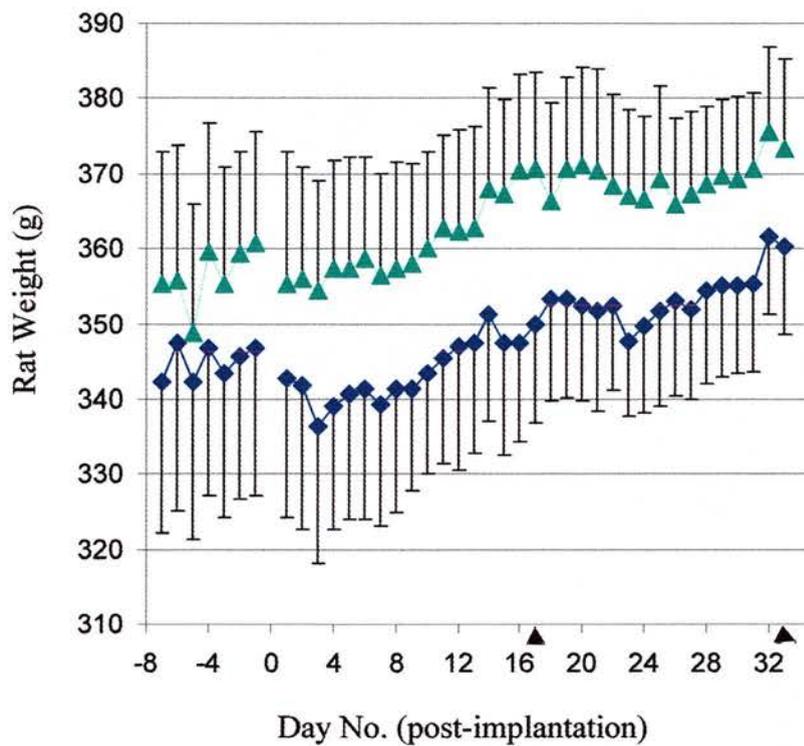
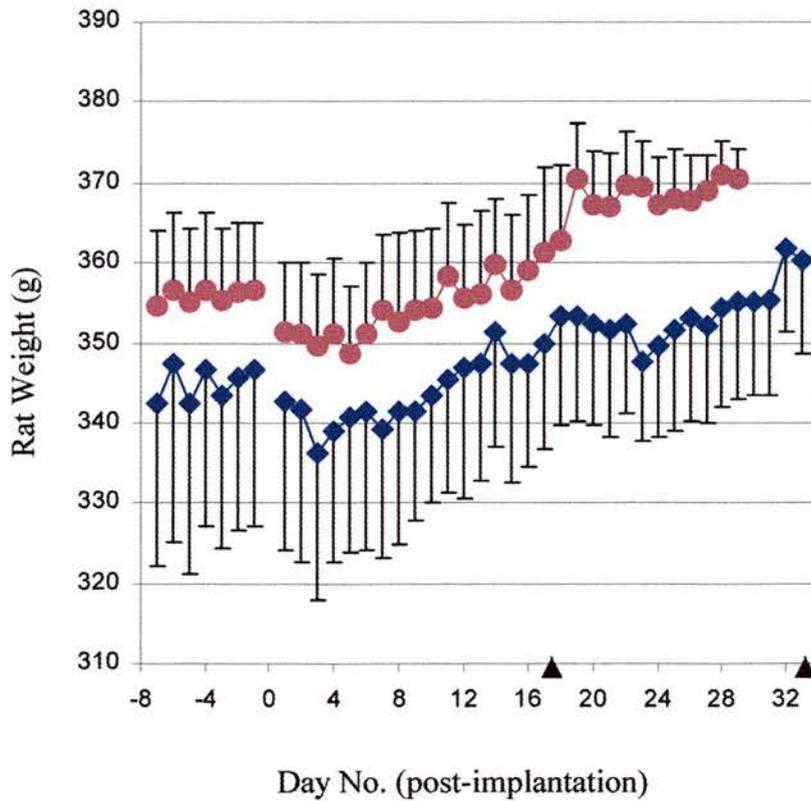
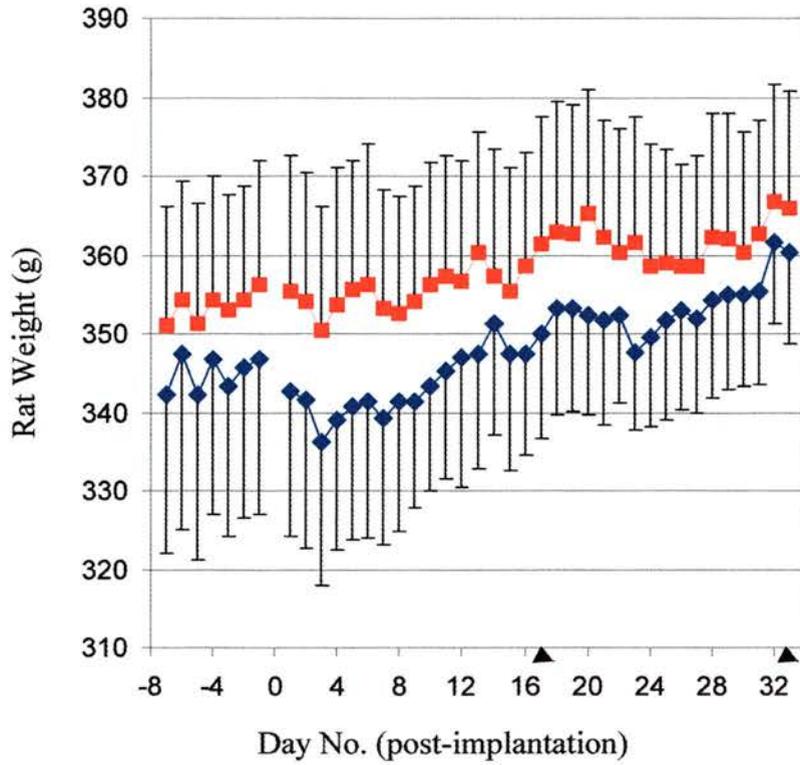


Fig. 2.2 Changes in body weight (g) of L-NAME-treated animals (green triangles) compared with Controls (blue squares).



Figs. 2.3 and 2.4 Changes in body weight (g) of AG-fed (red squares; top) and 1400W-fed (pink circles; bottom) animals compared with Controls (blue squares).

A baseline level was determined by making measurements for 7 days prior to tumour implantation (23 days before commencing drug feeding). All groups can be seen to lie within the specified 300-450g range prior to tumour implantation, and for the duration of the experiment. Tumour implantation surgery on day 0 caused a temporary (24 – 72 hr) drop in weight gain. Commencement of L-NAME administration on day +17 also caused a slight, 24 hour fall in weight gain in this group. Neither of the other drugs had any notable effect on animal weight and after day +3, all animals can be seen to gain weight steadily throughout the experiment.

2.4.2 Drinking Rate

The graphs below (Figures 2.5 – 2.7) illustrate the changes in drinking rate of the animals during the course of study one (mean \pm SEM). An arrow (day +17) again denotes the commencement of drug administration.

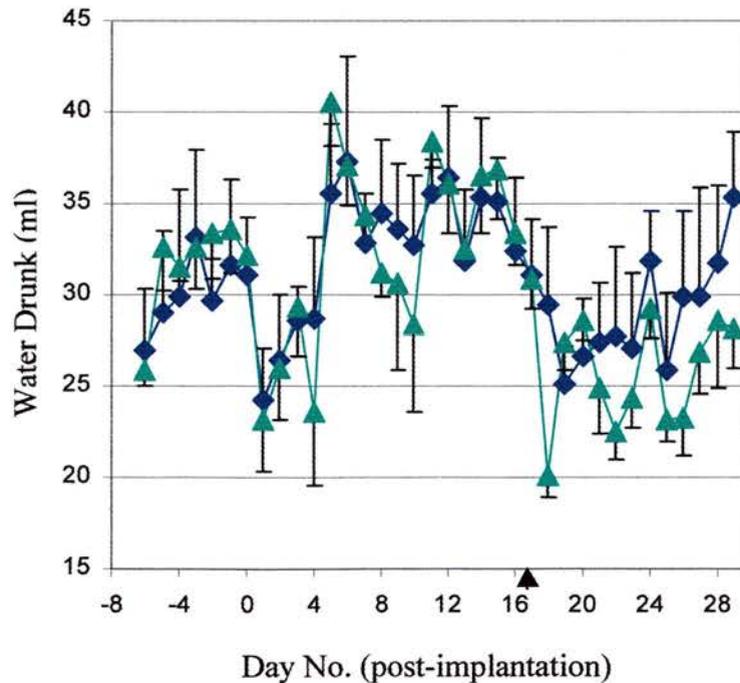
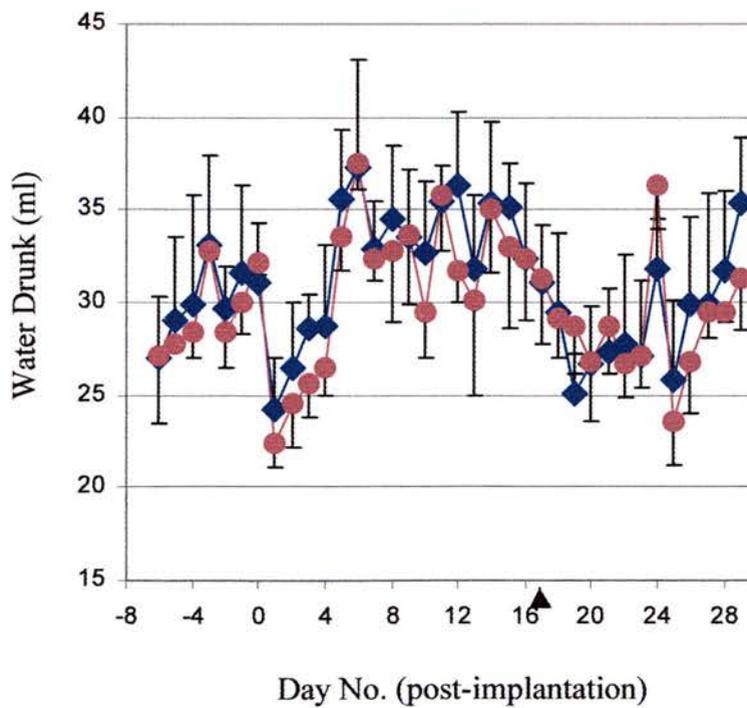
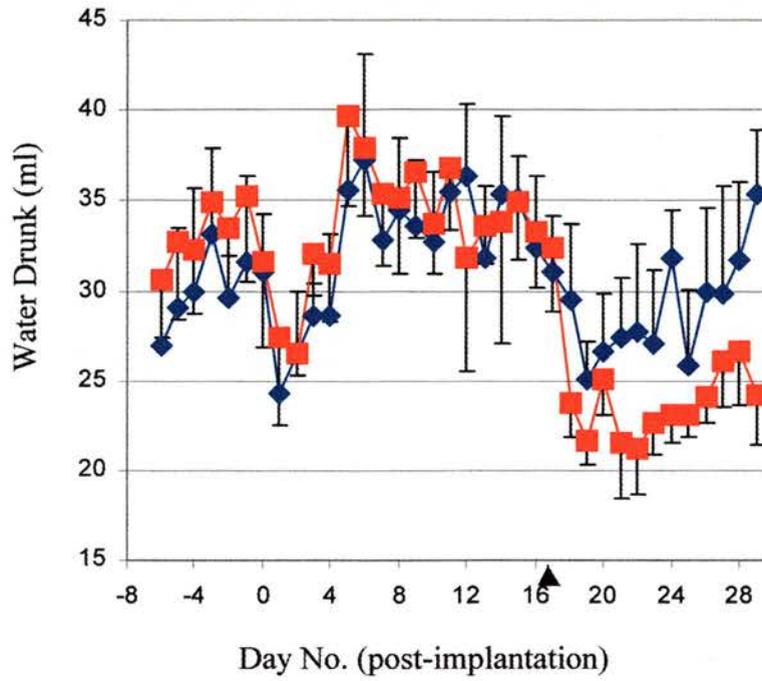


Fig. 2.5 Amount of water (ml) consumed by L-NAME-treated (green triangles) animals, compared with Controls (blue squares)



Figs. 2.6 and 2.7 Amount of water (ml) consumed by AG-treated (red squares; top) and 1400W-treated (pink circles; bottom) animals, compared with Controls (blue squares)

Once again a baseline level was established by monitoring water intake for 6 days prior to tumour implantation (22 days prior to drug administration). A transient reduction in drinking rate in all groups was observed on day +1, presumably the result of surgery on day 0. Administration of both L-NAME and AG on day +17 caused a similar temporary (48 hour) reduction in drinking rate, after which drinking returned to normal. 1400W administration did not bring about this effect. After day +19, and for the duration of the experiment thereafter, the drinking rate of all groups remained within the normal range for animals of this weight.

2.4.3 Blood Pressure

The graphs (Fig. 2.8 – 2.11) below illustrate blood pressure (systolic, mean, diastolic) (average \pm SEM) over the course of study one. Baseline figures were established by monitoring for 16 days prior to drug administration. Arrows denote the commencement and cessation of drug administration on days +17 and +33 respectively.

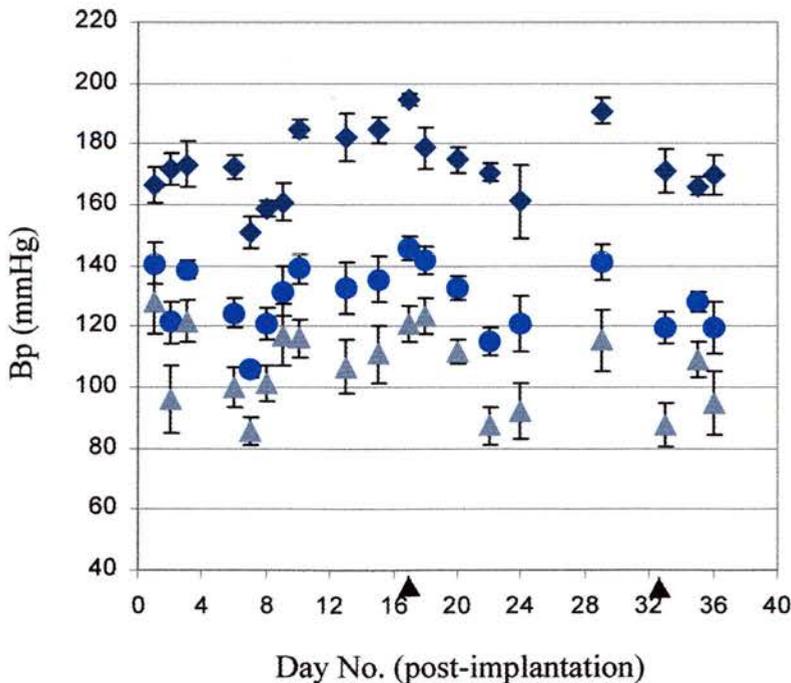
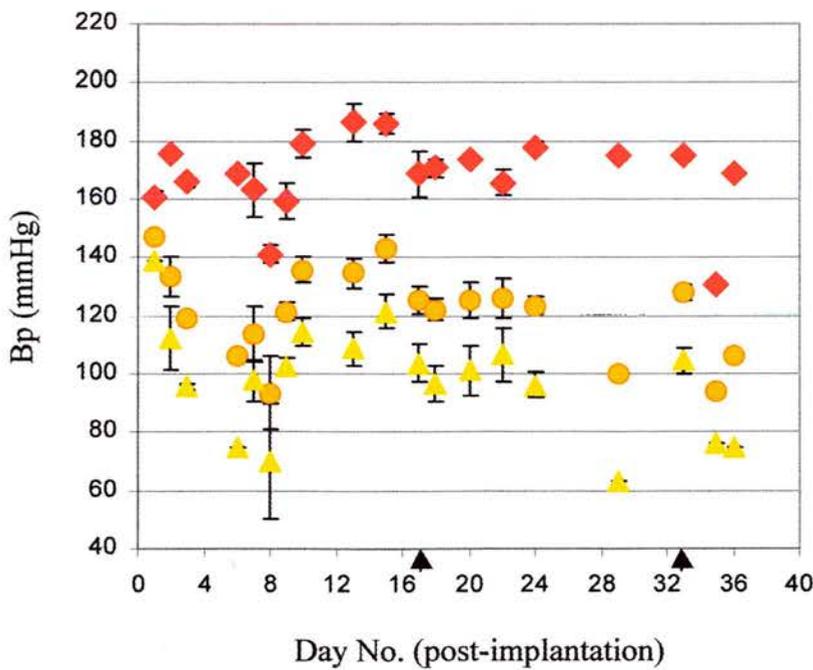
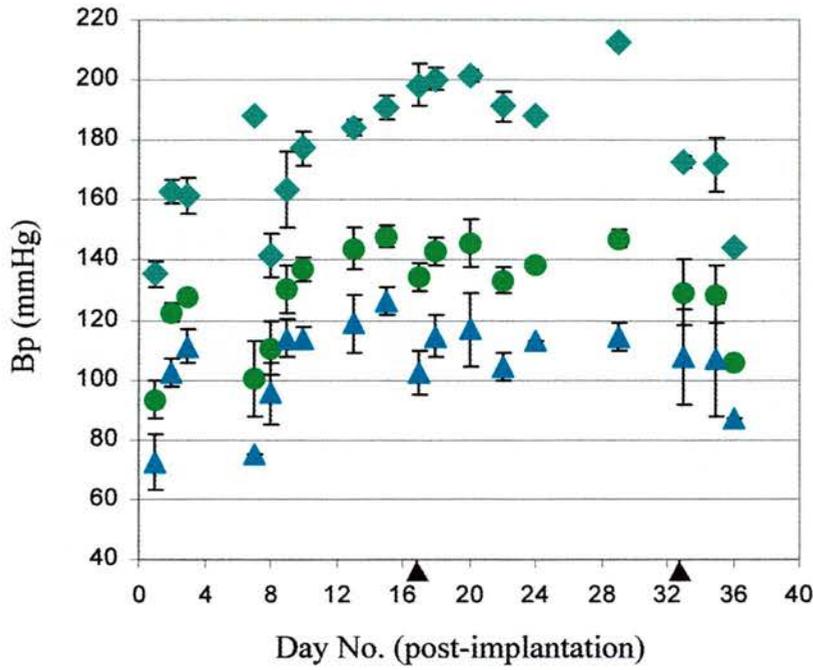


Fig. 2.8 Blood Pressure measurements (systolic – squares; mean – circles; diastolic – triangles) of Control animals.



Figs. 2.9 and 2.10 Blood Pressure measurements (systolic – squares; mean – circles; diastolic – triangles) of L-NAME-treated (top) and AG-treated (bottom) animals.

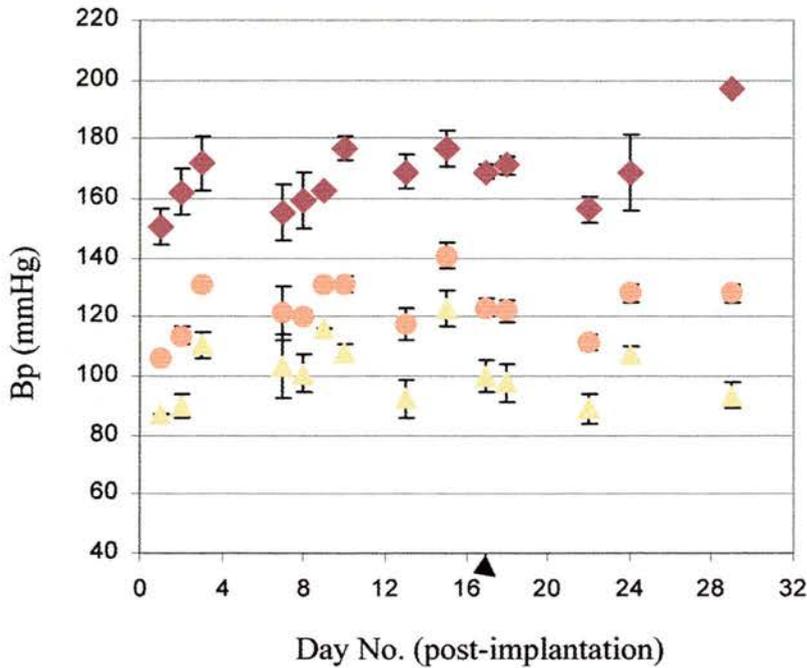


Fig. 2.11 Blood Pressure measurements (systolic – squares; mean - circles; diastolic– triangles) of 1400W-treated animals.

The tail cuff apparatus proved somewhat unpredictable, in some instances requiring six to eight measuring cycles before a consistent result could be obtained. Assuming a significance level of $p \leq 0.05$, analysis of variance (ANOVA) demonstrates that the systolic blood pressure of L-NAME-fed animals was significantly elevated relative to controls ($F = 6.03$, $p = 0.021$). Blood pressure of AG and 1400W animals was not elevated by the drug treatments (AG - $F = 0.41$, $p = 0.529$. 1400W - $F = 0.01$, $p = 0.925$).

2.4.4 Histology

Paraffin wax sections of mature tumours demonstrated several interesting characteristics of transformed cells. Histological H+E staining revealed numerous mitotic figures (see plates 2.6 and 2.7), indicating a high level of cellular proliferation. Tumour cell size and shape were highly pleomorphic and cell arrangement was chaotic. Two major cell morphologies appeared to be present, possibly reflecting the dual tumour origin of epithelia (carcinoma) and connective tissue (sarcoma) (see plates 2.1 and 2.2). Characteristic of sarcoma were directional 'streaming cords' of darkly-staining elongate cells containing grainy, elongate nuclei.

These were interspersed with pale staining, less dense cells which were significantly more rounded. Interspersed throughout much of the sections were large cells having the appearance of leukocytes. Many of these were in fact dying tumour cells containing granular pyknotic nuclei. They occurred across a range of sizes and contained one or more nuclear fragments. Various leukocytes were visible both within vessels and amongst the tumour cells, and a few tissue monocytes were evident, although the extent of immune infiltrate was scant.

Larger tumours contained extensive areas of necrosis within which dead and dying cells, apoptotic figures and cellular debris were evident. Necrosis appeared as pale eosinophilic staining (collagen) in H+E (see plates 2.11 – 2.13) and was present both within central (high solid pressure) regions of the tumour and also extending out towards more peripheral regions in some sections. As anticipated, necrotic areas were more numerous and extensive within larger tumours. Accordingly, control, AG- and 1400W-treated tumours displayed more necrosis than L-NAME-treated tumours whose growth had been held in check.

Necrotic regions were dominated by many large cell-free spaces bounded by pale cytoplasmic extensions of sparse mis-shapen tumour cells (see plate 2.3). Erythrocytes were dispersed throughout much of the necrotic region, spilled out between tumour cells from broken vessels (see plate 2.14). Progressive nuclear changes characteristic of apoptosis included pyknosis (the condensation of chromatin into a small, densely-staining mass), karyorrhexis (nuclear fragmentation) and finally karyolysis, in which the nucleus was lost altogether and the cell membrane had disappeared (cytolysis) (see plates 2.15 – 2.18 respectively). Masson's trichrome staining demonstrated necrosis well, even with the naked eye. The large areas of collagen which replaced dead cells were highlighted brilliant green (see plate 2.10). This stain similarly demarcated tumour and stromal compartments well, with green regions of stroma just visible surrounding dense tumour cells (see plate 2.3).

At the tumour margins, the capsule was visible in some areas as a green collagenous covering (Masson's trichrome) (plate 2.8). Transversely-sectioned skeletal muscle blocks surrounded much of the intact capsule (plate 2.9). Many blood vessels were visible within the peripheral regions of the tumour just beneath the capsule, and large muscular arteries were dispersed throughout the capsule.

Aside from the increased necrosis present within larger tumours, little histological difference could be ascertained between control and drug-treated tumours with these histological stains. Cellular and nuclear morphology appeared equally pleiomorphic. The reduced MVD within L-NAME-treated tumours relative to controls (see section 2.4.8) was not immediately evident.

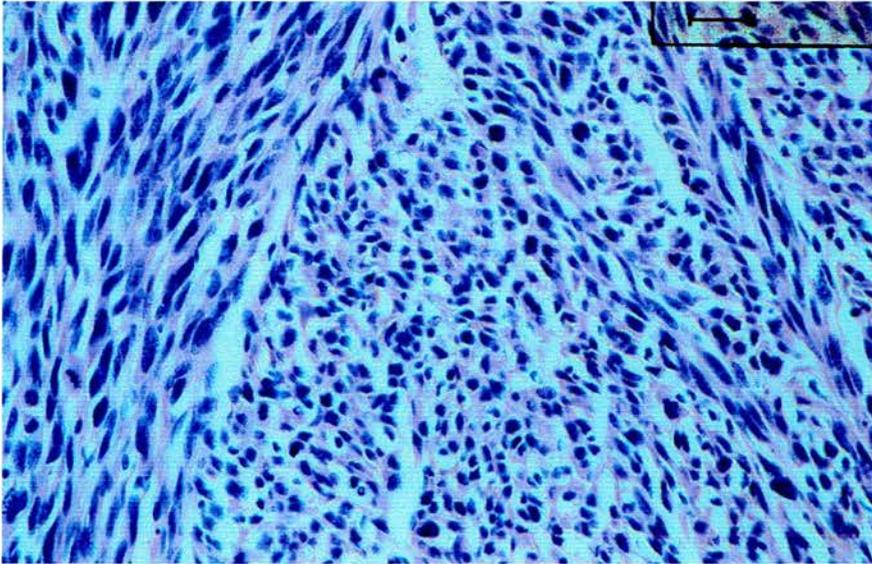


Plate 2.1 *General Tumour Structure (H+E, X400)*

Two distinct cell populations are apparent – elongated ‘streaming’ cells and a more ‘rounded’ population. Nuclear pleiomorphism is also evident (scalebar = 25 μ m).

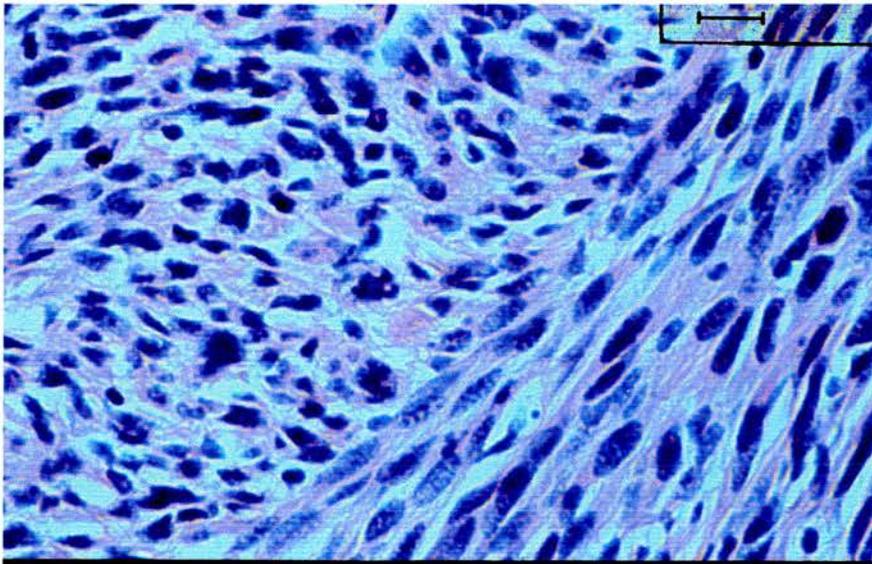


Plate 2.2 *General Tumour Structure (H+E, X630)*

A higher magnification than plate 2.1 clearly illustrates the cellular and nuclear pleiomorphism of the carcinosarcoma (scalebar = 16 μ m).

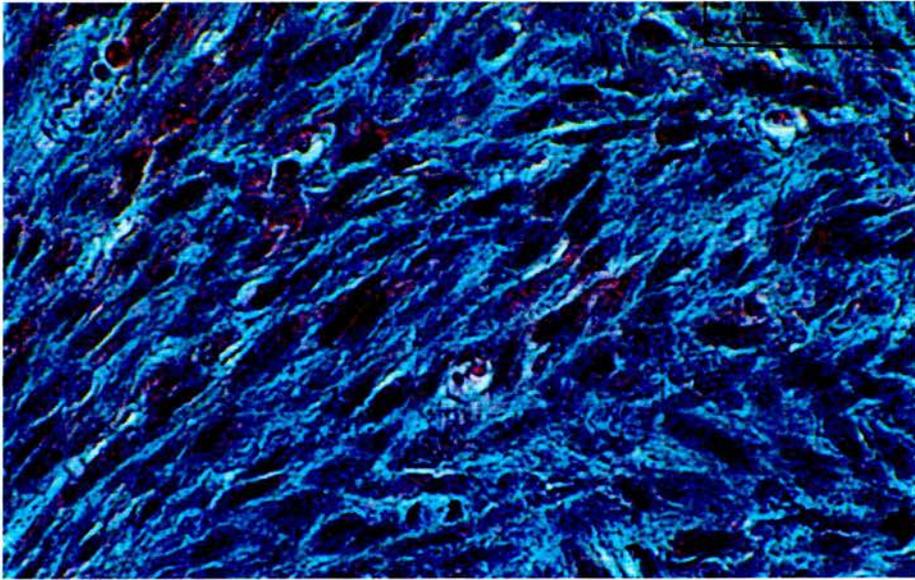


Plate 2.3 *Tumour Stroma* (Masson's trichrome, X630)

Collagenous tumour stroma is abundant surrounding dark-staining tumour parenchyma (scalebar = 16 μ m).

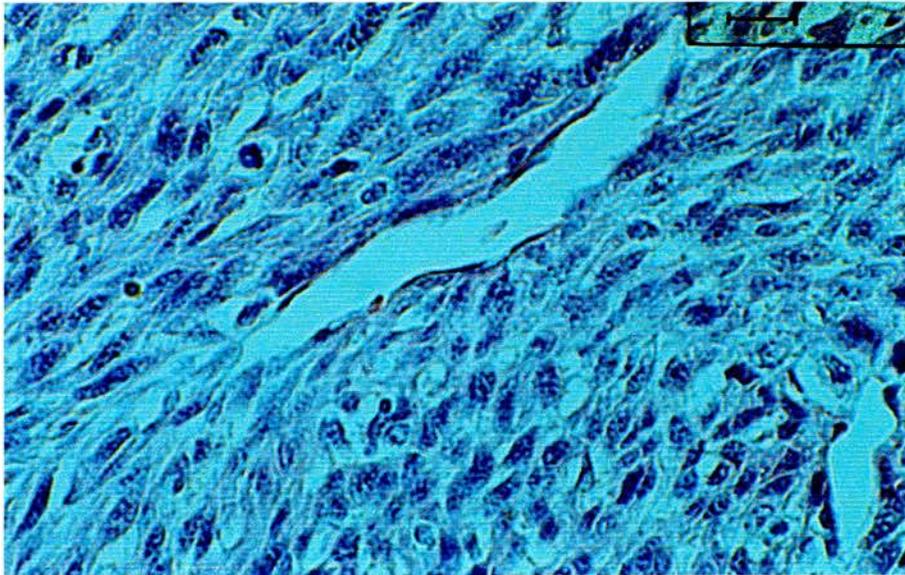


Plate 2.4 *Tumour Blood Vessel* (H+E, X630)

The endothelial cell lining is visible within blood vessels at this magnification (scalebar = 16 μ m).

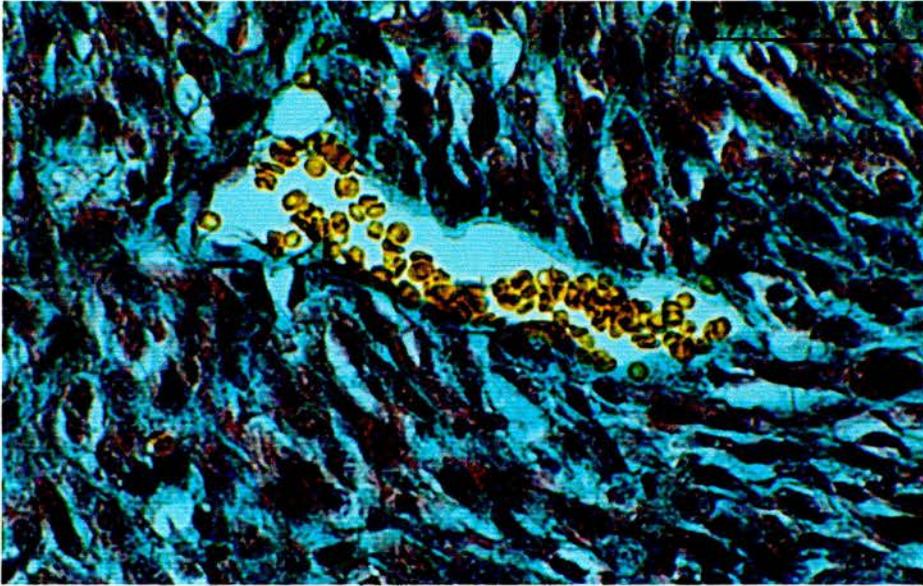


Plate 2.5 *Tumour Blood Vessel* (Masson's trichrome; X630)

Erythrocytes (yellow) are clearly visible packed within a large blood vessel. Haematoxylin-stained tumour cells can be seen to lie within the tumour stroma (green) (scalebar = 16 μ m).

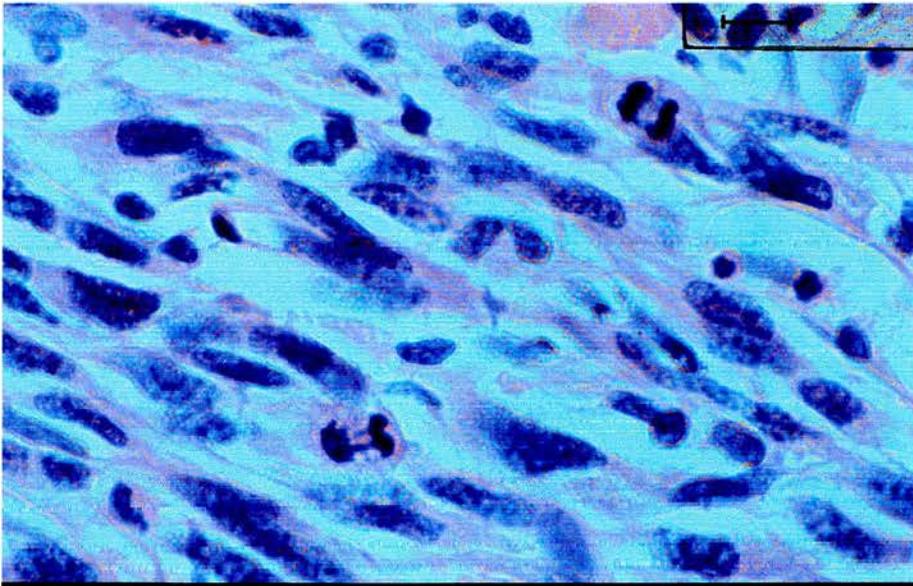


Plate 2.6 *Mitotic Figures* (H+E; X1000)

Two tumour cells undergoing mitosis (anaphase) are visible in this field of view (scalebar = 10 μ m).

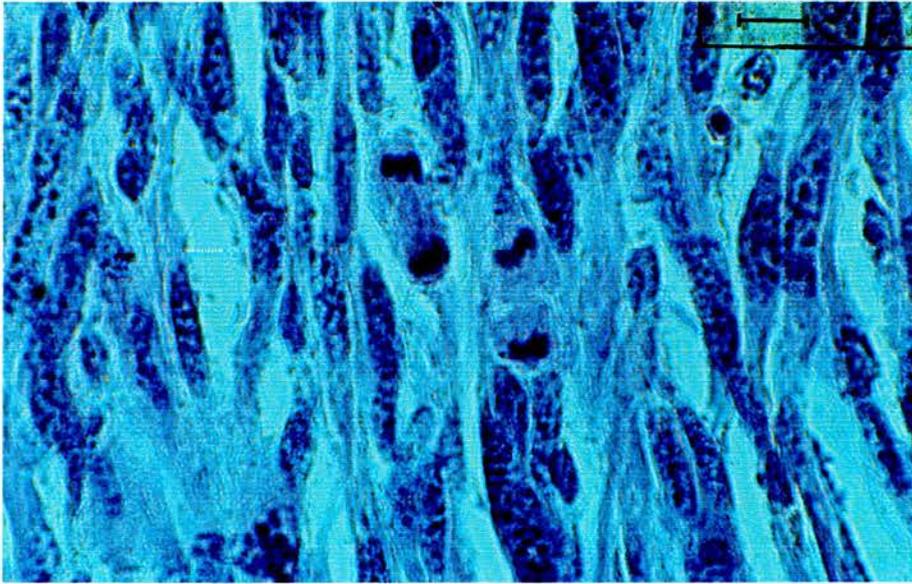


Plate 2.7 *Mitotic Figures* (H+E; X1000)

Two tumour cells undergoing mitosis (early telophase) are visible in this field of view (scalebar = 10 μ m).

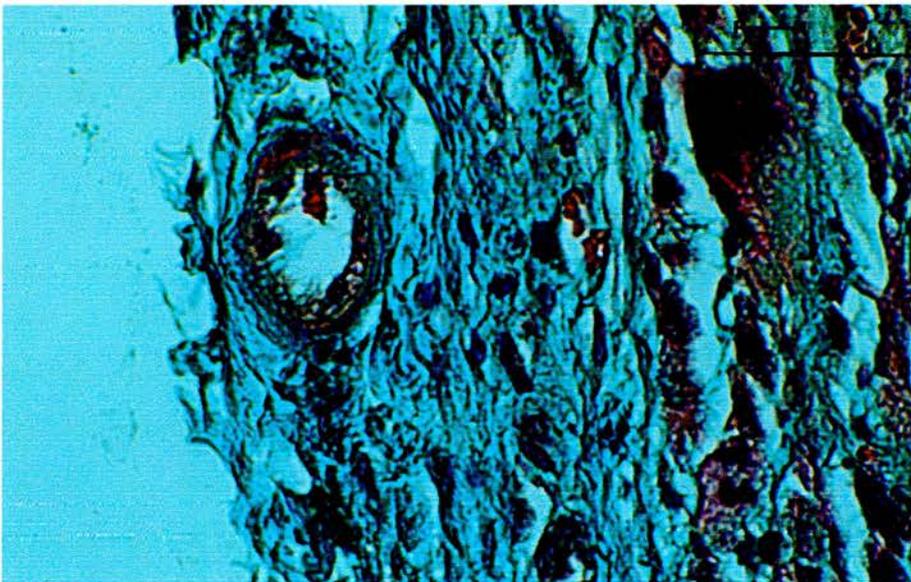


Plate 2.8 *Tumour Capsule* (Masson's trichrome; X630)

A large muscular blood vessel is evident within the dense green-staining capsule (scalebar = 16 μ m).

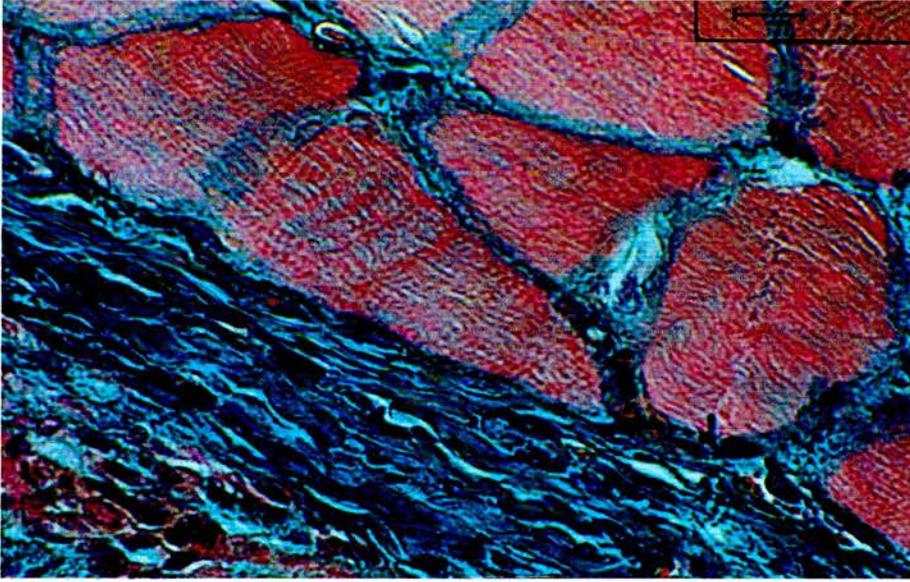


Plate 2.9 *Tumour Capsule* (Masson's trichrome; X630)

Haematoxylin-stained, transversely-sectioned skeletal muscle blocks can be seen lying outside the dense tumour capsule (scalebar = 16 μ m).

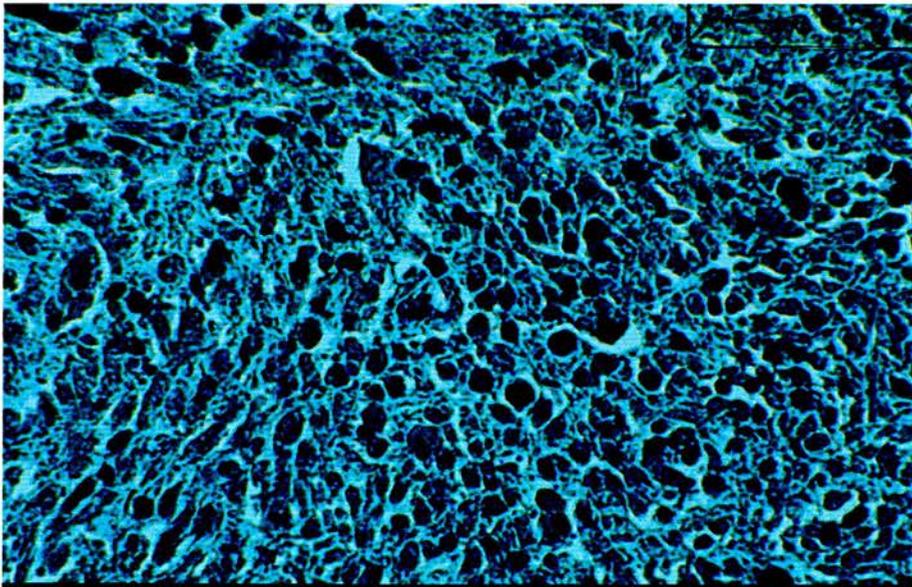


Plate 2.10 *Necrosis* (Masson's trichrome; X630)

Necrotic tumour region containing shrunken tumour cells with dense nuclei and an excess of collagenous tumour stroma (green) (scalebar = 16 μ m).

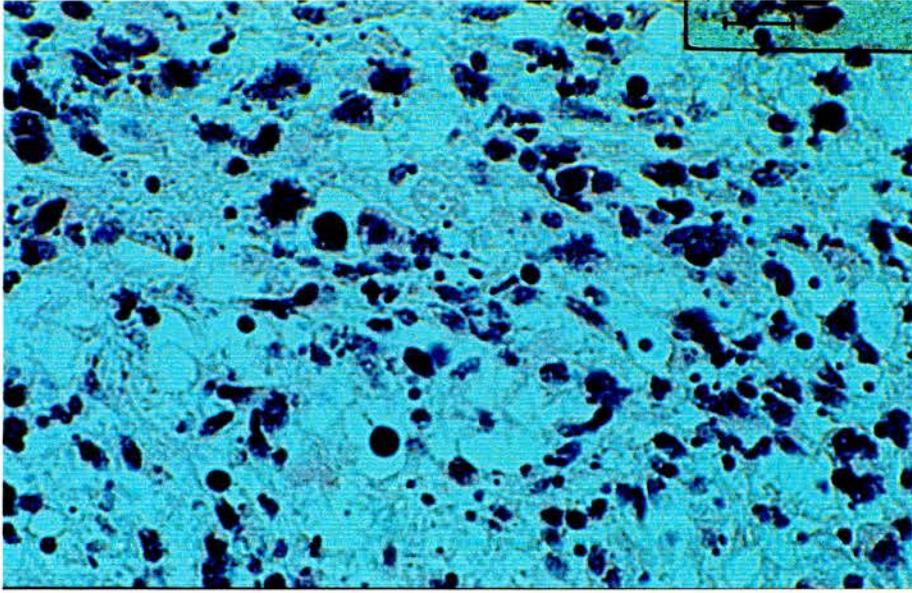


Plate 2.11 *Advanced Necrosis* (H+E; X630)
As for plate 2.10.

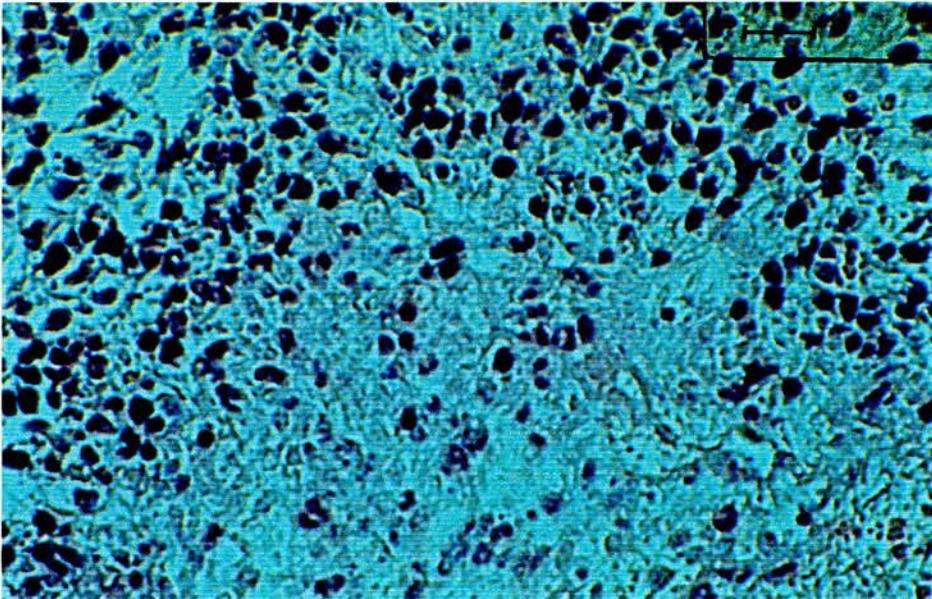


Plate 2.12 *Advanced Necrosis* (H+E; X630)
As for plate 2.10.

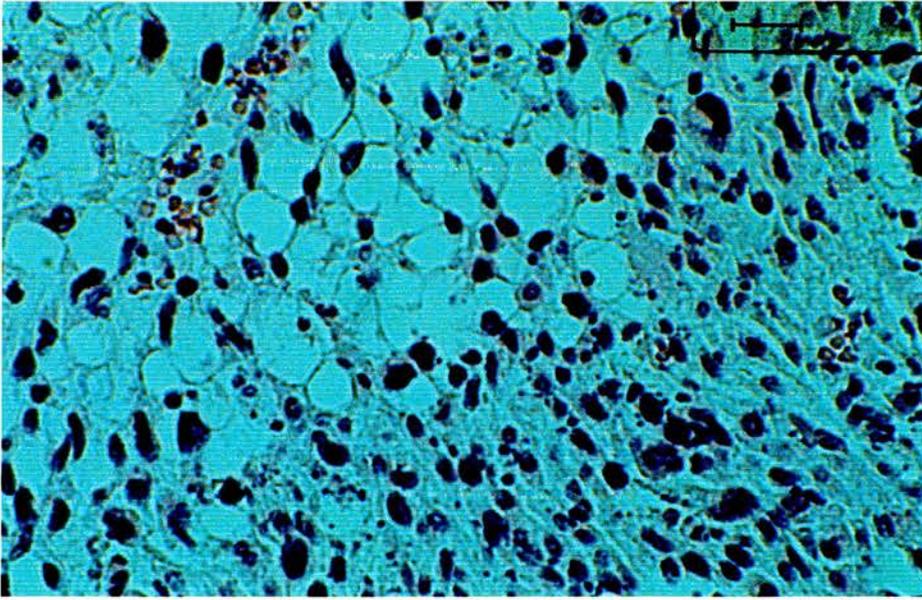


Plate 2.13 *Advanced Necrosis* (H+E; X630)

Large cell-free areas of tissue are evident at this advanced stage of necrosis (scalebar = 16 μ m).

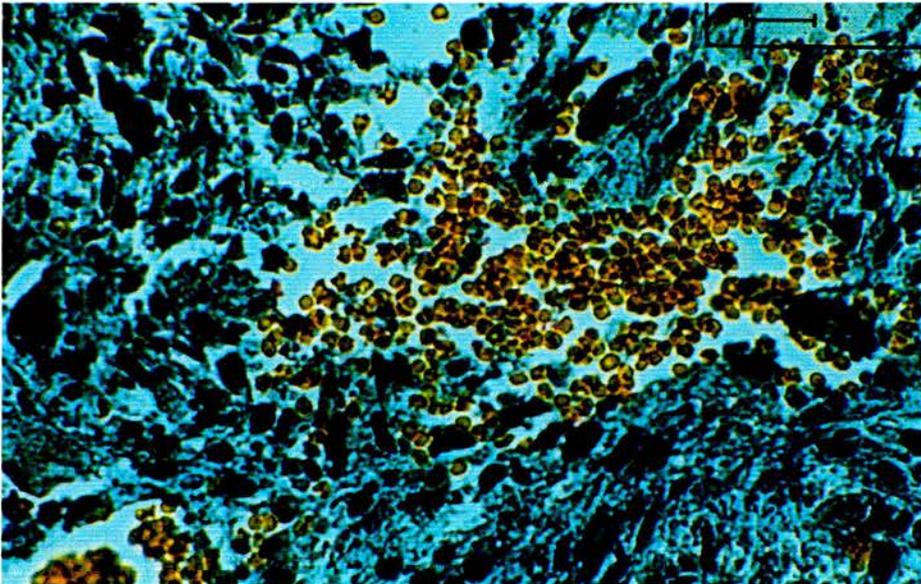


Plate 2.14 *Necrosis* (Masson's trichrome; X630)

Necrotic zone containing densely-staining pyknotic nuclei of dying tumour cells, and erythrocytes spilling out of broken blood vessels (scalebar = 16 μ m).

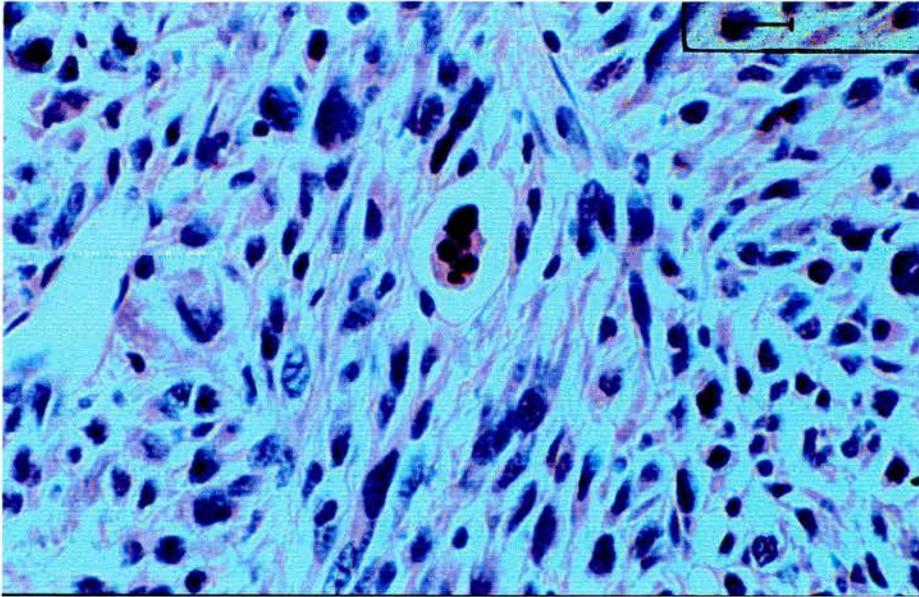


Plate 2.15 *Cell Apoptosis (early)* (H+E; X630)

A tumour cell with dense, shrunken, round fragments of a pyknotic nucleus is present in the centre of this field of view (scalebar = 16 μ m).

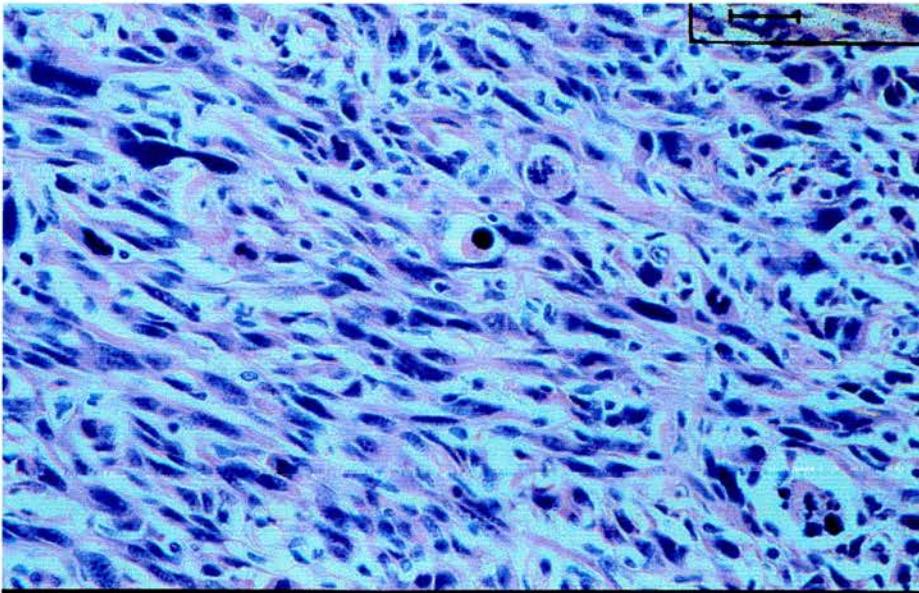


Plate 2.16 *Cell Apoptosis (mid)* (H+E; X400)

A tumour cell with fragmenting nucleus (karyorrhexis) is visible in the bottom right hand view. A cell with pyknotic nucleus is visible in the centre of the field of view (scalebar = 25 μ m).

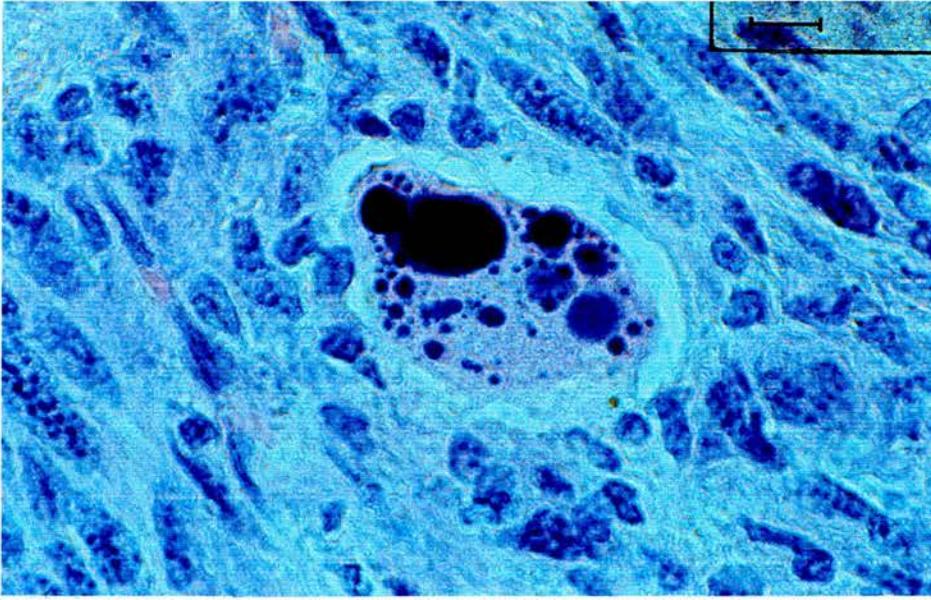


Plate 2.17 *Cell Apoptosis (mid)* (H+E; X1000)

At high power, the fragmenting cell nucleus is very apparent (scalebar = 10 μ m).

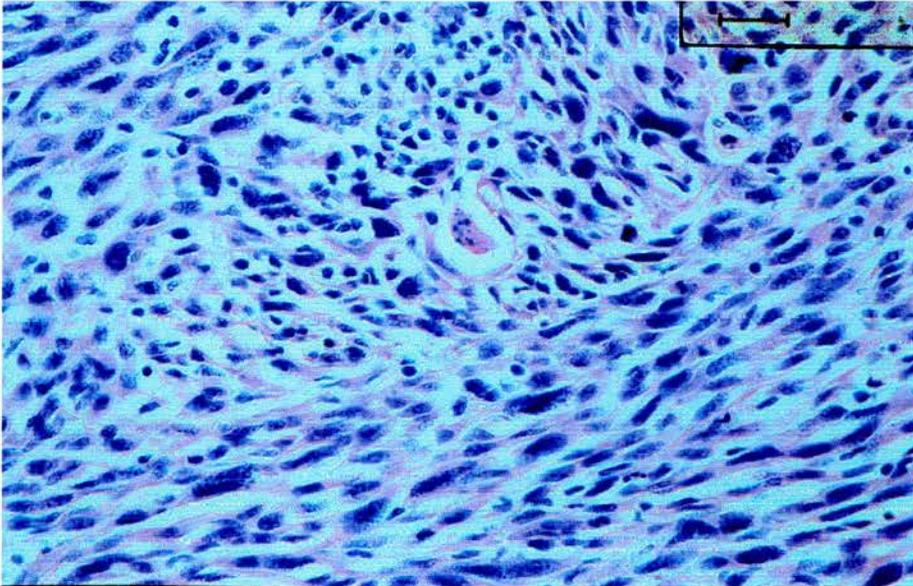


Plate 2.18 *Cell Apoptosis (late)* (H+E; X400)

A dead tumour cell with a disintegrated nucleus (karyolysis) and a dissolved cell membrane is visible in the centre of this field of view (scalebar = 25 μ m).

2.4.5 Immunohistochemistry

Snap-frozen tumour sections generally retained good tissue morphology as demonstrated with a simple trypan blue histological stain (see appendix 8.3.1). Immunohistochemical staining was performed on tumours from study one animals, which had been fed drugs continuously. Staining quality for each antibody was excellent, demonstrating high specificity and low background. All negative controls were similarly clean.

2.4.5.1 eNOS

Immunohistochemical staining for the eNOS enzyme (see appendix 8.3.2.1) was confined entirely to the endothelial lining of blood vessels. The anti-eNOS antibody therefore highlighted the vasculature across the tumour sections in a way analogous to anti-PECAM-1 (see section 2.4.8). Plates 2.19 – 2.22 illustrate anti-eNOS immunohistochemical tumour staining.

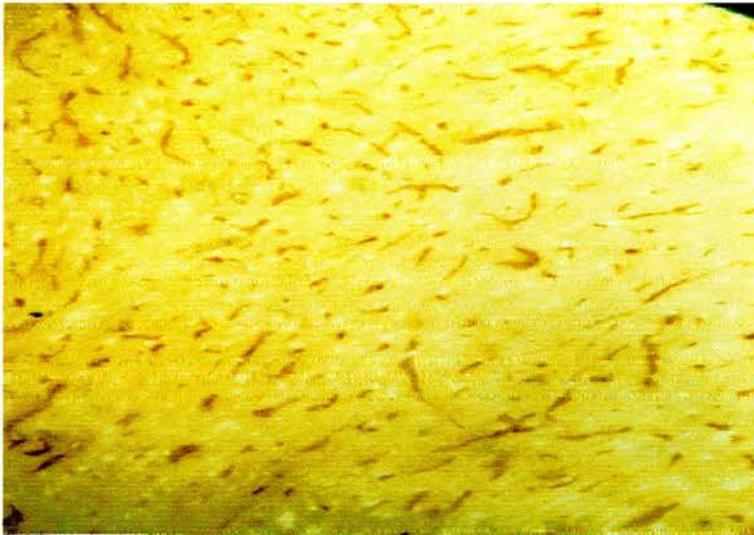


Plate 2.19 *Anti-eNOS staining of Control tumour X400*

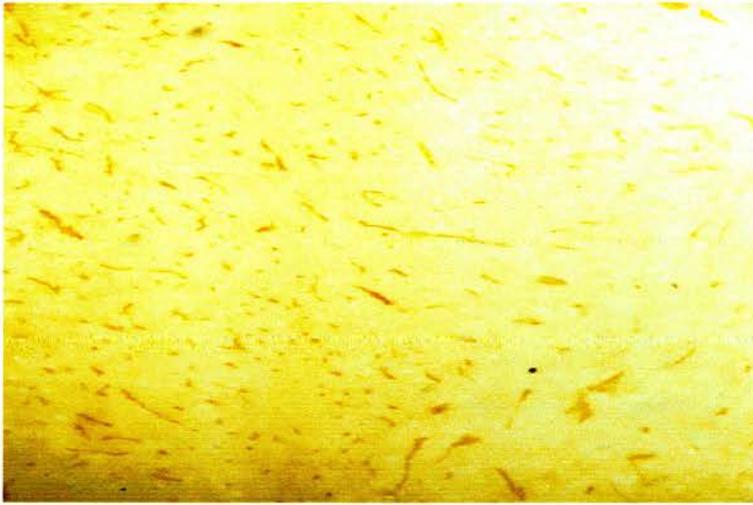


Plate 2.20 *Anti-eNOS staining of L-NAME-treated tumour X400*



Plate 2.21 *Anti-eNOS staining of AG-treated tumour X400*



Plate 2.22 *Anti-eNOS staining of 1400W-treated tumour X400*

The intensity of anti-eNOS staining in the L-NAME-treated tumour section was similar to that of the control, although the staining distribution was clearly reduced. However, bearing in mind the vascular-specificity of this stain, the diminished distribution may simply reflect a reduced MVD in this section relative to the control. The quality of the AG-treated section was not as good. However, anti-eNOS staining was clearly still confined to the vasculature, and both staining intensity and distribution appeared similar to the control. Unfortunately, the quality of the 1400W-treated tumour was very poor, containing a number of defrost artefacts. Staining intensity and distribution appear to be similar to the control, although this is difficult to conclude with confidence on account of the section damage.

2.4.5.2 *iNOS*

As for eNOS, immunohistochemical staining for iNOS was confined almost entirely to the vasculature. An occasional small cell, probably of immune origin, also stained positive. Staining was however, extremely weak across all sections. It required maximal antibody and chromogen incubation times and a high microscope magnification to be visualised (see appendix 8.3.2.2). Plates 2.23 – 2.25 illustrate anti-iNOS immunohistochemical staining.

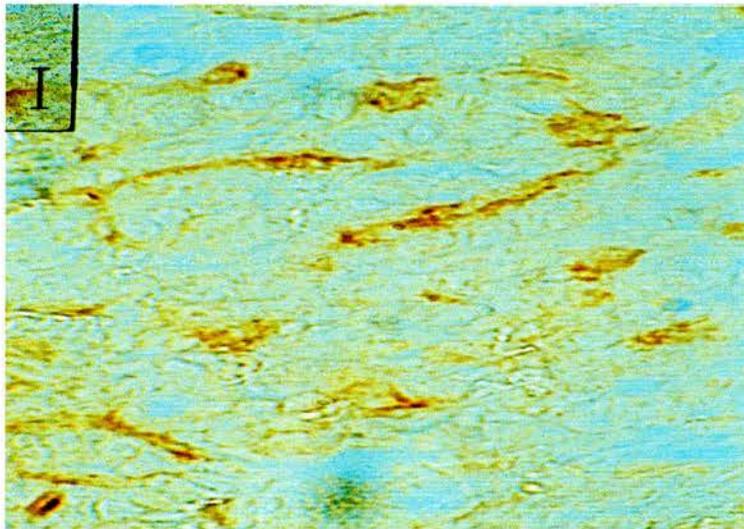


Plate 2.23 *Anti-iNOS staining of Control tumour X630 (scalebar = 16µm)*

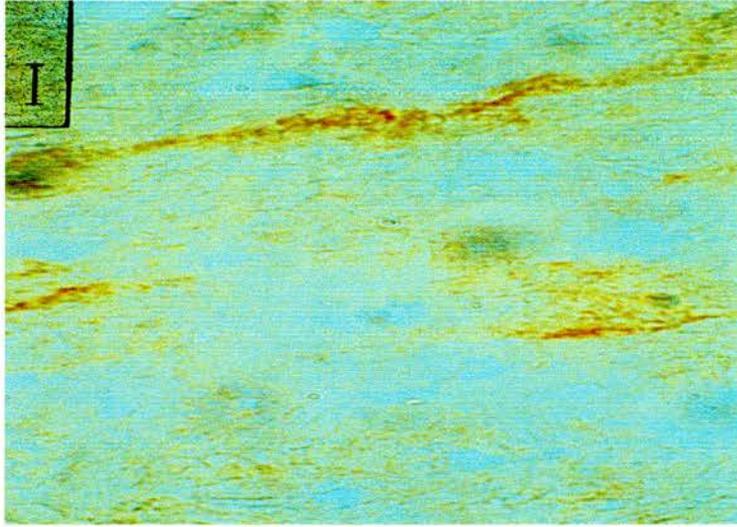


Plate 2.24 *Anti-iNOS staining of L-NAME-treated tumour X630 (scalebar = 16µm)*

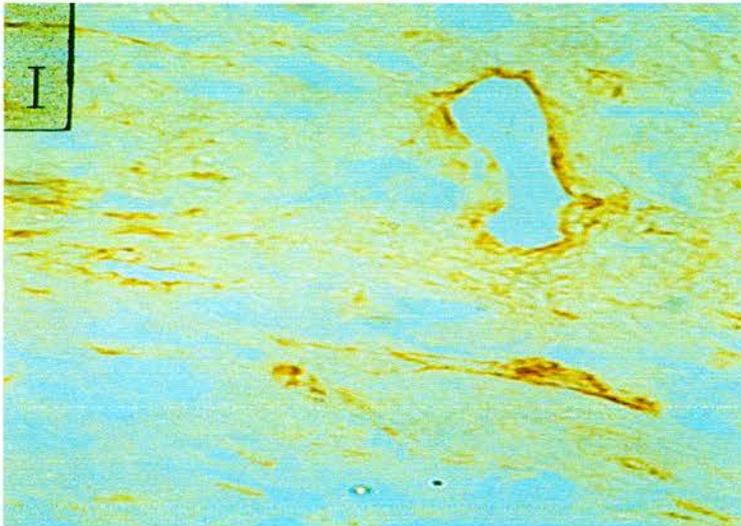


Plate 2.25 *Anti-iNOS staining of AG-treated tumour X400 (scalebar = 25µm)*

The weak nature of anti-iNOS immunostaining rendered it difficult to conclude, with confidence, quantitative differences between control and drug-treated tumour sections. Nevertheless, both the staining intensity and distribution in L-NAME-treated tumour sections appeared reduced relative to the control, possibly once again reflecting the reduced vascularisation of these tumours (see section 2.4.8). The staining intensity of the AG-treated section appeared similarly weak, although section damage may be in part, responsible for this. Anti-iNOS staining in 1400W

sections was almost undetectable. The occasional very weakly-staining vessel could be perceived with perseverance. Photography was not possible.

2.4.5.3 *KDR/Flk-1*

Contrary to the relatively EC-specific staining anticipated with this antibody, KDR/Flk-1 receptors were widely distributed across all tumour cells in these sections. Plates 2.26 – 2.30 illustrate anti-KDR/Flk-1 immunostaining. The 1400W-treated section (plate 2.30) is once again displaying poor morphology, but is included for reasons of comparison.

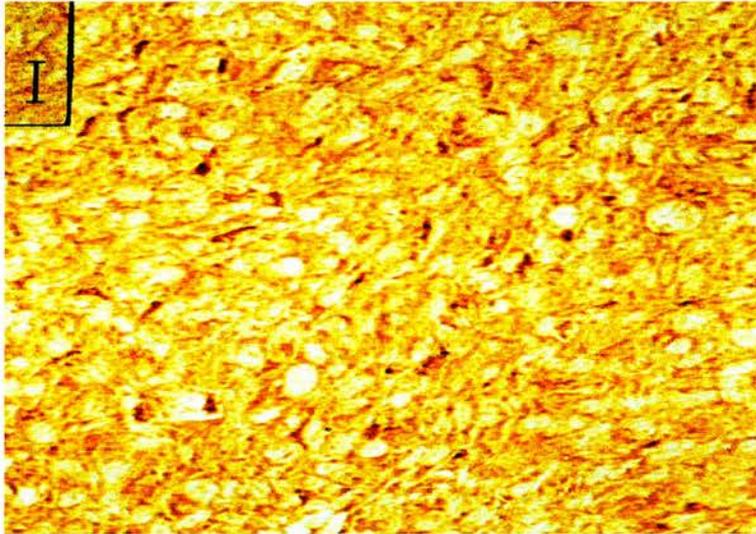


Plate 2.26 *Anti-KDR/Flk-1 staining of blood vessels in a control tumour X630*
(scalebar = 16µm)

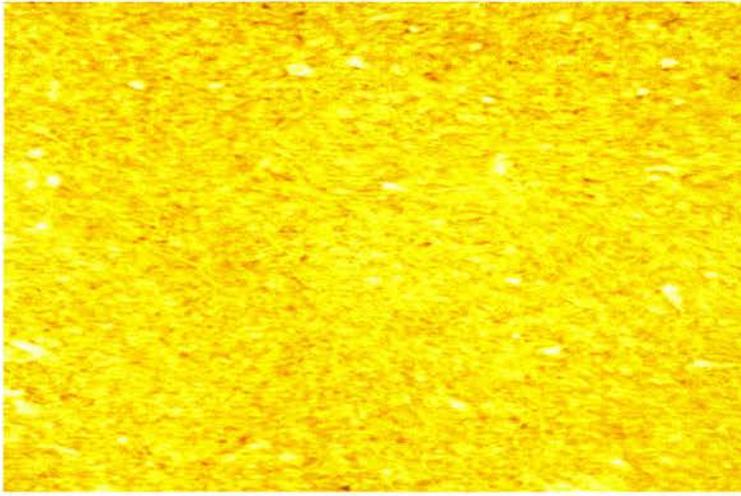


Plate 2.27 *Anti-KDR/Flk-1 staining of a Control tumour X400*

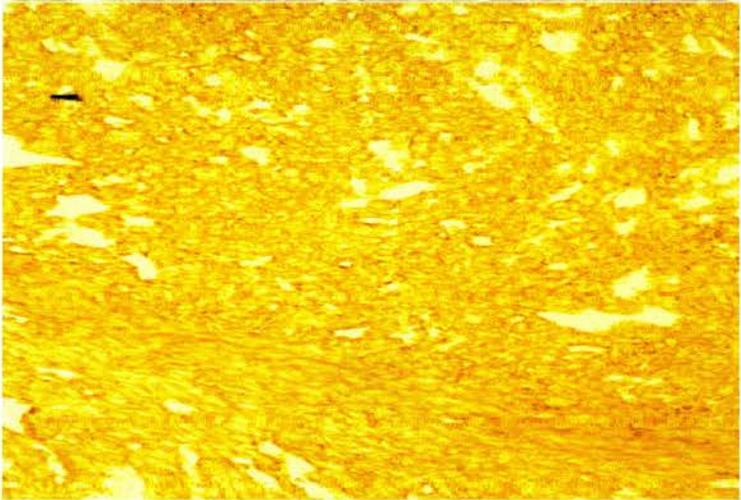


Plate 2.28 *Anti-KDR/Flk-1 staining of an L-NAME-treated tumour X400*

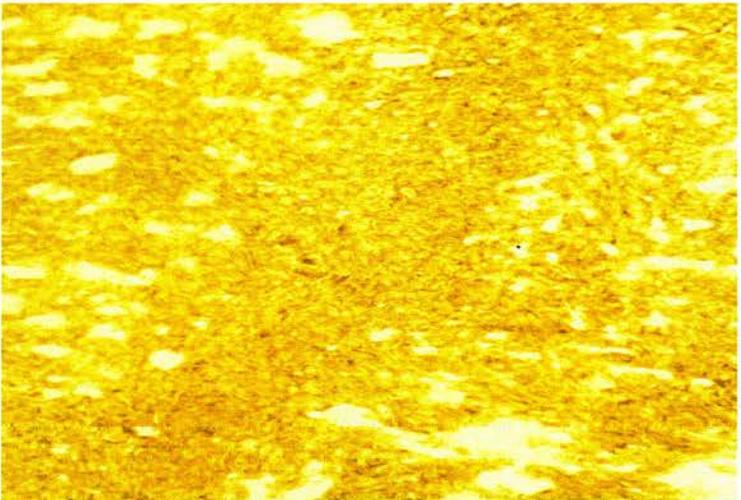


Plate 2.29 *Anti-KDR/Flk-1 staining of an AG-treated tumour X400*

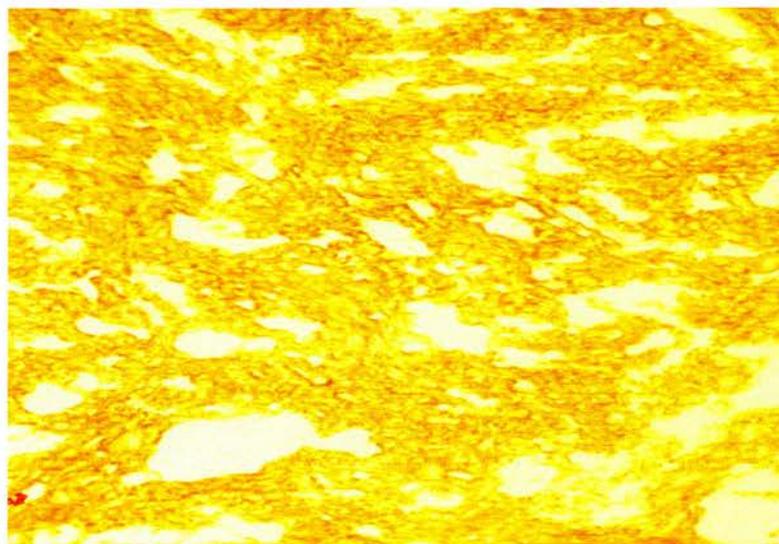


Plate 2.30 *Anti-KDR/Flk-1 staining of a 1400W-treated tumour X400*

Pale anti-KDR/Flk-1 staining can be seen across the entire tumour cell population. Immunoreactivity was most dense around the tumour cell membranes, but was also present throughout the cytoplasm. At higher magnification, intensely-staining blood vessels were visible amongst the paler tumour cells (see plate 2.26). Although these vessels were clearly more reactive with the anti-KDR/Flk-1 serum than the tumour cells, no quantitative difference could be observed in the intensity of vascular KDR/Flk-1 staining across the different tumour sections.

2.4.5 Biochemical Analysis

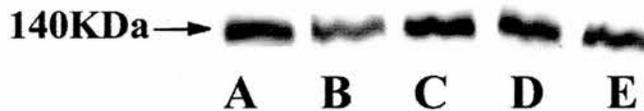
Control and drug-treated tumour extracts were prepared for protein gel electrophoresis (see appendix 8.4.1). Three sample sets, from study one animals - control, drug-treated continuously (17 days), and drug-treated with a withdrawal period (5 days) - were run in parallel. The protein content of each sample was estimated (see appendix 8.4.3) and samples were subsequently diluted to give a standard protein content (5 mg/ml protein). Duplicate samples were loaded and run on 6% gels, alongside MW standards. Coomassie brilliant blue gel staining was performed to confirm equal protein loading across all lanes. All gels analysed demonstrated equivalent banding intensities with this stain.

2.4.5.1 eNOS

Detection of eNOS in samples was optimal with a loading of 3µl of 5 mg/ml protein extracts. Nitrocellulose membranes were incubated overnight (4°C) with primary antibody (1:1000) and for 2 hrs (RT) with secondary (1:1000).

The results for control and drug-treated tumour extracts are shown in plates 2.31 to 2.33 below.

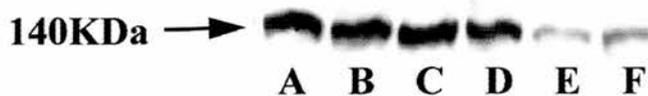
Plate 2.31 Control versus L-NAME-treated tumour extracts probed for eNOS (A-E)



Key – Control (lane A); L-NAME continuously (lanes B, C); L-NAME with withdrawal period (lanes D, E)

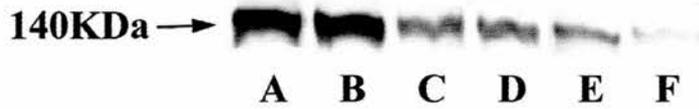
Relative to the control, it can be seen that neither L-NAME administration nor its subsequent withdrawal has any notable effect on eNOS expression in tumours.

Plate 2.32 Control versus AG-treated tumour extracts probed for eNOS (A-F)



Key - Control (lanes A, B); AG continuously (lanes C, D); AG with withdrawal period (lanes E, F).

As for L-NAME, AG administration has no significant effect on tumour eNOS expression. However, subsequent drug withdrawal causes a marked reduction in the level of expression.

Plate 2.33 Control versus 1400W-treated tumour extracts probed for eNOS (A-F)

Key - Control (lanes A, B); 1400W continuously (lanes C, D); 1400W with withdrawal period (lanes E, F).

Clearly 1400W administration causes a reduction in eNOS expression in tumours. Subsequent drug withdrawal, as for AG, appears to cause a further reduction in expression, although the heterogeneity of staining between extracts *E* and *F* preclude a concrete conclusion. Either way, expression does not return to control levels over the 5 day period of drug-withdrawal.

2.4.5.2 iNOS

Detection of iNOS in samples required heavier extract loading than for eNOS, demonstrating optimal staining when 7 μ l of 5 mg/ml protein extracts were loaded. Nitrocellulose membranes were incubated overnight (4°C) with primary antibody (1:200) and for 2 hrs (RT) with secondary (1:1000).

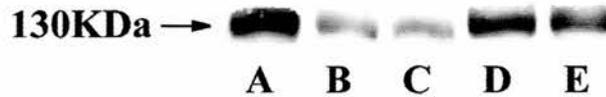
The results for control and drug-treated tumour extracts are shown in plates 2.34 to 2.36.

Plate 2.34 Control versus L-NAME-treated tumour extracts probed for iNOS (A-E)

Key - Control (lane A); L-NAME continuously (lanes B, C); L-NAME with withdrawal period (lanes D, E).

Clearly the administration of L-NAME causes a significant reduction in the expression of iNOS in tumours. Subsequent drug-withdrawal restores iNOS expression approximately back to control levels.

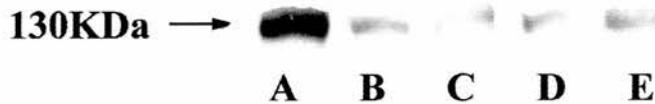
Plate 2.35 Control versus AG-treated tumour extracts probed for iNOS (A-E)



Key - Control (lane A); AG continuously (lanes B, C); AG with withdrawal period (lanes D, E).

In a similar way to L-NAME, administration of AG causes a reduction in iNOS expression within tumours which is restored back towards control levels after 5 days of drug-withdrawal

Plate 2.36 Control versus 1400W-treated tumour extracts probed for iNOS (A-E)



Key - Control (lane A); 1400W continuously (lanes B, C); 1400W with withdrawal period (lanes D, E).

The administration of 1400W causes a marked reduction in iNOS expression within tumours. Subsequent drug-withdrawal within the timeframe of this experiment does not restore this expression back to control levels.

2.4.5.3 PECAM-1/CD31

The anti-PECAM-1 antibody failed to react with tumour extracts in acrylamide gels.

2.4.6.4 KDR/Flk-1

Results obtained with the anti-KDR/Flk-1 antibody were extremely poor. Banding was weak, erratic and did not coincide with the correct MW region. Results were discarded.

2.4.6 Tumour Growth Rate

Daily measurements (mm^3) of tumour volume were made across all animals groups. The graphs (Fig. 2.12 – 2.16) below illustrate tumour growth (average volume \pm SEM) over the course of the two studies.

2.4.7.1 Study One

Arrows denote commencement and cessation of drug administration on days +17 and +33 respectively.

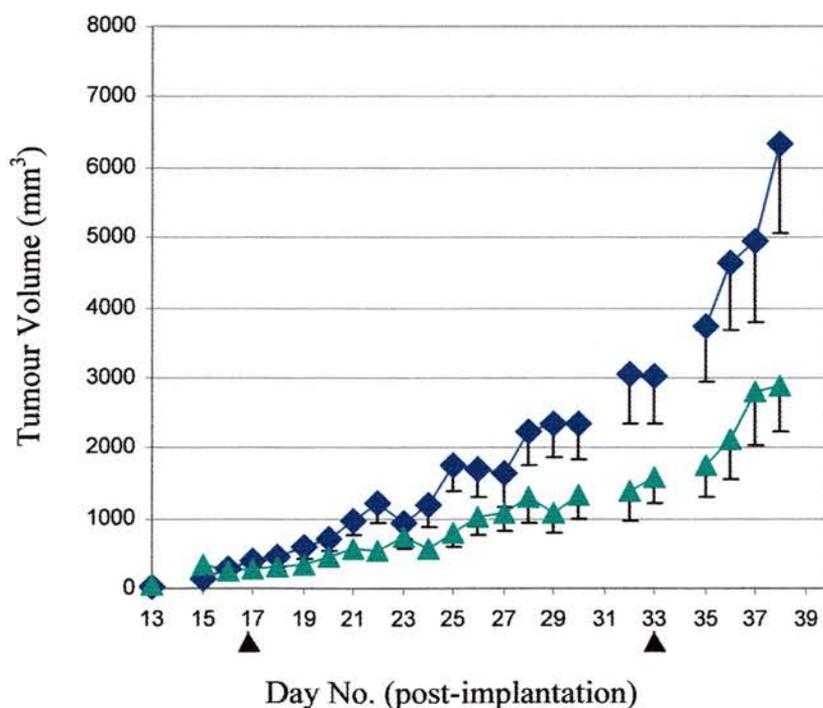
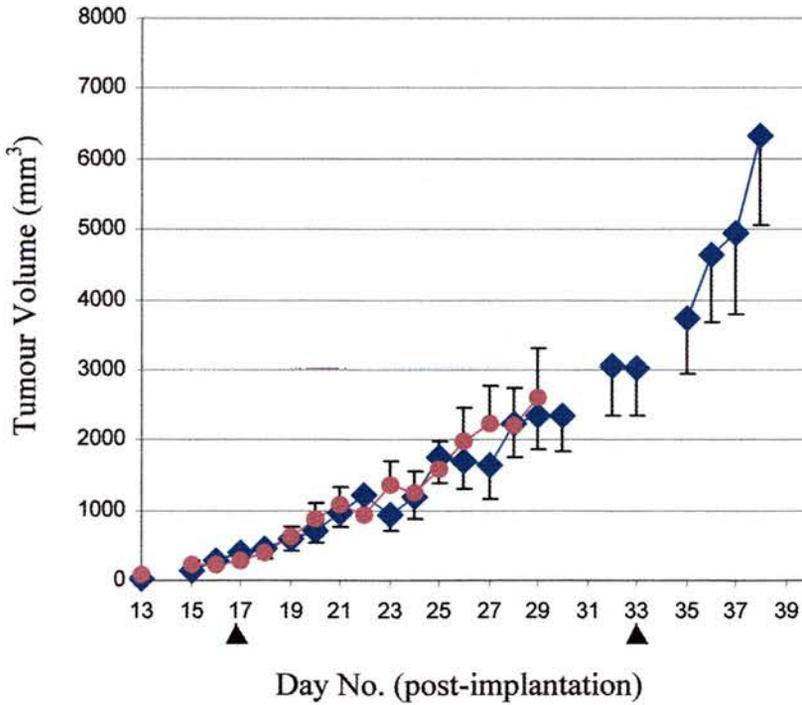
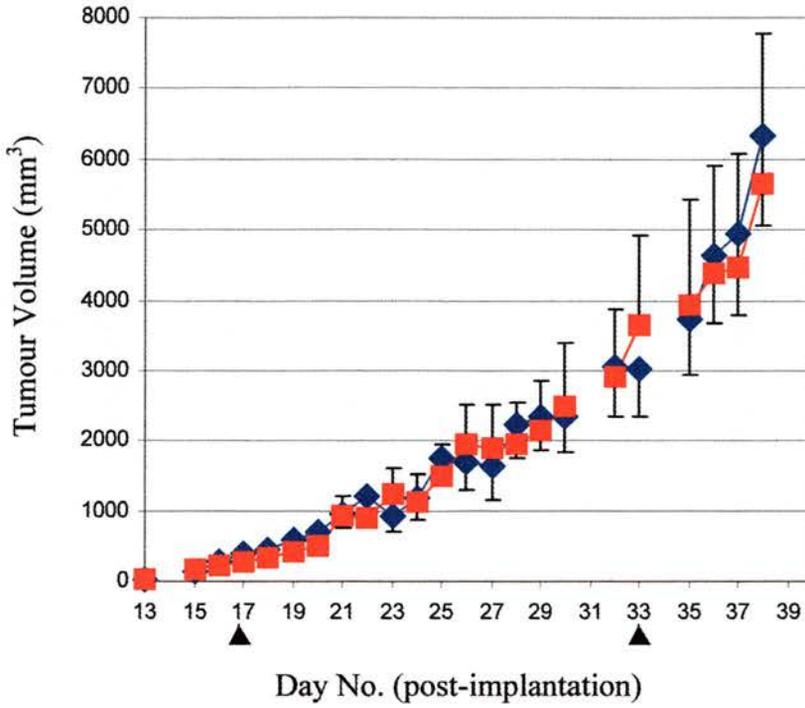


Fig. 2.12 Tumour volume (mm^3) of L-NAME-treated animals (green triangles) compared with Controls (blue squares)



Figs. 2.13 and 2.14 Tumour volume (mm³) of AG-treated animals (red squares; top) and 1400W-treated animals (pink circles; bottom) compared with Controls (blue squares).

It can be seen from figure 2.12 that L-NAME administration clearly had a marked inhibitory effect on the growth rate of tumours. Growth impairment was visible from ~ day 21, four days after commencing drug treatment, and was maintained throughout the duration of treatment. Two-way ANOVA, assuming a significance level of $p \leq 0.05$, showed that the growth of L-NAME-exposed tumours during drug-feeding (day +17 to +33) is significantly less than that of controls ($F = 4.370$, $p = 0.05$).

In contrast, figures 2.13 and 2.14 demonstrate that neither AG nor 1400W administration had any retarding effect on tumour growth. Two-way ANOVA demonstrates that growth was not significantly different from controls during the drug-feeding period (AG - $F = 0.015$, $p = 0.904$. 1400W - $F = 0.134$, $p = 0.719$).

2.4.7.2 Study Two

Drug commencement on day -7 (groups 2 and 4) or day +12 (groups 3 and 5) is not shown (it is off the graph axes). Drug cessation (all groups) on day +24 is denoted with an arrow.

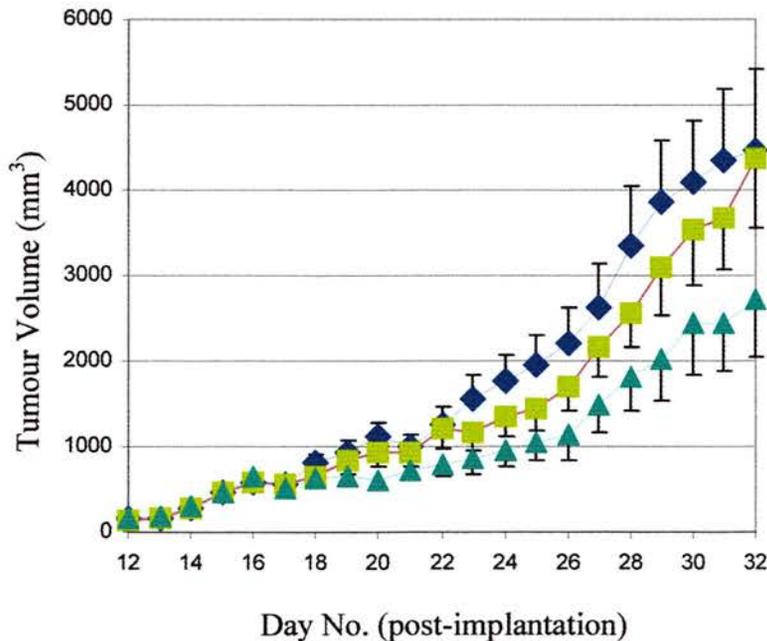


Fig. 2.15 Tumour volume (mm^3) of Control animals (group 1) (blue squares) and L-NAME-treated from day -7 (group 2) (green squares) and day +12 (group 3) (green triangles) animals.

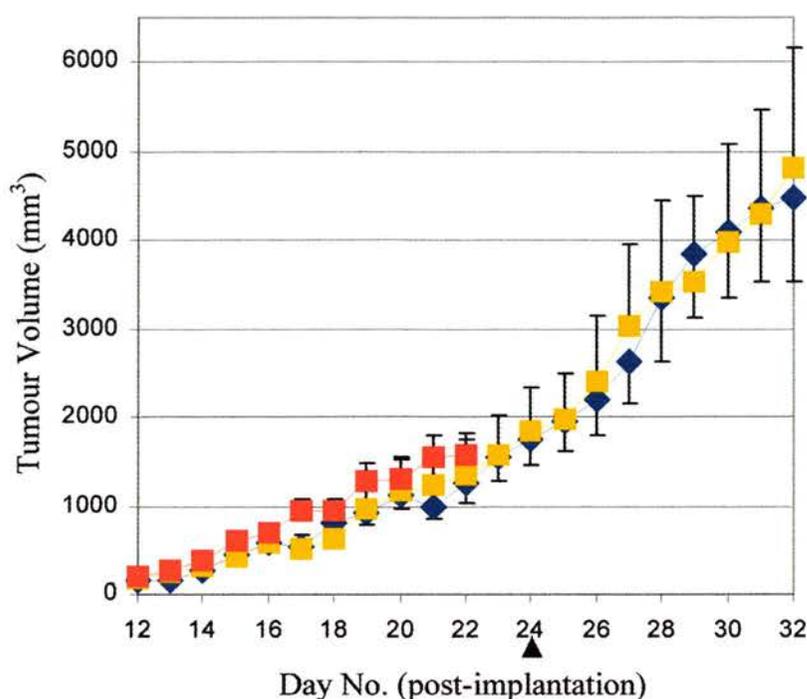


Fig. 2.16 Tumour volume (mm^3) of Control (group 1) (blue squares) and AG treated from day -7 (group 4) (orange squares) and day $+12$ (group 5) (red squares) animals.

It can be seen from figure 2.15 that L-NAME administration from day $+12$ (group 3) had a similar marked inhibitory effect on tumour growth as that seen with administration from day $+17$ (study one, Fig. 2.12). Slowed growth was evident from \sim day 18, five days after commencing drug treatment. Two-way ANOVA demonstrates that this growth retardation in L-NAME-fed animals was significant ($F= 5.923$, $p= 0.023$). The cessation of L-NAME administration caused a resumption in tumour growth rate back towards that of controls.

L-NAME administration prior to tumour implantation (day -7 , group 2), although retarding tumour growth relative to controls, was less effective than post-implantation administration. Slowed growth was evident only from \sim day 23. Two-way ANOVA showed that the growth of these tumours was not significantly different from that of controls ($F= 0.264$, $p= 0.612$).

Figure 2.16 confirms that AG-administration post-implantation (day $+12$, group 5) was ineffective at retarding tumour growth (see also fig. 2.13). Growth was not

significantly different from controls (2-way ANOVA $F= 0.795$, $p= 0.380$). Pre-implantation administration (day -7 , group 4) was similarly ineffective, also failing to reduce tumour growth rate significantly from control levels (2-way ANOVA $F= 0.296$, $p=0.592$).

Photographs (overleaf) of a control rat and one fed L-NAME following tumour implantation clearly illustrate the effectiveness of L-NAME-induced tumour growth-retardation in group 3 animals.



Plate 2.37 *Control Rat*



Plate 2.38 *L-NAME-fed Rat*

2.4.8 The Angiogenic Response

Although the use of paraffin wax-embedded tumour sections is generally recommended for MVD determination (Vermeulen *et al.*, 1996), dramatically less-effective anti-PECAM-1 staining (poor quality, weak) was obtained with these. This rendered accurate hot spot selection difficult. Therefore, in line with other researchers (Charpin *et al.*, 1995), cryostat sections of snap-frozen tumours were used instead (see appendix 8.3.2.4). These retained good morphology with minimal vessel fragmentation.

Successive H+E-stained tumour slices throughout half of a tumour block demonstrated remarkably similar vascular architecture throughout, reinforcing the validity of analysing a single tumour slice per block. The anti-PECAM-1 antibody gave sensitive and specific capillary staining, with almost undetectable background. The distribution of blood vessels throughout a tumour slice was variable although hot spots generally occurred within the peripheral regions. Sufficient practice made the process of consistent hotspot identification relatively straightforward.

An inherent problem in size matching of tumours was encountered between control and some L-NAME-treated tumours as a direct result of the effectiveness of drug treatment. Therefore, on account of the highly significant association between tumour size and vascular count (Horak *et al.*, 1992), a single tumour from each drug-treated group was selected for analysis - that which most closely matched the size of the control tumour. This unfortunately rendered statistical analysis of resulting Chalkley counts invalid. However, the individual sets of average count figures clearly demonstrate a reduced MVD in L-NAME-treated sections relative to controls (see Table 2.1). Additionally, photographs of representative tumour sections treated with anti-PECAM-1 immunoperoxidase staining provide clear visual evidence for the consequence of drug treatment on the angiogenic response.

Table 2.1 Chalkley Point Counts

DRUG REGIME	CHALKLEY POINT COUNTS			MEAN MVD
	Hotspot 1	Hotspot 2	Hotspot 3	
Control	8	10	7	8.3
L-NAME (17 days)	3	3	2	2.7
AG (17 days)	8	7	8	7.7
1400W (17 days)	9	6	5	6.6

Clearly, animals from study one which were fed L-NAME continuously for 17 days demonstrate markedly reduced intra-tumoural MVD and Chalkley point counts relative to control animals. In contrast, the tumour MVD of AG- and 1400W-fed animals is not dramatically different from that of controls, although that of the 1400W section must be interpreted with caution on account of section damage (see plate 2.36).

Plates 2.39 to 2.42 illustrate anti-PECAM-1 immunohistochemical staining of snap-frozen sections from the above tumours.

**Plate 2.39** Anti-PECAM-1 staining of a Control Tumour X360

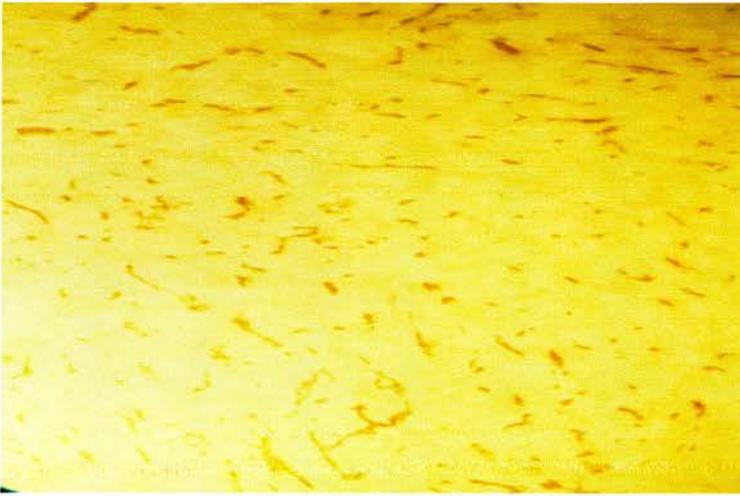


Plate 2.40 *Anti-PECAM-1 staining of an L-NAME-treated Tumour X360*

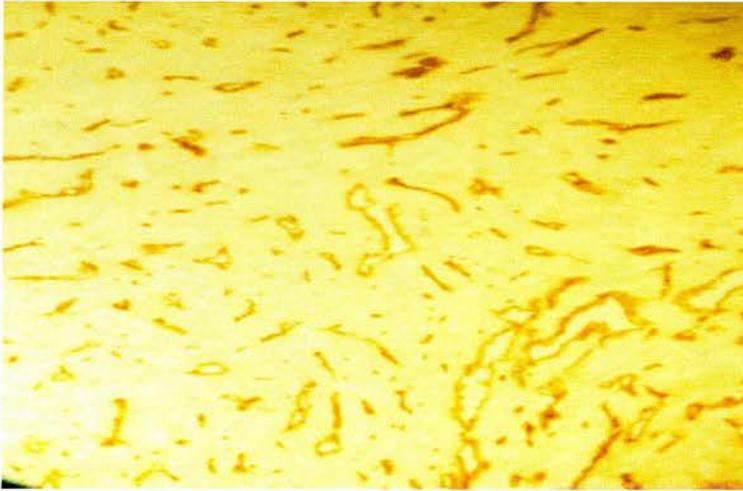


Plate 2.41 *Anti-PECAM-1 staining of an AG-treated Tumour X360*

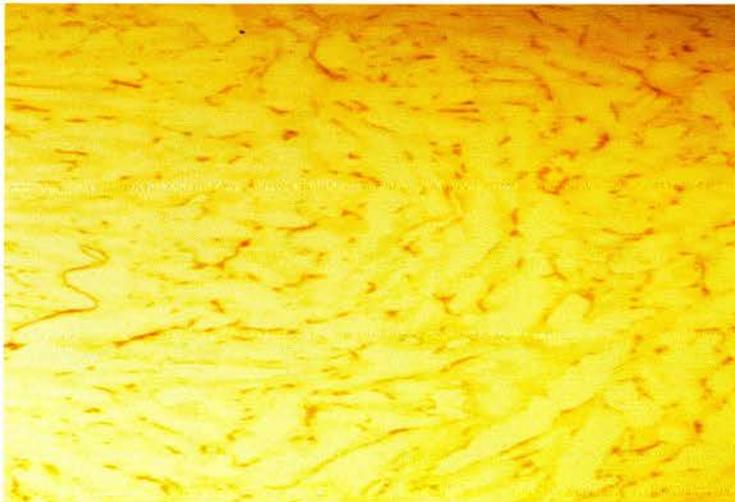


Plate 2.42 *Anti-PECAM-1 staining of a 1400W-treated Tumour X360*

The control tumour section was clearly highly vascularised. It was 'pitted' with a very high density of blood vessels, many of which were large and dilated. In contrast, the L-NAME-treated tumour contained markedly less vasculature. There was also a distinct absence of larger, dilated vessels in this section, with the majority of visible vessels appearing small and scattered. The AG-treated tumour was similar in vascularity to the control. Unfortunately, the 1400W-treated tumour was badly damaged with defrost artefacts, rendering an accurate assessment of the vasculature difficult. However, from the pattern of staining discernible, it appeared well vascularised, demonstrating a similar vessel distribution to the control section and including a population of larger, dilated vessels.

2.4.9 Metastasis

Metastatic tumour cells could be identified within lung/liver sections relatively easily, appearing as tightly-packed, densely-staining clusters of cells. In lung sections, metastasised tumour cells were very distinct within the pale, air-filled alveolar tissue. Metastases were most often found in the vicinity of blood vessels and could be seen being shed through the vessel wall in a number of sections. Occasional metastases were visible surrounding liver blood vessels. These were again very obvious amongst the pale-staining hepatocytes. Metastasis identification was confirmed by a pathologist (Dr Oliver Schmidt, School of Biology, University of St Andrews).

Plates 2.43 and 2.44 illustrate lung/liver sections from control and drug-treated animals.

Staining of the control group lungs revealed that all 3 control animals from study one, (100%), were bearing one or more lung metastases. L-NAME and AG-treated groups demonstrated similar secondary tumour formation, with 2 out of the 3 animals in each group (67%) bearing one or more lung colonies. Liver metastases were only observed in control and AG-treated animals. No analysis was performed in 1400W-treated animals.

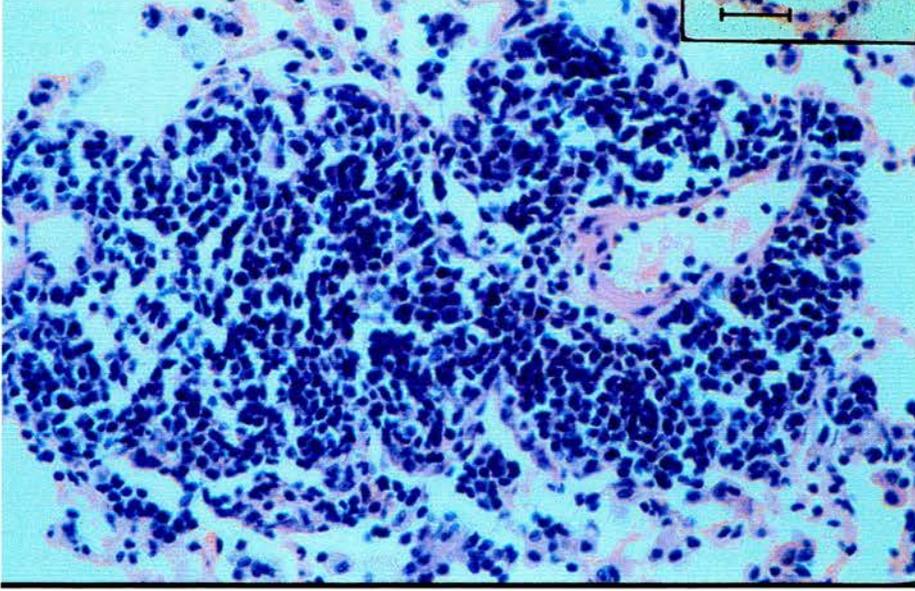


Plate 2.43 *Secondary Tumour Metastasis* (H+E; X400)

A large, solid mass of tightly-packed, compact, darkly-staining tumour cells amongst the alveolar tissue is evident (scalebar = 25µm).

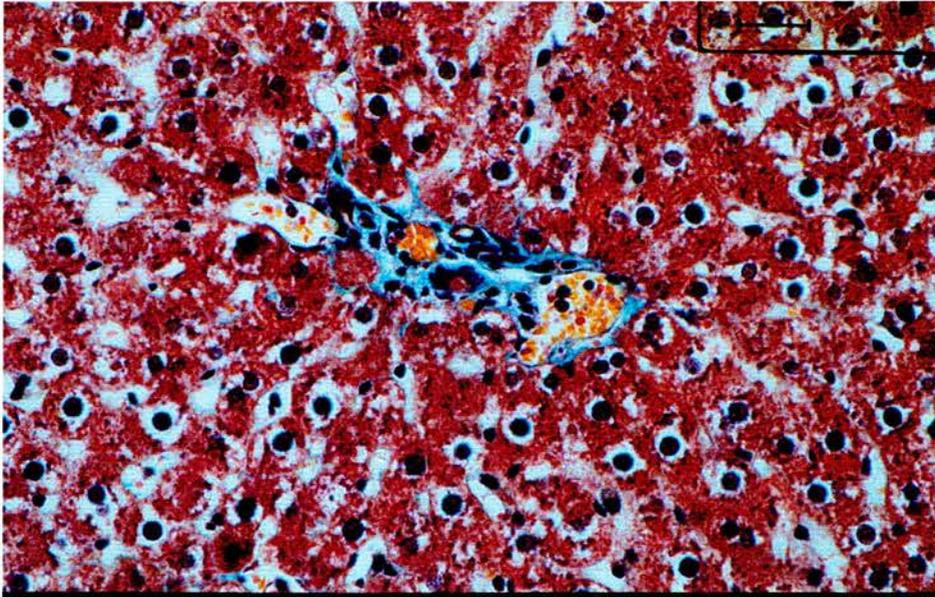


Plate 2.44 *Secondary Liver Metastasis* (Masson's trichrome; X400)

Two blood vessels filled with yellow-staining erythrocytes are visible within the centre of the field of view. Small, dark metastasising tumour cells can be seen to have escaped from these vessels and be forming a secondary tumour amongst the hepatocytes (scalebar = 25µm).

2.5 Discussion

2.5.1 NOS Inhibitors and Animal Wellbeing

The toxicity of the NOS inhibitors administered in this study was minimal, despite their continuous presence in the drinking water for up to 31 days. However, both L-NAME and AG induced a temporary reduction in drinking rate following initial administration. The transience of this effect suggests that the drugs were noxious to the animals at first. 1400W failed to induce this temporary reduction in drinking rate. No permanent reduction in drinking rate or weight change was however, noted in any group. This is in contrast to a number of other investigators who have noted a significant increase in body weight in animals administered AG chronically (Mattson *et al.*, 1998) and a progressive decrease in body weight in animals during L-NAME treatment (Zhao *et al.*, 1999). L-NAME has been shown to suppress food intake in rats (Squadrito *et al.*, 1993), mice (Morley & Flood, 1991) and chickens (Choi *et al.*, 1994). These effects either implicate L-NAME in the regulation of appetite (Vozzo *et al.*, 1999), or maybe, in the doses required to reduce food intake, a non-specific noxious effect was leading to aversion rather than a true suppressive effect on appetite. Either way, the dose of L-NAME which was effective in reducing angiogenesis and retarding tumour growth in this study did not affect food ingestion by the animals. In agreement with other observers (Gallo *et al.*, 1998; Ziche *et al.*, 1994), L-NAME did not have a long-term effect on the amount of ingested water either.

The observation that 1400W was well tolerated, failing to cause weight loss or affect animal wellbeing is in agreement with the findings of other observers (Thomsen *et al.*, 1997).

2.5.2 NOS Inhibitors and Haemodynamic Parameters

Problems with the blood pressure monitoring equipment rendered the technique, with a statistically-significant numbers of animals, extremely time-consuming. Blood pressure measurement was not therefore repeated for subsequent animal studies.

The blood pressure of all animals in this study remained within normal confines, with systolic pressure fluctuating around 150-200 and diastolic around 80-120 mmHg.

L-NAME-fed animals tended toward the upper end of this range, demonstrating statistically-significant mild hypertension relative to the other groups. This is in keeping with the results of others (Chyu *et al.*, 1992; Gardiner *et al.*, 1990 and 1990b; Sorrentino & Pinto, 1997) and indicates that the dose of L-NAME administered was effective in inhibiting eNOS.

Chronic AG infusion has been associated with an increase in MABP (Mattson *et al.*, 1998), but at the dose used here, AG-fed animals remained normotensive. This confirms the relative selectivity of AG for iNOS over eNOS in this system. 1400W-treated animals remained similarly normotensive, as expected with the administration of such a highly-selective iNOS inhibitor.

2.5.3 Tumour Histology and Necrosis

None of the drug regimes tested here had any noticeable effect on the histology of tumours. L-NAME-treated tumours demonstrated less necrosis than AG/1400W or control tumours by virtue of having their growth held in check. A distinct lack of immune infiltrate across all sections demonstrated that there was no host immune response to the presence of tumours. This is as expected for the use of isogenic animals and tumour tissue.

2.5.4 NOS and KDR/Flk-1 Localisation

The experimental tumour used here expressed both e- and iNOS, the former more substantially than the latter. Weak expression of iNOS could be attributed in part to the isogeneity of both host and tumour - the absence of an immune response to the implanted tumour will be expected to limit the presence of iNOS-inducing pro-inflammatory stimuli within the tumour environment. Expression of both NOS isoforms was confined exclusively to the vasculature. There was a conspicuous lack of macrophage iNOS expression, further demonstrating the lack of host immune response to the presence of the tumour.

Distribution of both e- and iNOS was markedly less in L-NAME-treated tumours than controls. However, bearing in mind the vascular specificity of NOS expression, this diminished distribution may simply reflect a reduced MVD in these sections

relative to controls. A striking absence of iNOS expression in 1400W-treated tumours reinforces the expressional changes evident in plate 2.36.

Intra-tumoural expression of KDR/Flk-1 receptors was conspicuously not confined to the endothelial cell population. Receptors were widespread across all tumour and endothelial cells in all sections, although blood vessels stained more darkly in some areas. Accordingly, the EC lining of small blood vessels in tumour stroma have previously been reported to label strongly for KDR/Flk-1 mRNA (Brown *et al.*, 1993). It is possible that a proportion of the positively-staining cells across these sections represent angioblasts, preparing to develop into ECs. These have previously been reported to express KDR/Flk-1 (Tufro-McReddie *et al.*, 1997).

The distribution of KDR/Flk-1 staining did not appear significantly different between control and drug-treated tumours. As reviewed in section 1.11.3, tumour cell KDR/Flk-1 expression has previously been documented by a number of observers. Clearly the experimental carcinosarcoma studied here can be added to the list of transformed tissues in which non-endothelial cell expression of this VEGF receptor occurs (see also section 4.6.3.3).

2.5.5 NOS Expression

The administration of NOS inhibitors induced considerable modification of the intra-tumoural expression of the NOS isoforms. It should be noted however, that the information gathered from Western blot analysis provides no information regarding the *activity* of the NOS isoforms and the consequential production of functional NO. It simply provides a measure of the amount of enzyme expressed.

The level of expression of eNOS was not affected by the administration of either L-NAME or AG. Surprisingly however, subsequent withdrawal of AG-feeding induced a ‘rebound’ reduction in eNOS expression. An explanation for this is elusive.

1400W induced a marked reduction in tumour eNOS expression which was not reversed with subsequent drug-withdrawal. The lack of effect of 1400W on blood pressure, however, suggests that the reduced expression of eNOS noted here is not translated into a reduction in NO production. That is, although the amount of eNOS

protein present is reduced by drug-treatment, a compensatory ‘activation’ of such enzyme as is expressed, may occur.

In contrast to its lack of effect on eNOS expression, L-NAME induced a marked reduction in iNOS expression which was reversed following drug-withdrawal. It could be hypothesised that L-NAME, by reducing iNOS substrate availability, may induce a concurrent reduction in enzyme expression which is reversed upon drug-withdrawal when the substrate limitation is removed.

AG has previously been observed to inhibit iNOS induction (Ruetten & Thiemermann, 1996b) and in agreement with this, it induced a reversible reduction of iNOS expression here. In contrast, isothioureas have not previously been found to affect iNOS induction (Szabo *et al.*, 1994). However 1400W dramatically reduced tumour iNOS expression here, an effect persisting even following its withdrawal.

2.5.6 Tumour Growth

In agreement with previous findings (Bisland, 1996; Kennovin *et al.*, 1994a), chronic oral administration of L-NAME after tumour implantation significantly retarded the growth rate of tumours. Marked differences in the extent of growth retardation were noted with different times of drug-administration, relative to tumour implantation. Post-implantation administration (days +12/+17) retarded growth maximally, in contrast to pre-implantation administration (day -7) which, although retarding growth relative to controls, was much less effective and failed to attain statistical significance. This time-dependency of effective treatment has been observed previously (Kennovin *et al.*, 1994a) and may be a consequence of the stage of tumour growth. An increase in experimental tumour NOS expression has been documented around days 12-14 post-implantation (Buttery *et al.*, 1993). Additionally, eNOS-transfected tumour cells implanted into animals have been shown to grow slowly at first but to increase their growth rate above that of controls between days +30 - +40 post-implantation (Liu *et al.*, 1998). Failure of early drug administration may therefore indicate that the amount of NO synthesised at earlier stages is not high enough to be influenced by NOS inhibitors. Clearly if NO potentiates tumour growth in the later stages, then a correspondingly later drug administration will be more effective in reducing growth.

In contrast to previous findings from this group (Bisland, 1996), iNOS-selective inhibitors were wholly ineffective at reducing tumour growth rate. Chronic, oral administration of either AG or 1400W, pre- (day -7) or post-tumour implantation (day +12/+17), failed to limit tumour size. Interestingly, mixed results have been obtained by other investigators with the use of AG for retarding tumour growth. Although it has been shown to reduce, significantly, the tumour growth of one particular line of iNOS-expressing cells (Ambs *et al.*, 1998) it is claimed to be ineffective in a more slow-growing iNOS-expressing tumour line. One study found that AG *increased* tumour growth (Lind *et al.*, 1997).

The failure of 1400W to retard tumour growth in this study is in variance with the findings of others (Thomsen *et al.*, 1997). It may however be that the dose provided here was insufficient. A dose of 7mg.Kg.hr^{-1} (168mg.Kg.day^{-1}) fails to produce a significant reduction in tumour growth in mice (Thomsen *et al.*, 1997). The maximum ingested dose of 99mg.Kg.day^{-1} achieved in this study (see appendix table 8.3) is clearly well below this threshold. It is also possible that oral administration of 1400W is not effective. This is the first study to administer the drug via this route.

Overall, based on the findings presented here, it is tempting to speculate that eNOS is perhaps of more significance than iNOS in the promotion of tumour growth. The situation however, is clearly more complex and is discussed more fully in chapter six.

2.5.7 Angiogenesis

Tumours exposed to L-NAME demonstrated markedly less vasculature than their corresponding controls. The constraint of analysing a single tumour section from each drug-treated group clearly limits the ability to draw strong, quantitative conclusions from the Chalkley point counts. However, a remarkable reduction in MVD was also evident from microscopic examination of PECAM-1-stained immunohistochemical sections from L-NAME-treated animals. This effectively corroborated the anti-angiogenic response indicated by the Chalkley point counts. Clearly the vasculature of the tumour is highly susceptible to systemic NOS inhibition in the proliferative state, and L-NAME is an orally-active anti-angiogenic compound in this rodent model.

Previous investigators have attenuated the angiogenic response in rabbits by systemic L-NAME administration of 0.5 and 1 mg.ml⁻¹, an approximate ingested dose of 100 and 200 mg.day⁻¹ per rabbit, respectively (Gallo *et al.*, 1998; Ziche *et al.*, 1994).

The results presented here demonstrate that L-NAME administration in much lower doses (ingested dose of 30-45mg.day⁻¹) is highly effective in limiting solid tumour angiogenesis and growth.

Chalkley point counts and PECAM-1-staining indicated that AG did not influence the MVD of tumours. This is in keeping with its failure to retard the growth rate of tumours, but is in opposition to the findings of others (Ambs *et al.*, 1998). Other investigators have concluded that the doses of AG required to elicit a considerable inhibition of iNOS (and thus elicit beneficial biological effects) in rats, are rather high – 15-45 mg.Kg⁻¹ *i.v.* (Wu *et al.*, 1995). However, problems of toxicity may be encountered with chronic, high-dose administration (Sothan & Szabo, 1996). Even if the failure to introduce sufficient AG is responsible for the lack of an anti-angiogenic effect observed here, it does not explain the failure of AG to retard tumour growth. Previous investigators have achieved growth-retardation with AG at the same doses used here (Bisland, 1996).

Chalkley point counts and PECAM-1-staining indicated that 1400W failed to influence the MVD of tumours. This is in keeping with its failure to retard the growth rate of tumours and is considered more fully in chapter six.

2.5.8 Metastasis

By the time of sacrifice, after 33 days of tumour growth, all control animals (100%) demonstrated one or more metastatic lung tumours. This is testimony to the aggressive growth characteristics of this tumour. The density of the microvessel bed within a tumour is likely to provide a direct measure of the size of the vascular window through which tumour cells must pass to spread to distant body sites. Accordingly, L-NAME-treated animals displayed less metastases than control – 67%. However, since AG-fed animals demonstrated a similarly-reduced level, the relationship is clearly more complex than simply tumour vascularity. Of note was the fact that the only colonies of liver secondaries evident were confined entirely to

control and AG-fed animals. Whether the livers of L-NAME-fed animals contained tumour cells which were not evident (being held in a non-vascularised state) remains undetermined. Since the establishment of metastases is directly related to the number of tumour cells shed into the circulation (Liotta *et al.*, 1974), it can be anticipated that larger tumours represent the most significant risk for secondary tumour formation.

Chapter Three

NO Scavengers and the Solid Tumour

3.1 Introduction

The findings of the previous chapter illustrate that NO is clearly involved in the angiogenic response of the experimental solid *p22* carcinosarcoma. Reducing NO, through the systemic administration of the NOS inhibitor L-NAME, produced a marked inhibition of intra-tumoural angiogenesis and a potent tumour growth-retarding effect. The research presented in this chapter examines the anti-tumour efficacy of an alternative NO-depleting regime, NO *scavenging*. The NO-scavenging compounds used here have previously been tested in rodent and swine models of septic shock (Fricker *et al.*, 1997; Fricker, 1999). They demonstrated beneficial effects in these models, through their potent elimination of NO. The research presented here is the first to assess these compounds in an *in vivo* solid tumour model.

Two chemically-related polyaminocarboxylate-ruthenium (III) complexes: potassium chloro[hydrogen(ethylenedinitrilo)tetraacetato]ruthenate (*AMD6221*) and aqua[hydrogen(ethylenedinitrilo)tetraacetato]ruthenium (*AMD6245*) (see section 1.4.5.2), were tested in experiments designed to do the following:

1. Study the effect of NO scavenging on animal general well-being, by direct observation (survival, body weight, drinking rate). This provided a gauge of possible drug toxicity.
2. Test the efficacy of the NO scavenging regime, by measuring nitrite (NO_2^-) and nitrate (NO_3^-) levels ($=\text{NO}_x$) in blood plasma at the time of sacrifice.
3. Examine the effect (if any) of drugs on the histology (including necrosis) of primary experimental solid tumours.
4. Examine the localisation of eNOS, iNOS and KDR/Flk-1 within control and drug-treated tumours, by immunohistochemistry.
5. Determine any effect of drugs on the expression of e- and iNOS isoforms within control and drug-treated tumours.
6. Examine the differential effects of the two NO scavengers on the growth rate of tumours.

7. Determine whether NO scavenging influences the angiogenic response of tumours by measuring intra-tumoural MVD.
8. Assess the effect of drugs on the formation of metastatic tumours in the lung and liver of animals.

Based on the results of chapter two, drug administration *post*-tumour implantation was examined. The impact of a period of drug-withdrawal was similarly explored.

3.2 Materials and Methods

3.2.1 Methods

3.2.1.1 Animal Tumour Model

The *in vivo* tumour model used was the same as previously (see section 2.2.1.1 and appendix 8.1). In this study however, only 2 tumour nodules were grown in each animal, one towards the front and one towards the rear of the dorsal surface. Tumour volume was measured daily, with hand-held skin-fold calipers.

3.2.1.2 NO Scavengers

The water solubility of the two compounds was relatively poor, and their oral bio-availability (membrane permeability) was questioned. They were therefore administered by *i.p.* injection. This combined administration into a highly-vascularised compartment, facilitating absorption, with relatively slow absorption, to compensate for their rapid clearance. Daily injections were given to maintain blood levels.

AMD6221 was provided as a sterile solution (50mg.ml⁻¹ in 0.2M PO₄, pH 7.4). This was used for the first 4 days of drug treatment. From the fifth day onwards, following some degradation of the compound, fresh solution was made up from solid compound. AMD6245 was provided as a solid which was similarly dissolved at 50mg.ml⁻¹ in sterile 0.2M PO₄ buffer. This was made up fresh each day, immediately

prior to use as it was not air stable, rapidly forming a dimer in the presence of oxygen. All drugs were sterile filtered prior to injection.

AMD6245 provided daily at 50mg.Kg^{-1} for 7 days has been shown to have beneficial effects in a rat model of colitis (S.Fricker, per.comm). Drugs were therefore administered to animals as a single daily *i.p.* injection at a dose of 50mg.Kg^{-1} .

3.2.1.3 Measurement of NO in Blood Plasma

The Griess test was performed on samples of blood harvested from animals at the time of sacrifice. This is a two step procedure consisting of the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) using nitrate reductase (NR), followed by spectrophotometric determination of NO_2^- as a coloured azo dye product.

Blood samples (~8mls) were obtained from the thoracic cavity of animals killed by anaesthetic overdose. These were immediately centrifuged at 13,000 RPM for 10 mins at RT and 2ml aliquots of the supernatant plasma were pipetted into cryovials and snap frozen. Prior to sampling, plasma was concentrated by freeze drying for 24-48 hours. Samples (2ml) containing low NO_x (from drug-treated animals) were reconstituted in 0.5ml Milli-Q water (4X concentration) and those with higher NO_x (control animals) were reconstituted in 8ml Milli-Q water (4X dilution). The Griess test was performed as described in appendix 8.5.

3.2.1.4 Tumour Harvesting

At the time of sacrifice, tumours were harvested as previously, with portions snap frozen for immunohistochemistry and angiogenesis scoring and a single tumour per animal dissected out and processed for histological examination. However, the equivalent tumour portions which were diced and snap-frozen for homogenate production were first separated into cortical (outer 3mm) and core regions, to analyse separately.

3.2.1.5 Tumour Histology

The gross structure of control and drug-exposed tumours was examined in paraffin-wax sections. (see appendix 8.2).

3.2.1.6 Immunohistochemistry

The localisation of eNOS, iNOS and KDR/Flk-1 within control and drug-treated tumours was examined using immunoperoxidase staining of snap-frozen, cryostat sections (see appendix 8.3).

3.2.1.7 Biochemical Analysis

The expression of e- and iNOS proteins in core and cortical regions of control and drug-exposed tumour extracts was quantified by 6% SDS-polyacrylamide gel electrophoresis and Western Blotting techniques (see appendix 8.4).

3.2.1.8 Angiogenesis Scoring – Microvessel Density (MVD) Determination

Quantification of angiogenesis in control and drug-treated tumours was performed as previously, in snap-frozen tumour sections (see appendix 8.3) utilising an anti-PECAM-1/CD31 antibody and Chalkley point counting method (see section 1.9.7.2). Tumours for analysis were matched in terms of front or rear growing position, and were again size-matched as closely as possible.

3.2.1.9 Secondary Tumour Formation – Lung and Liver Metastasis

The metastasis of tumours to the two most common secondary organ sites was examined using histological stains. A representative section of lung and liver was removed from each animal at the time of sacrifice and processed for paraffin-wax embedding (see appendix 8.2). The number of animals per drug group demonstrating one or more metastatic foci was determined and expressed as a percentage of the total group.

3.2.2 Materials

3.2.2.1 Drugs

Both AMD6221 and AMD6245 were obtained from Anormed Inc, Vancouver, CA.

All other materials are as for Chapter 2 (see section 2.2.2).

3.3 Experimental Protocol

Rats were separated into 3 groups of 8 animals/group. Group 1 were control animals. Groups 2 and 3 received daily *i.p.* injections of AMD6245 and AMD6221 respectively. Taking day 0 to represent the day of tumour implantation, rat body weight and drinking rate were monitored from day -10. Tumours were visible from day +7 and measurable from day +10. Drug injections were commenced on day +10, for 18 consecutive days (until day +27). At this time, 3 animals in each group were sacrificed as examples of animals which had received NO scavengers continuously. The remaining 5 rats per group underwent a seven day period without drug injections and were sacrificed on day + 35.

3.4 Results

3.4.1 Body Weight

The graph overleaf (Fig. 3.1) illustrates the changes in body weight of the animals during the course of the experiment (mean \pm SEM). Arrows denote the commencement and cessation of drug administration on days +10 and +28 respectively.

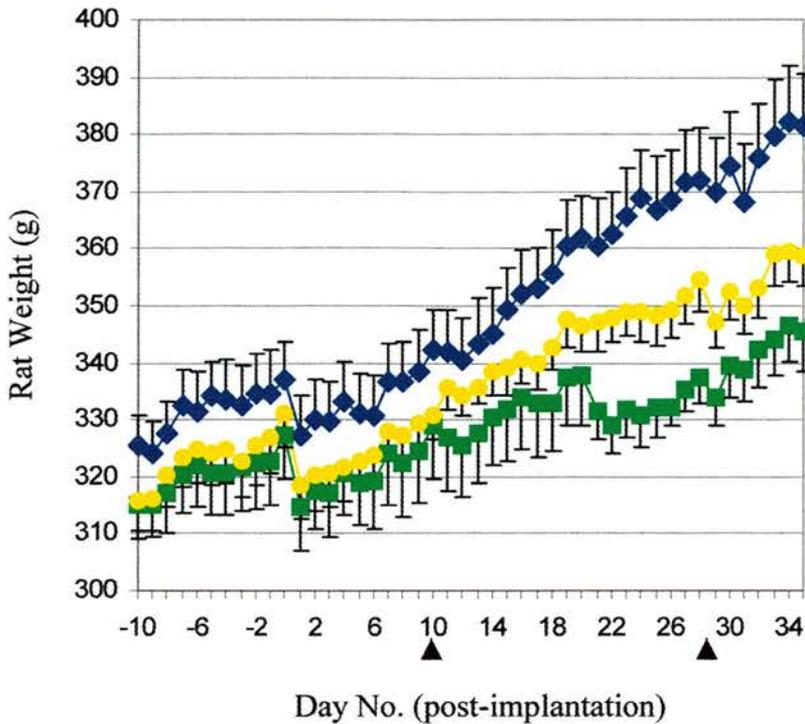


Fig. 3.1 Changes in body weight (g) of AMD6221- (yellow circles) and AMD6245-treated (green squares) animals, compared with Controls (blue squares).

A baseline level was determined by making measurements for 10 days prior to tumour implantation (20 days before commencing drug administration). All groups can be seen to lie within a narrow weight range (300-350g) prior to tumour implantation and to remain within the 300-450g range for the duration of the experiment.

A temporary (24hr) reduction in weight occurred in all groups immediately after implanting the tumour (day +1). Animals treated with AMD6245 (but not AMD6221) showed a further transient weight loss around day +10, coinciding with the onset of drug administration. With the exception of a 48hr drop in weight on day +21 in the AMD6245-administered group, all groups can be seen to gain weight steadily throughout the experiment.

3.4.2 Drinking Rate

The graphs below (Figs. 3.2 and 3.3) illustrate the drinking rate (mean \pm SEM) of animals over the course of the experiment. Arrows denote commencement and cessation of drug administration on days +10 and +28 respectively.

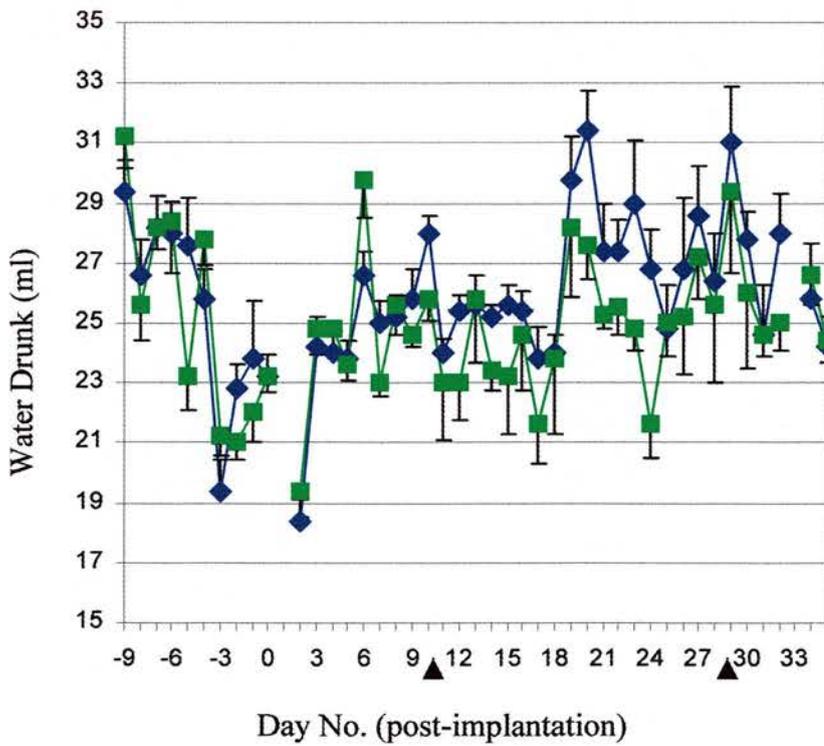
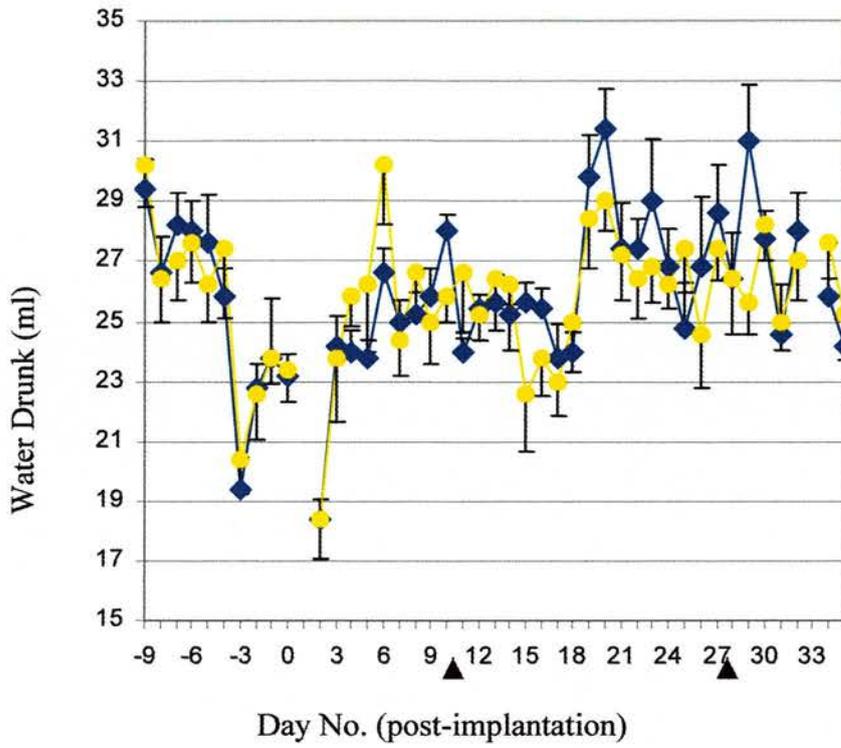
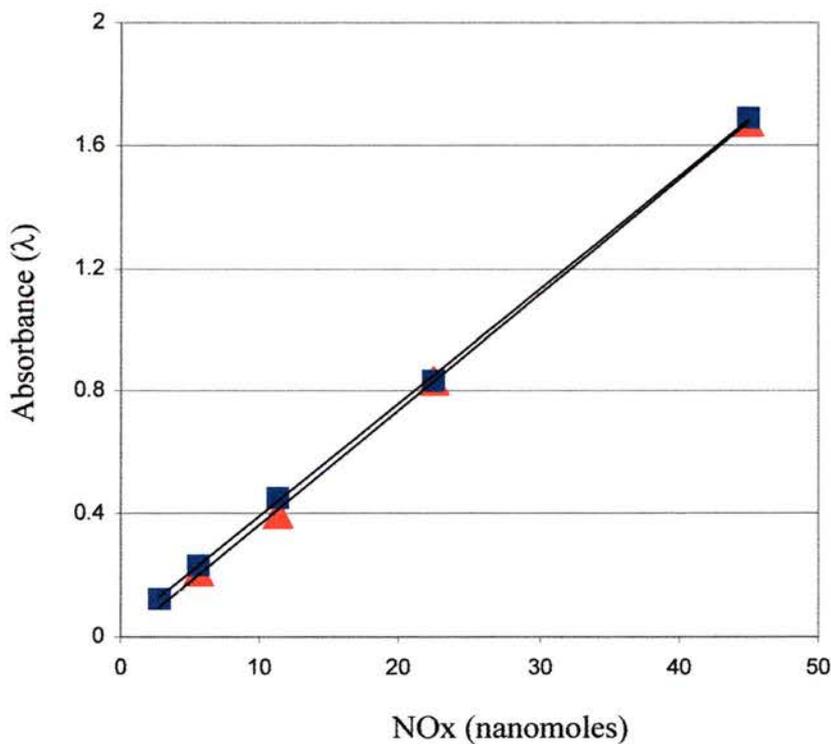


Fig. 3.2 and 3.3 Amount of water (ml) consumed by AMD6221-(yellow circles; top) and AMD6245-treated (green squares; bottom) animals, compared with Controls (blue squares).

Once again baseline values were established by monitoring water intake for 10 days prior to tumour implantation. A transient (48hr) reduction in drinking rate occurred in all groups on day +1 i.e. shortly after surgical tumour implantation on day 0. After this point, and for the duration of the experiment, drinking rate remained within the expected confines for animals of this weight.

3.4.3 Griess Assay

Standard curves for nitrate and nitrite, displayed below (Fig. 3.4) show that the conversion of nitrate to nitrite under these experimental conditions is almost 100%.



$$\text{NO}_3^- \quad y = 0.0374x - 0.0069. \quad R^2 = 0.9998$$

$$\text{NO}_2^- \quad y = 0.0369x + 0.0232. \quad R^2 = 0.9996$$

Fig. 3.4 The Conversion of Nitrate (NO_3^-) (red triangles) to Nitrite (NO_2^-) (blue squares)

A problem was encountered in making the measurements, due to insufficient yield of plasma and difficulty of dissolving freeze-dried samples into the required (small) volume of water. A number of erroneous absorbance readings were obtained which had to be removed from the subsequent calculations and statistics. However, the trend was as expected for compounds that are known to have NO scavenging ability.

Based on the gradient of the standard NO_3^- curve above, control animals sacrificed at the time of drug-withdrawal contained levels of $7.75\mu\text{moles/l}$ plasma. Control animals sacrificed at the end of the experiment 7 days later showed similar levels, $7.15\mu\text{moles/l}$ plasma. Animals receiving AMD6245 continuously showed much reduced levels of $3.88\mu\text{moles/l}$ plasma. After withdrawing drug-treatment, the concentration rose to $6.87\mu\text{moles/l}$ plasma, tending back towards that of control animals. Animals receiving AMD6221 continuously also showed reduced levels compared with controls, $5.09\mu\text{moles/l}$ litre. No data is available for the AMD6221-withdrawal group.

Assuming a significance level of $p \leq 0.05$, t-tests assuming equal variance show that animals treated with AMD6245 continuously showed significantly lower NO_x levels relative to controls ($t = 4.486$, $df = 8$, $p = 0.002$). However, there was no significant difference between the two after 7 days of drug-withdrawal ($t = 0.791$, $df = 6$, $p = 0.459$). The reduction in plasma $[\text{NO}_x]$ in animals administered AMD6221 continuously just failed to reach statistical significance ($t = 2.234$, $df = 7$, $p = 0.060$).

3.4.4 Histology

Paraffin-wax sections of mature tumours demonstrated similar histological detail as those from control/NOS-inhibitor-fed animals in the previous chapter (see section 2.4.4). Tumours from animals administered NO scavengers were markedly smaller than control tumours and, accordingly, contained less extensive necrosis.

3.4.5 Immunohistochemistry

Immunohistochemical staining of snap-frozen tumour sections was good quality, demonstrating high specificity and low background. All negative controls were similarly clean.

3.4.5.1 *eNOS*

As observed in the immunohistochemical staining from the previous chapter (section 2.4.5.1), staining for eNOS was highly-specific for the tumour vasculature. Plates 3.1 to 3.3 (overleaf) illustrate anti-eNOS staining from a control animal and from animals administered drugs continuously. Plates 3.4 to 3.6 illustrate staining from a control animal and from animals administered drugs then allowed a drug-withdrawal period.

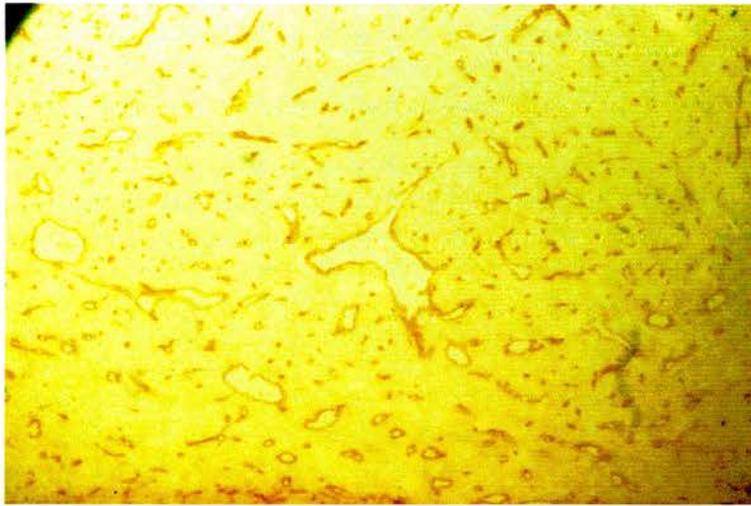


Plate 3.1 *Anti-eNOS staining of Control tumour X360*

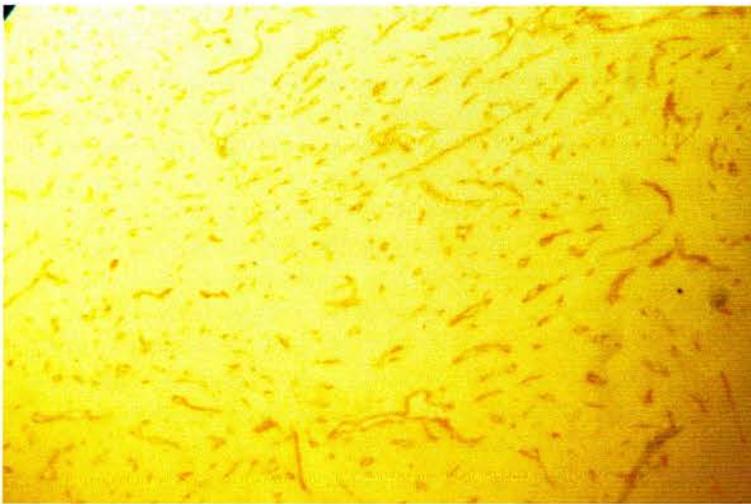


Plate 3.2 *Anti-eNOS staining of AMD6221-treated (continuously) tumour X360*



Plate 3.3 *Anti-eNOS staining of AMD6245-treated (continuously) tumour X360*

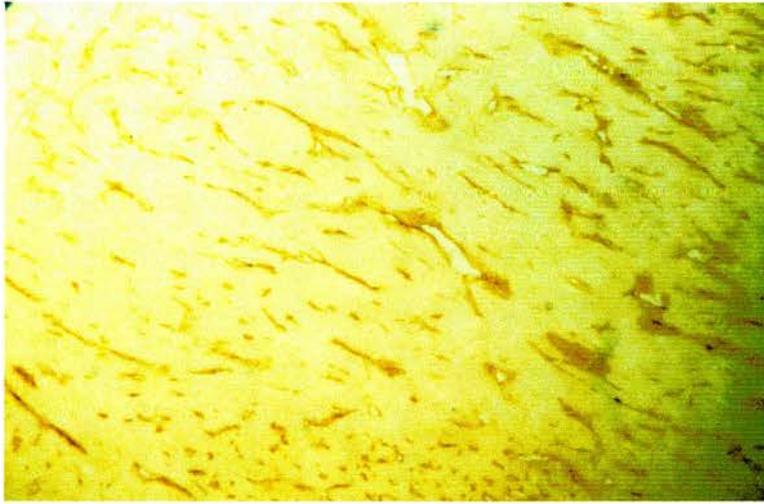


Plate 3.4 *Anti-eNOS staining of Control tumour (at time of drug-withdrawal) X360*

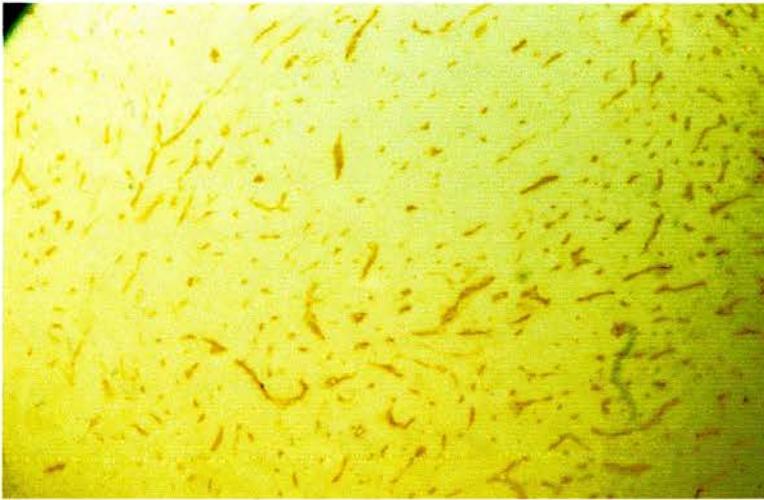


Plate 3.5 *Anti-eNOS staining of AMD6221-treated (with withdrawal) tumour X360*

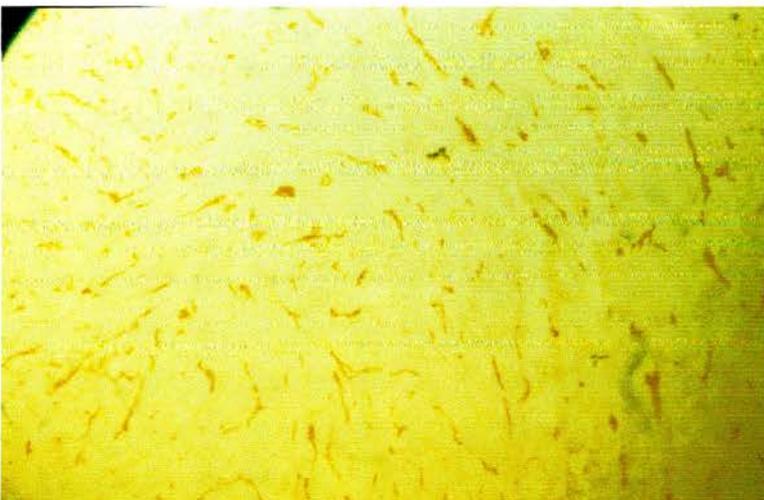


Plate 3.6 *Anti-eNOS staining of AMD6245-treated (with withdrawal) tumour X360*

The intensity of staining for eNOS appeared similar across all sections. However, sections from drug-treated animals display a marked reduction in the distribution of eNOS stain, relative to controls. Bearing in mind the vascular specificity of the stain, this diminished distribution may simply reflect a reduced MVD in these sections. Staining was also reduced in the core region of tumours relative to more peripheral, cortical areas. Seven days of drug withdrawal did not cause any obvious changes in eNOS distribution.

3.4.5.2 *iNOS*

Again, similar to findings in the previous chapter (section 2.4.5.2), staining for iNOS was extremely weak, requiring high antibody concentrations and long incubation times. This meant that it was not possible to discern accurately, differences between control and drug-treated sections. Nevertheless, staining was specific and clean, once again being confined entirely to the vasculature. Plates 3.7 to 3.9 illustrate staining from a control animal and from one each of a continuously-administered AMD6221 and AMD6245 animal.

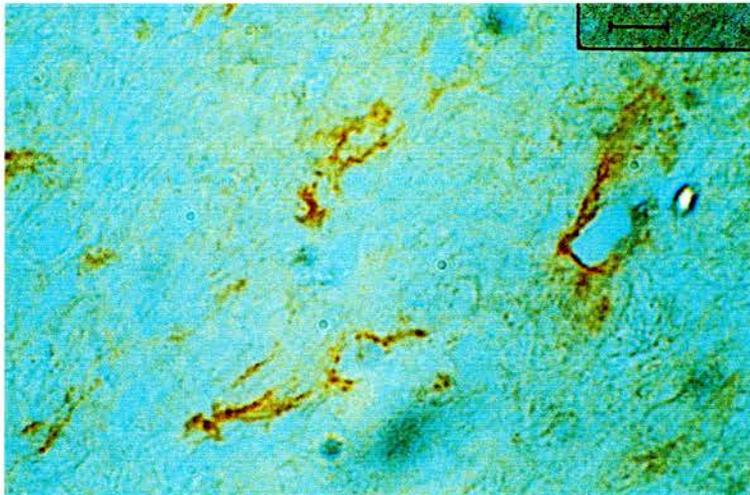


Plate 3.7 *Anti-iNOS staining of Control tumour X630 (scalebar = 16µm)*

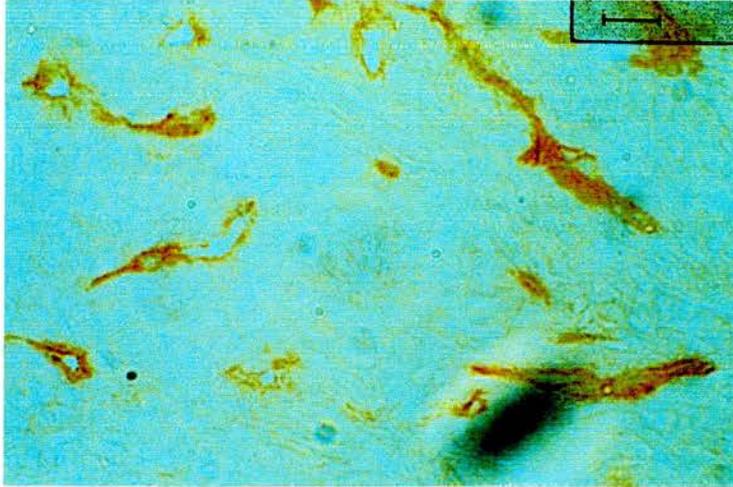


Plate 3.8 *Anti-iNOS staining of AMD6221-treated tumour X630 (scalebar = 16µm)*

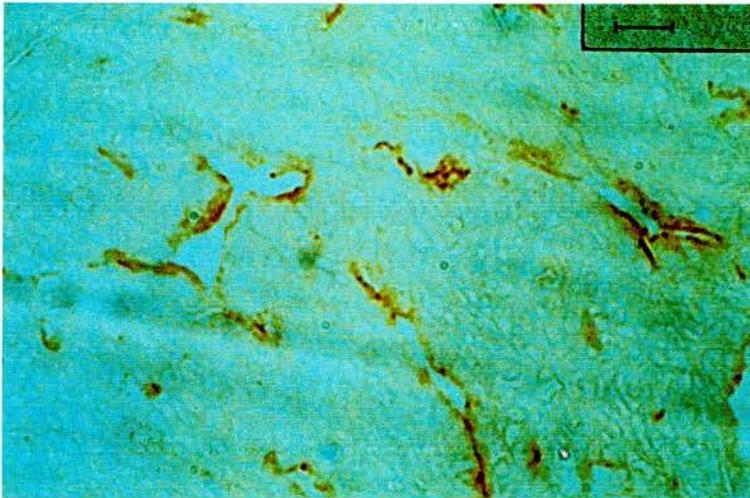


Plate 3.9 *Anti-iNOS staining of AMD6245-treated tumour X630 (scalebar = 16µm)*

3.4.5.3 *KDR/Flk-1*

KDR/Flk-1 receptors were distributed throughout the tumour sections. Staining was most intense at the tumour cell membranes and on blood vessels, but copious amounts of antigen were also present throughout the cytoplasm of tumour cells. Fig. 3.10 (overleaf) illustrates KDR/Flk-1 staining in a control animal.

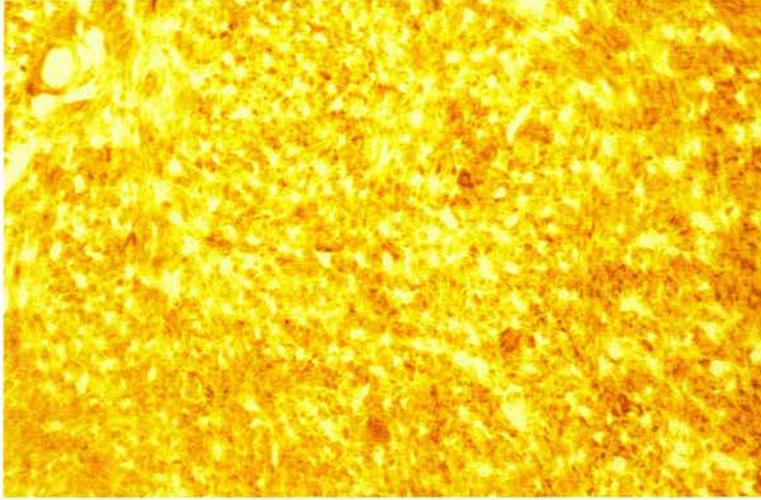


Plate 3.10 *Anti-KDR/Flk-1 staining of a Control tumour X400*

Staining was similar across all tumour sections and no quantitative differences could be determined between those from control and drug-treated animals.

3.4.6 Biochemical Analysis

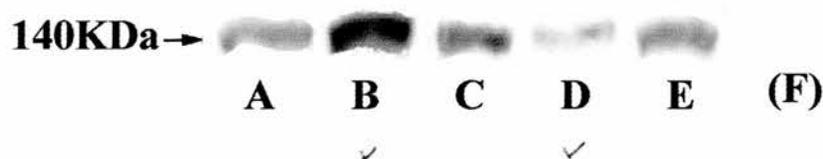
Control and drug-treated tumour extracts were prepared for protein gel electrophoresis (see appendix 8.4.1). Three sample sets – control, drug-treated continuously (18 days) and drug-treated with a withdrawal period (7 days) – were run in parallel. Tumour core and invasive cortical edge (outer 3mm) were homogenised separately to examine differential levels of activity across the different tumour regions. The protein content of each sample was estimated (see appendix 8.4.3) and samples were subsequently diluted to a standard protein content ($2\text{mg}\cdot\text{ml}^{-1}$ protein). Duplicate samples were loaded and run on 6% gels, alongside MW standards. Coomassie brilliant blue gel staining was performed to confirm equal protein loading across all lanes. All gels analysed demonstrated equivalent banding intensities with this stain.

3.4.6.1 eNOS

Detection of eNOS in samples was optimal with a loading of 20ul of 2mg.ml⁻¹ protein extracts. Nitrocellulose membranes were incubated overnight (4°C) with primary antibody (1:800) and for 2 hrs (RT) with secondary (1:800).

The results for control and drug-treated tumour extracts are shown in plates 3.11 and 3.12.

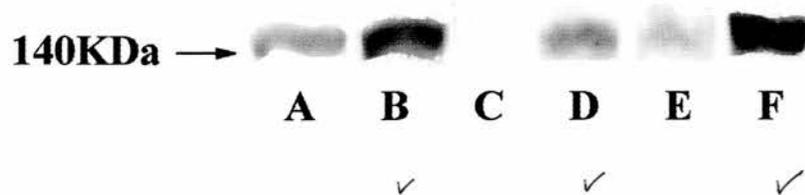
Plate 3.11 Control versus AMD6221-treated tumour extracts probed for eNOS (A-E)



Key - Control core region (lane A); Control cortical region (lane B); AMD6221-treated core region (lane C); AMD6221-treated cortical region (lane D); AMD6221-treated after drug withdrawal core region (lane E); AMD6221-treated after drug withdrawal cortical region (lane F – no data).

The control tumour sample can be seen to express eNOS at a much higher level in the cortex relative to the core region. This is consistent with the pattern on immunohistochemical staining which showed greater MVD (see section 3.4.8) and eNOS staining (see section 3.4.5.1) in tumour cortex relative to more central regions. AMD6221-treatment reduced cortical (but not core) eNOS expression. Drug-withdrawal similarly has no major effect on core eNOS expression. No data is available for the corresponding cortical region.

Plate 3.12 Control versus AMD6245-treated tumour extracts probed for eNOS (A-F)



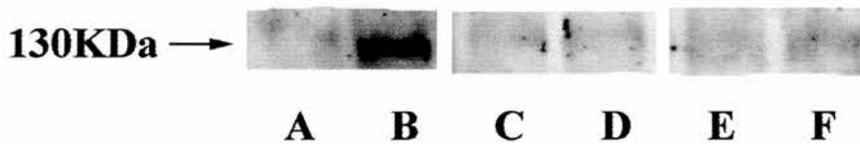
Key - Control core region (lane A); Control cortical region (lane B); AMD6245-treated core region (lane C); AMD6245-treated cortical region (lane D); AMD6245-treated after drug withdrawal core region (lane E); AMD6245-treated after drug withdrawal cortical region (lane F).

AMD6245-treatment reduced both core and cortical eNOS expression. Note that eNOS could not be detected in the core region following treatment (lane C). eNOS immunoreactivity returned after a period of drug-withdrawal. Cortical eNOS expression was comparable to that seen in control (untreated) tumours.

3.4.6.2 iNOS

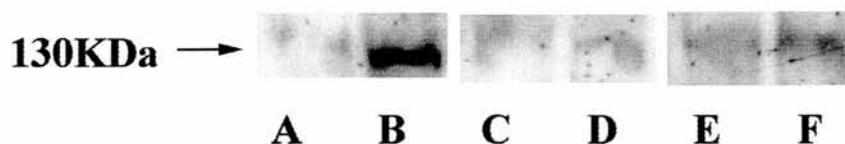
Expression of iNOS in the tumour samples was weak, and detection therefore poor-quality. Gel sample loading was maximal at 20 μ l of 2mg.ml⁻¹ protein extract. Nitrocellulose membranes were incubated overnight (4°C) with primary antibody (1:200) and for 2hrs (RT) with secondary (1:800). The results for control and drug-treated extracts are shown in plates 3.13 and 3.14.

Plate 3.13 Control versus AMD6221-treated tumour extracts probed for iNOS (A-F)



Key - Control core region (lane A); Control cortical region (lane B); AMD6221-treated core region (lane C); AMD6221-treated cortical region (lane D); AMD6221-treated after drug withdrawal core region (lane E); AMD6221-treated after drug withdrawal cortical region (lane F).

As for eNOS expression, the control tumour shows much greater iNOS expression in the cortical region than the core. Treatment with AMD6221 dramatically reduced iNOS expression in the tumour cortex. A period of drug-withdrawal began to restore this cortical expression, but not to levels approaching those of the control sample.

Plate 3.14 Control versus AMD6245-treated tumour extracts probed for iNOS (A-F)

Key - Control core region (lane A); Control cortical region (lane B); AMD6245-treated core region (lane C); AMD6245-treated cortical region (lane D); AMD6245-treated after drug withdrawal core region (lane E); AMD6245-treated after drug withdrawal cortical region (lane F).

Treatment with AMD6245, similarly to AMD6221, reduced cortical iNOS expression dramatically. A period of drug withdrawal began to restore this expression.

3.4.6.3 PECAM-1/CD31

The anti-PECAM-1 antibody once again failed to react with tumour samples in acrylamide gels.

3.4.6.4 KDR/Flk-1

Results obtained with the anti-KDR/Flk-1 antibody were disappointing, similar to those in the previous chapter. They were discarded.

3.4.7 Tumour Growth Rate

Daily measurements (mm^3) of tumour volume were made across all animal groups. The graph (Fig. 3.5) overleaf illustrates tumour growth (average volume \pm -SEM) over the course of the experiment. Arrows denote the commencement and cessation of drug administration on days +10 and +28 respectively.

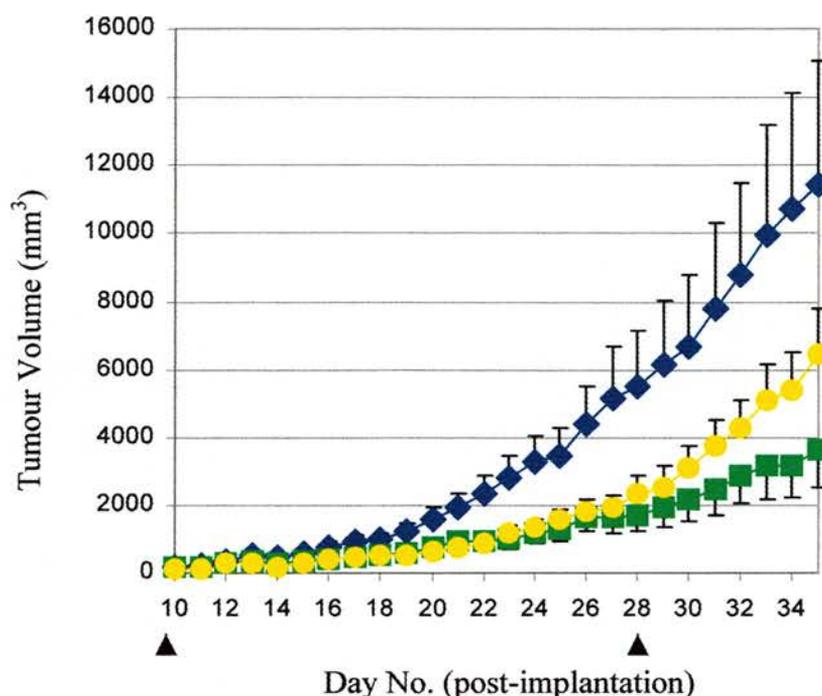


Fig. 3.5 Tumour volume (mm^3) of AMD6221- (yellow circles) and AMD6245-treated (green squares) animals, compared with Controls (blue squares)

Daily injections of AMD6221 and AMD6245 slowed the growth of solid tumours to differing degrees. The beginnings of growth suppression were evident from \sim day +16. Cessation of drug treatment on day + 28 caused the growth of AMD6221-treated tumours to increase somewhat, back towards control values. However, the growth of AMD6245-treated tumours remained low even in the absence of drug administration.

Two-way ANOVA showed that the growth of AMD6245-treated tumours during drug treatment was significantly less than that of controls ($F= 5.059$, $p = 0.037$). Following drug withdrawal, tumours just failed to retain statistically-significant growth retardation ($F= 3.688$, $p = 0.071$). The growth of AMD6221-treated tumours during drug-treatment however, was not significantly different from that of controls ($F = 3.089$, $p = 0.098$). After drug-withdrawal, tumour grow retardation was even less significant ($F = 0.631$, $p = 0.438$).

Photographs of tumour-bearing rats (Plates 3.15-3.17), taken on the final day of drug-administration (day +28) are shown overleaf.



Plates 3.15 to 3.17 *Control rat (top); AMD6221-treated (middle); AMD6245-treated (bottom)*

3.4.1 The Angiogenic Response

Cryostat sections of snap-frozen tumours were used for MVD determination. The quality of anti-PECAM-1 staining was good, with almost undetectable background. As for the L-NAME-treated tumours analysed in section 2.4.8, size-matching of control and drug-treated tumours was problematic. A single tumour from each of the drug-treated groups was therefore selected for Chalkley point analysis, that which most closely matched the size of the control tumour.

Chalkley point analysis was also performed on tumours from animals which had undergone a drug-withdrawal period. However, the results of this must be interpreted with caution as there were necessarily, notable differences in tumour size between control and drug-treated tumours by this time (see day +35 in fig.3.5).

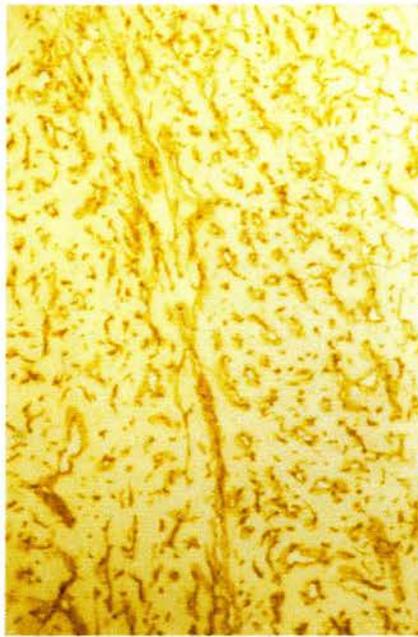
Photographs of PECAM-1 immunoperoxidase staining of tumour sections provide clear visual evidence for the consequence of drug treatment on the angiogenic response (see plates 3.18 to 3.29).

Table 3.1 *Chalkley Point Counts*

DRUG REGIME	CHALKLEY POINT COUNTS			MEAN MVD
	Hotspot 1	Hotspot 2	Hotspot 3	
Control (at end of drug administration)	11	13	15	13
AMD6221 (at end of drug administration)	5	5	6	5.3
AMD6245 (at end of drug administration)	2	4	3	3
Control (at end of drug withdrawal)	9	9	12	10
AMD6221 (at end of drug withdrawal)	7	8	8	8
AMD6245 (at end of drug withdrawal)	4	4	6	4.7

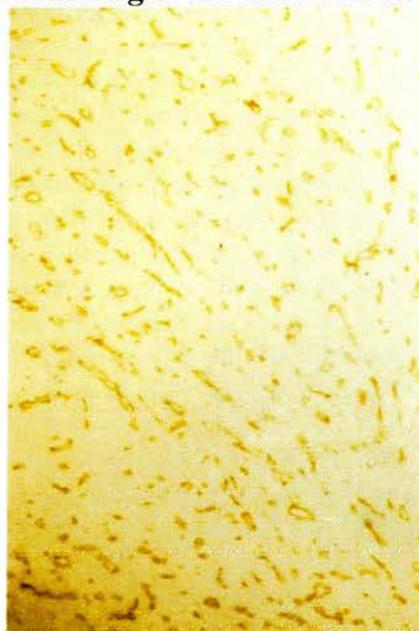
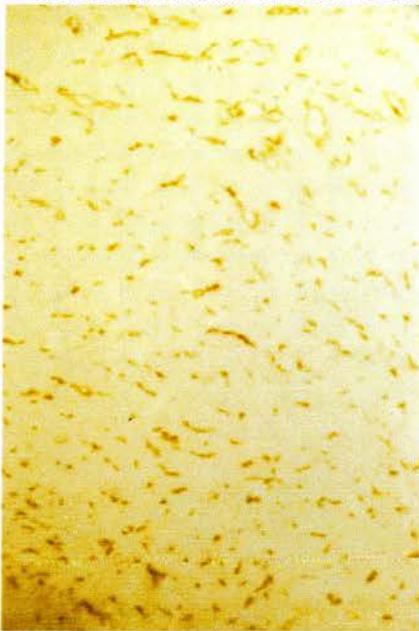
Control tumours clearly demonstrate high vessel counts relative to drug-treated tumours. AMD6221-treated tumours appear slightly more vascularised after one week of drug-withdrawal, but still demonstrate Chalkley point counts below those of controls. In contrast, drug-withdrawal generates only a slight increase in MVD in AMD6245-treated tumours.

Photographs of tumour sections reflect these counts well. Plates 3.18 to 3.26 (overleaf) are representative sections from animals fed drugs continuously. Sections from control tumours (top row) can be seen to be 'pitted' with a very high density of vessels within both core and cortical regions. Many appear long and branching. Tumours from drug-treated animals (AMD6245 - middle row; AMD6221 – bottom row) are dramatically less vascularised. Vessels were more concentrated at the periphery and are smaller, with less dilation and branching.

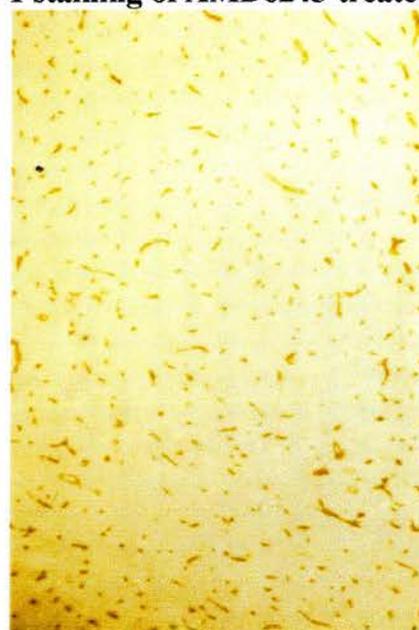
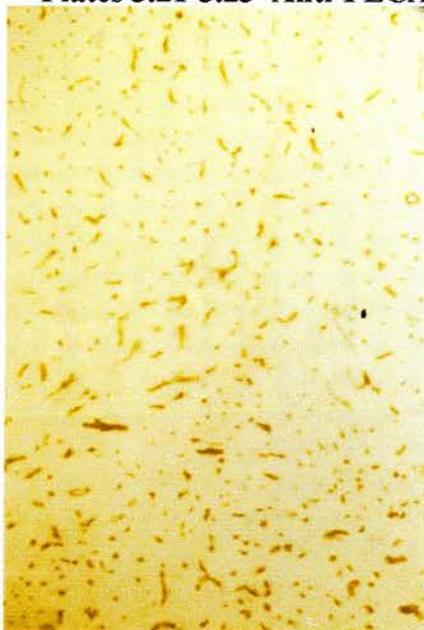


Plates 3.18-3.20 Anti-PECAM-1 staining of Control Tumour

X360



Plates 3.21-3.23 Anti-PECAM-1 staining of AMD6245-treated (continuously) Tumour X360



Plates 3.24-3.26 Anti-PECAM-1 staining of AMD6221-treated (continuously) Tumour X360

Plates 3.27 to 3.29 are PECAM-1/CD31-stained tumour sections from animals which underwent a period of drug withdrawal.

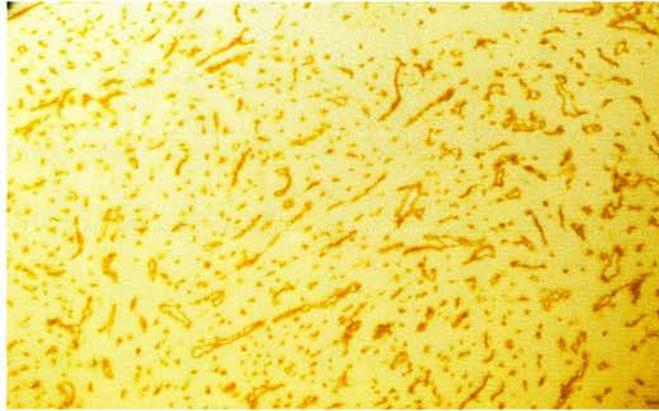


Plate 3.27 *Anti-PECAM-1 staining of Control Tumour X360*

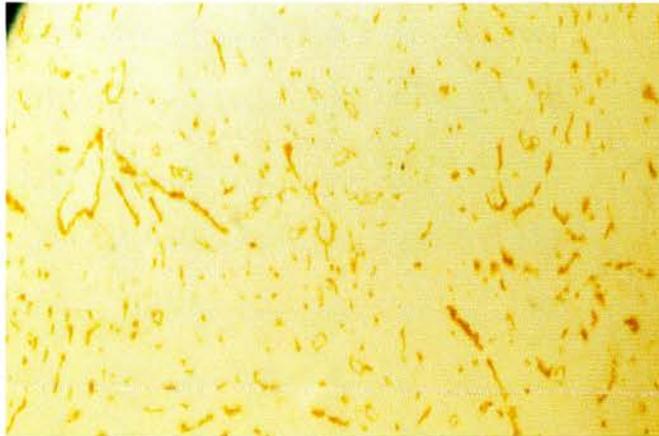


Plate 3.28 *Anti-PECAM-1 staining of AMD6245-treated (after drug withdrawal) tumour X360*

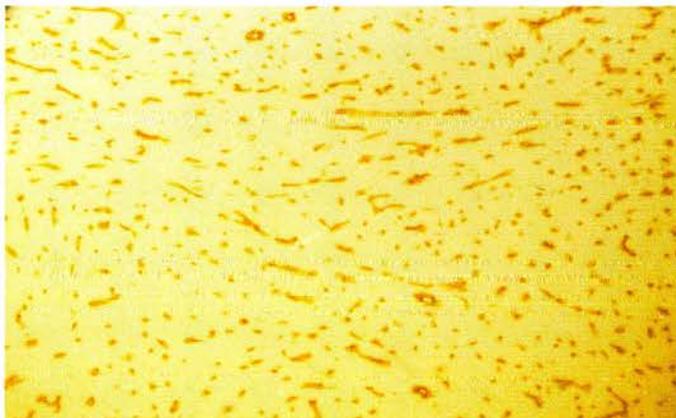


Plate 3.29 *Anti-PECAM-1 staining of AMD6221-treated (withdrawal) tumour X360*

The control section (Plate 3.27) clearly demonstrated a high MVD. However, it appeared to show a slight reduction in vasculature relative to the equivalent sections from 7 days previously (plates 3.18 – 3.20). This was possibly due to tissue necrosis as the increasing tumour mass outstrips its vascular supply. Despite seven drug-free days, the AMD6245- (Plate 3.28) and AMD6221- (Plate 3.29) treated sections still demonstrated low MVD, although vascularisation is clearly re-establishing in the AMD6221-treated section.

3.4.1 Metastasis

Histological staining revealed secondary tumour spread in paraffin sections of lung removed from animals at the time of sacrifice. Unlike NOS inhibitor-exposed sections, no metastases were visible in the liver.

Staining of the control lung revealed that 71% of lung samples (5 out of 7 animals) contained one or more metastatic foci. The AMD6245 group revealed 63% occurrence (5 out of 8 animals) and the AMD6221 group revealed 50% occurrence of secondary spread (4 out of 8 animals). Plates 3.30 and 3.31 (overleaf) illustrate lung and liver metastatic colonies from control animals.

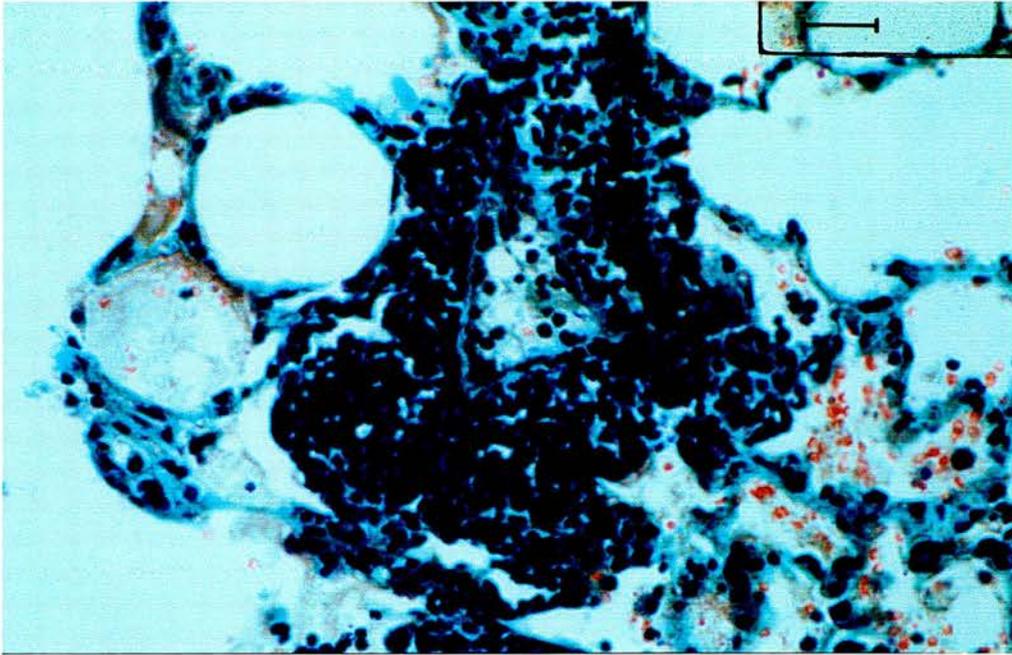


Plate 3.30 *Secondary Lung metastasis* (Masson's trichrome; X400)

A large, solid mass of tightly-packed compact, darkly-stained tumour cells is evident (scalebar = 25 μ m).

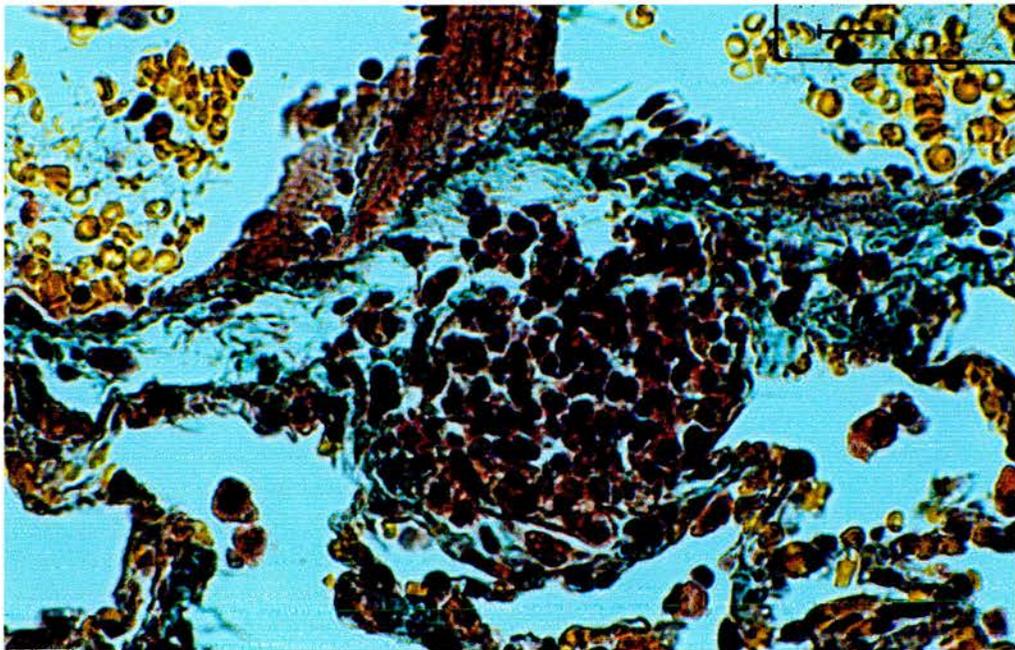


Plate 3.31 *Secondary Lung metastasis* (Masson's trichrome; X630)

A compact 'cannonball' lung tumour escaping from a blood vessel (scalebar = 16 μ m).

3.5 Discussion

3.5.1 NO Scavengers and Animal Wellbeing

The toxicity of the NO scavengers was minimal, despite daily injections of drugs for 18 consecutive days. No permanent weight loss or reduction in drinking rate was noted in any group, demonstrating that both drugs were well tolerated. It is interesting that only AMD6245 treatment induced a temporary drop in weight gain upon initial drug commencement. This may reflect its relatively greater NO scavenging activity (see section 3.5.2). An explanation for the further temporary weight loss of the AMD6245-treated group on day +21 is lacking. It was not however, accompanied by a fall in drinking rate or a change in tumour growth rate.

3.5.2 Efficiency of NO Scavenging

Griess assaying demonstrated that both drugs effectively scavenged NO in this system, with drug-treatment reducing plasma [NO_x] levels to below those of control animals. AMD6245-treatment was more effective at scavenging NO than AMD6221, with the two drugs reducing plasma [NO_x] levels by 50% and 34% respectively. The AMD6245-induced reduction was statistically-significant. An explanation for the increased potency of AMD6245 remains undetermined since both compounds have demonstrated equivalence in their NO-binding affinity in aqueous solution (Davies *et al.*, 1997). However, it may be that AMD6221 was less stable in aqueous solution than was initially realised. Some compound degradation may therefore have occurred over the course of the experiment. As anticipated, after 7 days of AMD6245-withdrawal, plasma [NO_x] was increasing back towards control values.

3.5.3 Tumour Histology and Necrosis

Neither of the NO scavengers had any notable effect on the histological structure of tumours. There was however, less necrosis within drug-treated sections than in controls, probably reflecting the overall smaller volume of the former following treatment.

3.5.4 NOS and KDR/Flk-1 Localisation

The distribution of e- and iNOS in tumours was limited exclusively to the vasculature, as in the previous chapter (section 2.4.5). Conspicuously less eNOS was distributed across both AMD6221 and AMD6245-treated tumours, relative to controls. This may reflect the reduced intra-tumoural MVD of these sections (see section 3.4.8). Drug-withdrawal produced no noticeable increase in eNOS distribution. This is in keeping with the MVD of drug-treated sections, which was not restored to control levels over the 7 drug-free days.

KDR/Flk-1 distribution was once again, widespread across both the endothelial and tumour cell populations, in a non-specific fashion.

3.5.5 NOS Expression

As observed with the use of NOS inhibitors in chapter two, the NO scavenging regime effectively modified the intra-tumoural expression of the NOS isoforms.

eNOS was expressed at a much higher level in the invasive edge of tumours relative to more central portions. This is consistent with the pattern of immunohistochemical staining which demonstrated greater MVD (see section 3.4.8) and eNOS staining (see section 3.4.5.1) in tumour cortex relative to more central regions. AMD6221 treatment did not affect core expression of eNOS, but it reduced cortical expression considerably. The removal of AMD6221 had no effect on the expression of eNOS in tumour core. Unfortunately no data is available for cortical eNOS expression following drug-withdrawal.

In contrast, AMD6245 reduced both core and cortical expression of eNOS. Reduction in the tumour core was particularly dramatic. Expression across all tumour regions was restored back towards control levels following AMD6245 removal.

Previous researchers (Beirith *et al.*, 1999), examining two different ruthenium scavengers, *trans*-[RuCl₂(nic)₄] and *trans*-[RuCl₂(i-nic)₄], have concluded that these compounds failed to increase MABP, suggesting that they didn't interfere with eNOS. It remains undetermined whether the reduction in eNOS expression observed with AMD6221 and AMD6245 treatment was translated into a reduction in eNOS activity.

As for eNOS, the expression of iNOS was greater in the cortical region of tumours than in the core. It was difficult to ascertain changes in the expression in tumour core on account of the very low levels of staining. However, in cortical regions treatment with both compounds induced markedly-reduced iNOS expression. Although the subsequent withdrawal of drugs began to restore expression, control levels were not attained over the course of 7 drug-free days.

3.5.6 Tumour Growth

Both of the compounds tested here had potent anti-tumour effects in this *in vivo* rodent model. The rate of tumour growth was significantly retarded in the AMD6245-treated group, but just failed to achieve significance in the AMD6221-treated group.

This greater efficacy of the hydrated complex, AMD6245, presumably reflects its more potent scavenging of NO. Growth retardation with AMD6245 treatment was maintained for 7 days after the cessation of drug treatment, although this just failed to maintain statistical significance.

3.5.7 Angiogenesis

Tumours exposed to both AMD6221 and AMD6245 demonstrated markedly less vasculature compared with their corresponding controls. Similar to the previous chapter, the constraint of analysing a single tumour section from each drug-treated group limits the ability to draw strong quantitative conclusions from the Chalkley point counts. However, the dramatic reduction in MVD was also apparent from a microscopic examination of PECAM-1 stained immunohistochemical tumour sections. Clearly both compounds are effective anti-angiogenic agents in this rodent model. To my knowledge, this is the first demonstration of the anti-angiogenic potential of an NO scavenging regime.

Of the two compounds, AMD6245 caused the greatest reduction in tumour vascularity. This is in keeping with its more potent reduction in intratumoural NOS expression and its more marked and long-lasting retardation of tumour growth, and presumably once again reflects its greater NO scavenging ability.

3.5.8 Metastasis

By the time of sacrifice, after 28 days of tumour growth, 71% of control animals demonstrated one or more lung metastases. Animals treated with AMD6221 and AMD6245 for 18 of these 28 days demonstrated 50% and 63% metastatic occurrence. Clearly this implies a further benefit of the NO scavenging regime. However, findings must be interpreted cautiously, since reduced metastatic spread may reflect the smaller size of primary tumours in treated animals.

Chapter Four

The Expression of Angiogenic Proteins in the Cultured Endothelial Cell - The Influence of Tumour Cells and Pericytes

4.1 Introduction

As an extension of the *in vivo* research described over the previous chapters, *in vitro* studies were undertaken here, with endothelial cells in culture. The aim of these experiments was to mimic the *in vivo* environment of both tumour cells and pericytes, and to examine their effect on the expression of proteins involved in angiogenesis, namely: iNOS, eNOS, KDR/Flk-1 and PECAM-1/CD31.

Initial experiments were designed to do the following:

1. Establish and propagate one each of a human micro- and macrovascular endothelial cell line (PART A).
2. Examine the expression and distribution of iNOS, eNOS, KDR/Flk-1 and PECAM-1/CD31 in cultures of the above cells, by indirect immunofluorescence (PART B).

Having established successful *in vitro* cell cultures, and characterised the normal expression of the proteins of interest, experiments were designed to:

3. Examine the effect on the expression of iNOS, eNOS, KDR/Flk-1 and PECAM-1/CD31 of incubating HDMEC in culture media conditioned by tumour cells (TCM) and retinal pericytes (PCM) (PART C).

PART A

Endothelial Cell Culture

4.2 Introduction

As reviewed in section 1.13.2, it is important to work with an appropriate EC culture when extrapolating *in vitro* observations to a given pathology. Human dermal microvascular ECs (HDMEC) were therefore established as a model of the capillary endothelium present within the blood vessels of experimental tumours. Macrovascular human coronary artery ECs (HCAEC) were selected to mimic the endothelium to which fluid forces associated with flow are primarily applied *in vivo* (see chapter 5). Both cell lines were purchased from commercial suppliers and consideration is given here to the process of establishing these cultures.

4.3 Cell Propagation

4.3.1 Human Coronary Artery Endothelial Cells

Propagation of HCAEC in commercially-optimised culture medium was relatively straightforward (see appendix 8.6) and a stock of young cells was successfully established (see plate 4.1).

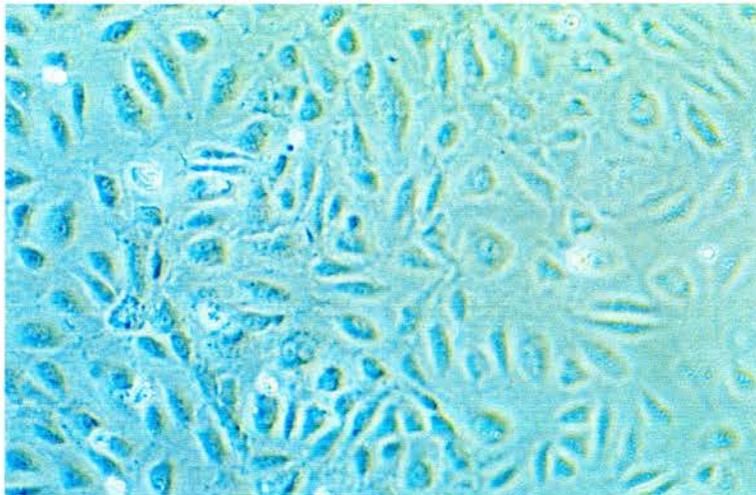


Plate 4.1 *Human coronary artery endothelial cells in culture (phase contrast, high power)*

4.3.2 Human Dermal Microvascular Endothelial Cells

In comparison with other cultured EC lines, microvascular ECs are fastidious to propagate, and some information regarding their specialised requirements and complex phenotype is pertinent to the studies undertaken here (also see appendix 8.6).

HDMEC were grown on surfaces coated with a type I collagen solution. Attachment and growth in the absence of this substrate was poor. Initial population expansion was performed in specialised medium produced by the suppliers, after which the cells were weaned onto supplemented MCDB131. This medium was originally developed for endothelial culture (Knedler and Ham, 1987) and it supported growth well. The serum requirement of the cells was tested, and 5% dialysed foetal bovine serum (FBS) was found to be optimal. This low serum requirement minimised interference by the ill-determined extraneous factors present in serum, providing better-defined experimental conditions.

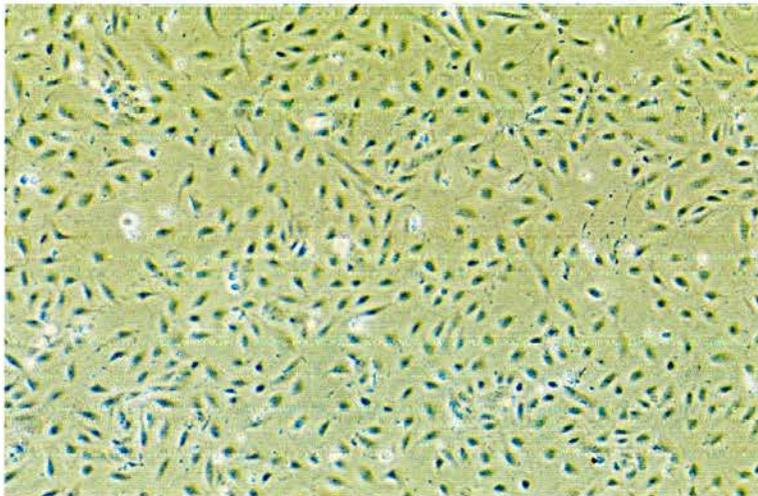
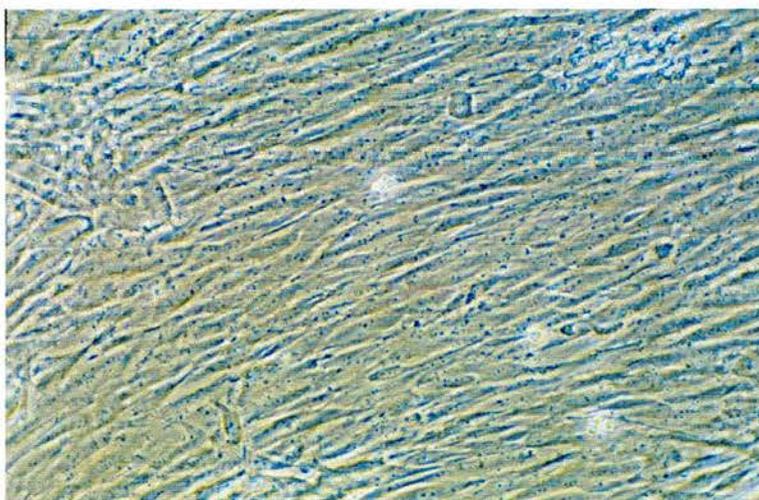
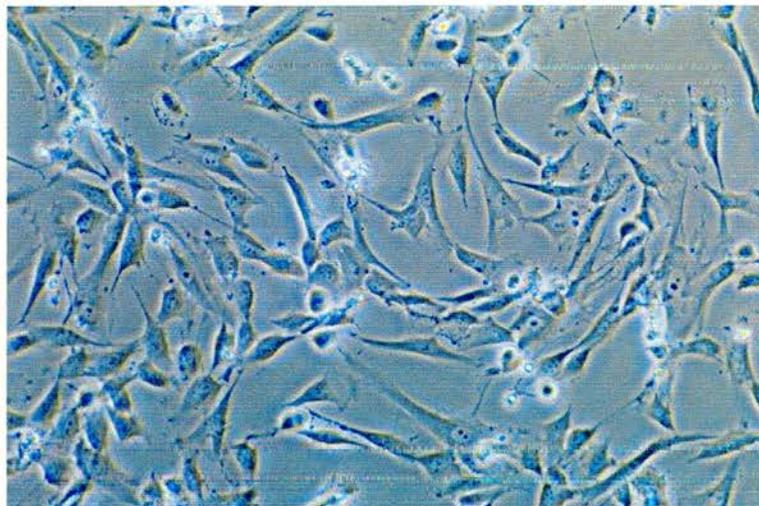


Plate 4.2 *Human dermal microvascular endothelial cells in culture (passage 3)
(phase contrast, low power)*

At passage 6 of culture, the cells lost their cobblestone morphology and became spindle-shaped and fibroblast-like (see plate 4.3). They formed bundles, aligning at confluence to resemble tree bark and finally appearing to overgrow the monolayer (see plate 4.4). For this reason, and on account of a documented loss of endothelial

cell-specific markers at this stage (Ruszczak, 1996), all experiments were performed on cells younger than passage 6.



Plates 4.3 and 4.4 *Human dermal microvascular endothelial cells at passage 6. Top – subconfluent (high power); Bottom – confluent (high power).*

Normal cells have a limited capacity to divide in culture and after a finite number of cell divisions undergo replicative senescence (Campisi, 1996; Maier *et al.*, 1990; Smith & Pereira-Smith, 1996). The addition of VEGF to culture medium has been documented both to reverse senescence and to return cells to a more characteristic morphology (Wantanabe *et al.*, 1997). Expense prevented the routine use of VEGF in this study, and consequently HDMEC cultured to high passage numbers (>p15) lost any resemblance to ECs, becoming progressively larger (plate 4.5), a characteristic sign of senescence (Maier *et al.*, 1990), before finally dying.

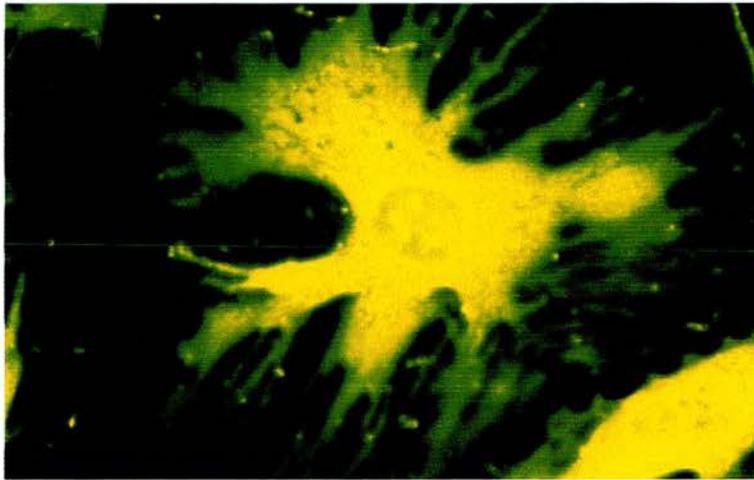


Plate 4.5 *A human dermal microvascular endothelial cell undergoing replicative senescence (labelled with anti-actin antibody; X630)*

PART B

Indirect Immunofluorescence

4.4 Introduction

Indirect immunofluorescence was used to determine both the presence and distribution of the proteins of interest in cell cultures. Immunoreactivity for iNOS, eNOS, KDR/Flk-1 and PECAM-1/CD31 was tested in both HDMEC and HCAEC.

4.5 Materials and Methods

4.5.1 Methods

4.5.1.1 Cell Culture

HDMEC (< passage 6) were seeded onto collagen-coated standard 4cm² glass coverslips at a density of 10,000 cells/cm². HCAEC (< passage 8) were seeded onto uncoated coverslips at a density of 20,000 cells/cm² (see appendix 8.6). Each coverslip received 2ml of culture medium and was incubated in 5%CO₂/air, with fresh medium added on alternate days, until cells reached confluence (~ 4 – 6 days).

4.5.1.2 Indirect Immunofluorescence

Confluent coverslips were examined under an inverted microscope for cell integrity and the absence of bacterial contamination. They were rinsed twice with phosphate buffered saline with Ca²⁺ and Mg²⁺ (PBSc) and fixed by a 10 minute submersion in 1% formaldehyde. Cells were then immunostained (see appendix 8.7) and mounted onto slides. Slides were viewed with a Zeiss Axioplan fluorescence microscope and photographed promptly before fluorescence fading occurred.

4.5.2 Materials

4.5.2.1 Cell Culture

HDMEC were purchased as a proliferating 2nd passage culture (Cell Applications Inc., San Diego). They were grown in *MCDB131 medium* (GibcoBRL) with

supplements of 5% dFBS, 5mM L-glutamine, 5ul/ml Gentamicin (all from GibcoBRL), 1µg/ml hydrocortisone and 10ng/ml epidermal growth factor (EGF) (both from Sigma). Collagen for surface coating was 'attachment factor solution' (Cell Applications Inc.).

Cryopreserved HCAEC were purchased at 3rd passage (Biowhittaker). They were grown in *microvascular endothelial growth medium-2* (EGM-2-MV; Biowhittaker) with supplied supplements (for 500ml) of 5% FBS, 0.5ml EGF, 2.0ml bFGF, 0.5ml VEGF, 0.5ml ascorbic acid, 0.2ml hydrocortisone, 0.5ml IGF-1, 0.5ml heparin, 0.5ml gentamicin, 0.5ml amphotericin B (Biowhittaker) and 5mM L-glutamine (GibcoBRL).

4.5.2.2 Immunological Reagents

4.5.2.2.a Primary Antibodies

Monoclonal mouse anti-human iNOS (Santa Cruz Biotech.). *Monoclonal mouse-anti-human eNOS* (Transduction Labs). *Monoclonal mouse anti-human KDR/Flk-1* (Santa Cruz Biotech.). *Monoclonal mouse anti-human CD31* (Dako).

4.5.2.2.b Secondary Antibodies

Goat anti-mouse fluorescein-tagged secondary (GAMF) (Jackson Labs.)

4.6 Results

4.6.1 iNOS

Anti-iNOS antibodies failed to stain either sub-confluent or confluent cultures of HDMEC or HCAEC, suggesting that these cell types do not express iNOS in the absence of stimulation (i.e. pro-inflammatory cytokines).

4.6.2 eNOS

4.6.2.1 Human Coronary Artery Endothelial Cells

Bright immunofluorescent staining of eNOS required overnight incubation (4°C) with primary antibody at 1:20 and 1 hr (RT) incubation with secondary antibody at 1:100.

Staining was striking, with particulate eNOS expression localised predominately to the nucleus, and some staining around the outer cell membrane.

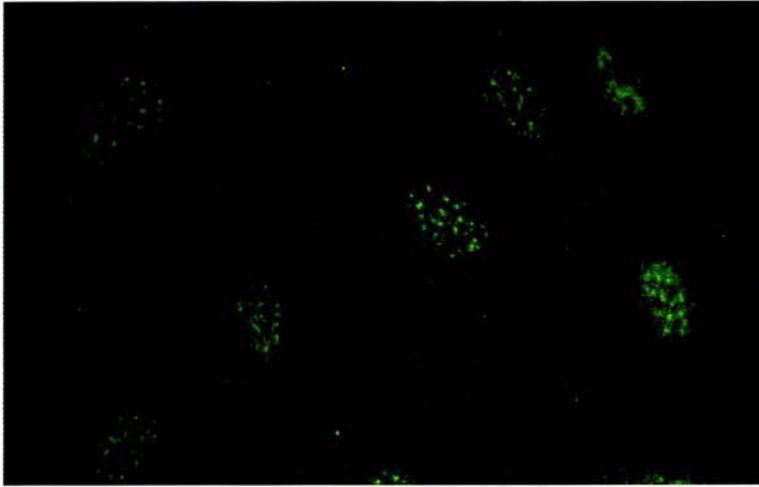


Plate 4.6 *Anti-eNOS Immunostaining of HCAEC X630*

4.6.2.2 Human Dermal Microvascular Endothelial Cells

Immunofluorescence was optimal with overnight incubation (4°C) with primary antibody at 1:20 and 1 hr (RT) incubation with secondary antibody at 1:100. The distribution of eNOS was similar to that of HCAEC, with particulate staining also evident around the outer cell membrane.

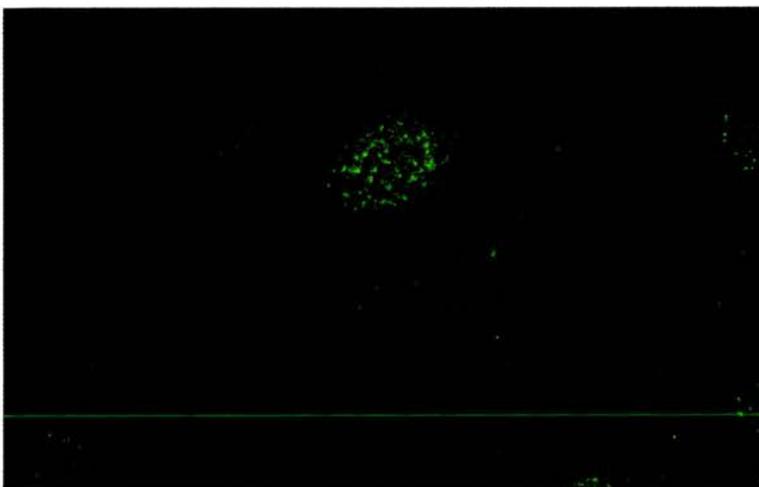


Plate 4.7 *Anti-eNOS Immunostaining of HDMEC X1000*

4.6.3 KDR/Flk-1

4.6.3.1 Human Coronary Artery Endothelial Cells

Anti-KDR/Flk-1 staining was very weak (Plate 4.8), requiring overnight incubation (4°C) with primary antibody at 1:50 and 1 hr (RT) incubation with secondary antibody at 1:100. Receptor expression appeared to be down-regulated in cells approaching confluence, and consequently fluorescence was weak. Immunostaining was therefore performed on cultures which were ~60% confluent. Staining was widespread and particulate. It was bright in the cell nucleus but also visible in the cytoplasm of cells and around the plasmalemma.

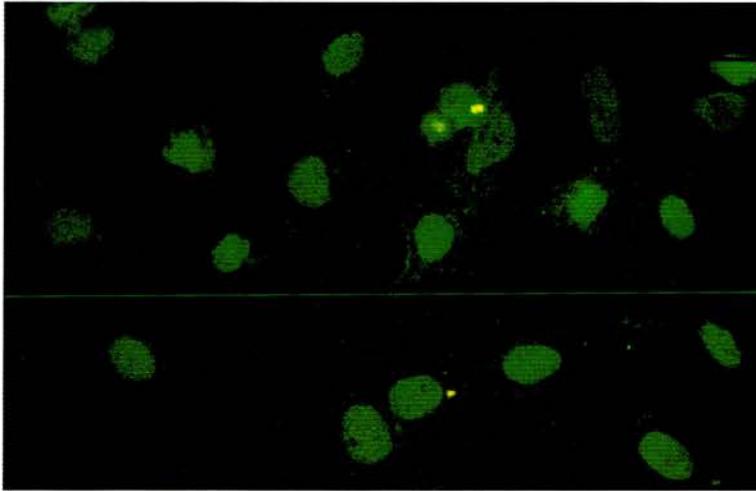


Plate 4.8 *Anti-KDR/Flk-1 Immunostaining of HCAEC X400*

4.6.3.2 Human Dermal Microvascular Endothelial Cells

Anti-KDR/Flk-1 reactivity was weak, requiring overnight incubation (4°C) with primary antibody at 1:50 and 1 hr (RT) incubation with secondary antibody at 1:100. Receptor expression again appeared to be down-regulated in confluent cells. The distribution of immunostaining was similar to that in HCAEC and a high power microscopic examination of cells demonstrated particularly-striking staining along the outer membrane of cells (Plate 4.9).

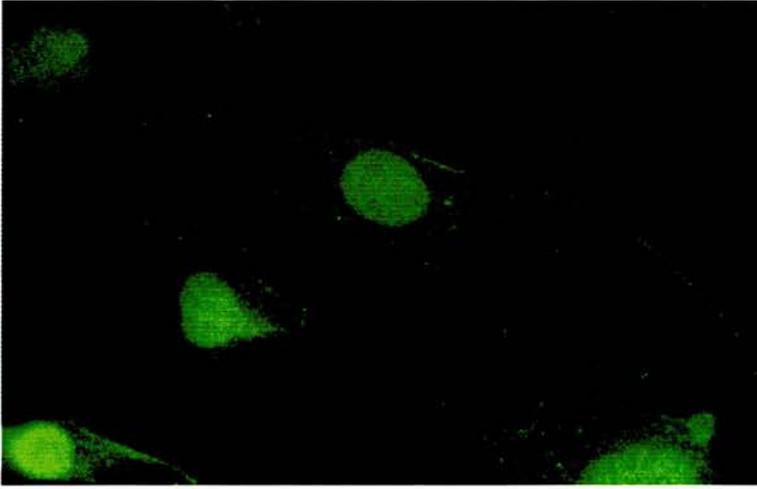


Plate 4.9 *Anti-KDR/Flk-1 Immunostaining of HDMEC X630*

4.6.3.3 HT-29 Adenocarcinoma

In the previous two chapters, it was shown that the experimental *p22* carcinosarcoma which was used for all *in vivo* tumour work, demonstrated a surprisingly widespread expression of KDR/Flk-1 receptors (see sections 2.4.5.3 and 3.4.5.3). Based on these findings, it was decided to examine the KDR/Flk-1 status of the HT-29 human adenocarcinoma, which was cultured for the production of tumour-conditioned medium (see part C). Indirect immunofluorescence was performed on subconfluent monolayers of HT-29. The results are shown in plate 4.10.

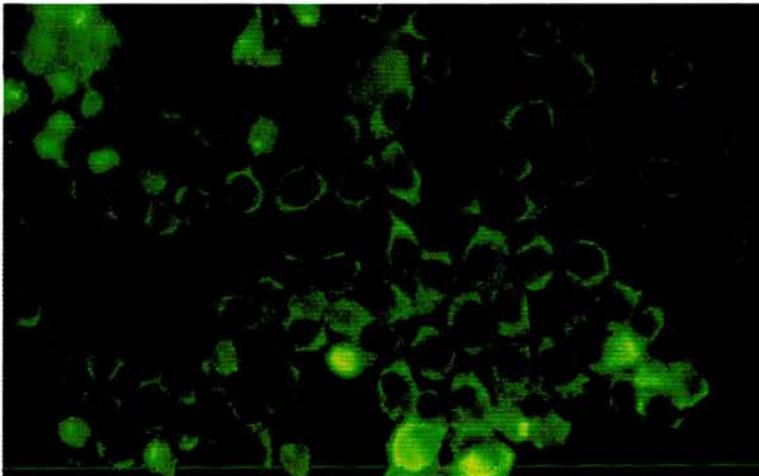


Plate 4.10 *Anti-KDR/Flk-1 Immunostaining of HT-29 X400*

Clearly this tumour line demonstrates a widespread expression of KDR/Flk-1 receptors. Immunostaining was bright and particulate, with dense immunoreactivity throughout the cell cytoplasm and around cell membranes.

4.6.4 PECAM-1/CD31

4.6.4.1 Human Coronary Artery Endothelial Cells

Immunofluorescence of PECAM-1 was intense. Optimal staining required 1 hr incubation (RT) with primary antibody at 1:200 and 30min (RT) incubation with secondary antibody at 1:200. Expression was strongest at the intercellular junctions of cells which were approaching confluence.

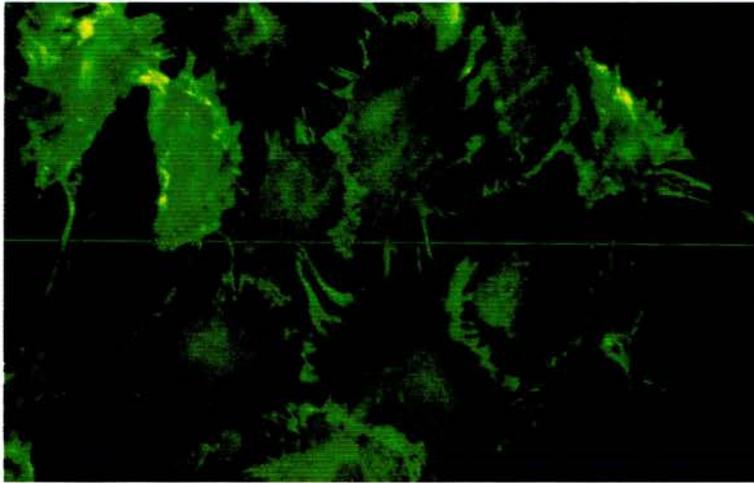


Plate 4.11 *Anti-PECAM-1/CD31 Immunostaining of HCAEC X630*

4.6.4.2 Human Dermal Microvascular Endothelial Cells

The pattern of PECAM-1 immunoreactivity in HDMEC was the same as in HCAEC, being confined to the intercellular junctions. Staining was however, pale.

4.7 Discussion

Indirect immunofluorescence proved a useful tool to characterise the expression of the proteins of interest by the two EC types used here.

4.7.1 iNOS

Failure to detect basal iNOS immunoreactivity in micro- or macrovascular cell types has been noted by other investigators (Hoffman *et al.*, 1999). Accordingly, both micro- and macrovascular ECs produce low levels of nitrite under un-stimulated conditions (Balligand *et al.*, 1995; Geiger *et al.*, 1997). Indeed, a failure to induce iNOS gene expression, even with pro-inflammatory cytokines or LPS, has been documented in some human large vessel ECs (MacNaul & Hutchinson, 1993; Werner-Felmeyer *et al.*, 1993). It appears that both species- and organ-specific differences exist in the expression of iNOS and the release of NO by ECs (Koller *et al.*, 1991; Lamas *et al.*, 1992; Lang *et al.*, 1999; Mayer *et al.*, 1989). Culture-induced phenotypic changes have also been shown to influence iNOS expression (Lang *et al.*, 1999).

4.7.2 eNOS

In contrast to iNOS, eNOS expression was apparent in both cell types. Contact inhibition of proliferation is a recognised down-regulator of eNOS expression in cell culture (Arnal *et al.*, 1994). Nevertheless, immunoreactivity remained positive in fully-confluent cultures of both cell types here. Other investigators have reported eNOS mRNA and protein to be completely absent in cultured, but not fresh, rat microvascular ECs (Lang *et al.*, 1999). Clearly a culture-induced loss of eNOS expression was not apparent in HDMEC and HCAEC.

eNOS expression was evident around the outer cell membrane of both cell types. This is in keeping with localisation of the enzyme to the plasmalemma and caveolae (Hecker *et al.*, 1994; Shaul *et al.*, 1996).

4.7.3 KDR/Flk-1

From the limited number of published investigations, findings for *in vitro* KDR/Flk-1 expression are varied. A range of both murine (Millauer *et al.*, 1993; Oelrichs *et al.*, 1993) and normal human macro- and microvascular ECs have been shown to express KDR (Hewett & Murray, 1996b). In contrast, a lack of KDR mRNA has been reported in new-born human foreskins (Gupta *et al.*, 1997), a rich source of microvascular ECs. Serially-passaged HUVECs and bone-marrow microvascular ECs have also been reported gradually to lose expression of Flk-1/KDR (Hewett & Murray, 1996b).

KDR/Flk-1 immunoreactivity was achieved in both HDMEC and HCAEC here, although this required long antibody incubation periods, suggesting that the receptor was not present in abundance. Indeed, the elevated oxygen concentrations present under standard culture conditions (cell culture gas of 5%CO₂/air contains ~ 20% O₂) have shown both KDR/Flk-1 and flt-1 reactivity to be barely detectable, implicating this as a factor causing their downregulation under ‘standard’ culture conditions (Tufro-McReddie *et al.*, 1997). Despite the apparent receptor down-regulation at confluence observed here, both post-confluent, quiescent monolayers and sparse (20% confluent) cultures have previously been reported to express the receptor (Hewett & Murray, 1996b).

As expected, immunostaining was particularly strong around the outer membrane of cells. However, nuclear and cytoplasmic fluorescence was also apparent, presumably representing receptor synthesis and trafficking.

Anti-KDR/Flk-1 immunostaining of HT-29 adenocarcinoma revealed widespread expression of the receptor, especially throughout the cell cytoplasm. Staining may represent non-functional receptors or receptor subunits which are unable to bind VEGF. Nevertheless, this human tumour line clearly represents a further tissue which demonstrates non endothelial cell-specific expression of a VEGF receptor.

PART C

The ‘Angiogenic Environment’ *in vitro* – Conditioned Cell Culture Medium

4.8 Introduction

Tumour cells and pericytes are major players in the process of tumour angiogenesis (see chapter one). In an attempt to mimic part of the ‘angiogenic environment’ within a tumour *in vitro*, HDMEC were grown in the presence of cell culture media which had been ‘conditioned’ by both types of cell. These will contain the ‘paracrine’ substances produced by both cell types. The effect of tumour- and pericyte-conditioned media on the expression of iNOS, eNOS, KDR/Flk-1 and PECAM-1/CD31 was examined by SDS-PAGE and Western Blotting. The outcomes were compared in both the presence and absence of serum.

4.9 Materials and Methods

4.9.1 Methods

4.9.1.1 Preparation of Tumour-Conditioned Medium (TCM)

Cryopreserved HT-29 (human colon adenocarcinoma) cells were seeded at a density of 20,000 cells/cm² into standard T-75 tissue culture flasks. They were cultured in Earle’s MEM supplemented with (for 500ml) 10% FBS, 5ml non-essential amino acids, 2mM L-glutamine and 5ml penicillin/streptomycin. Once 90% confluent, monolayers were rinsed (PBSc) and medium was replaced with *serum-free* MEM. Incubation was for 24 hrs, after which time medium was removed, centrifuged (1000G, 10mins) to remove cellular debris and frozen at –20°C. At the time of use, medium was defrosted and supplemented with 50U/ml Polymyxin B Sulfate to block the effects of contaminating endotoxin which could affect iNOS expression erroneously. 5% dialysed foetal bovine serum (dFBS) was added at this stage if applicable (see section 4.10).

4.9.1.2 Preparation of Pericyte-Conditioned Medium (PCM)

PCM from a 24hr-incubation with bovine retinal pericytes was obtained pre-prepared. This was stored frozen at -20°C until the time of use, when it was supplemented with 50U/ml Polymyxin B Sulfate and 5% dFBS if applicable (see section 4.10).

4.9.1.3 Cell Culture and Incubation

HDMEC (< passage 6) were seeded onto collagen-coated 60mm (28cm^2) petri dishes at a density of $10,000\text{ cells/cm}^2$ (see appendix 8.7). Each petri dish received 6ml of normal cell culture medium, with fresh medium added on alternate days. Cells were incubated in 5% CO_2 /air until $\sim 80\%$ confluence was reached. At this point, monolayers were rinsed (PBSc) and medium replaced with 6ml of the appropriate test culture medium (see section 4.10). Incubation with control and conditioned media was for 24hours.

4.9.1.4 Cell Harvesting

Following the 24hr test incubation, cell morphology was examined with an inverted microscope. Culture medium was removed from the petri dishes, monolayers were rinsed with PBSc to remove any residual medium, and an ice-cold cell lysate was made (appendix 8.4.2) for subsequent protein gel electrophoresis.

4.9.1.5 Biochemical Analysis

The protein content of each cell lysate was estimated (see appendix 8.4.3) and samples were subsequently diluted to give a standard protein content ($400\mu\text{g/ml}$). Samples were run on 6% gels (see appendix 8.4.4) alongside MW standards and the expression of iNOS, eNOS, KDR/Flk-1, PECAM-1/CD31 was quantified. Coomassie brilliant blue gel staining was performed to confirm equal protein loading across all lanes. All gels analysed demonstrated equivalent banding intensities with this stain.

4.9.2 Materials

4.9.2.1 Cell Culture

Cryopreserved *HT-29* and pre-prepared *PCM* were kindly supplied by Prof D.G. Hirst, University of Ulster. All *cell culture medium* and *supplements* were obtained from GibcoBRL. *Polymyxin B Sulfate* was purchased from Sigma.

4.9.2.2 Immunological Reagents

4.9.2.2.a Primary Antibodies

All *primary antibodies* were as for section 4.5.2.2.a. *Cruz marker molecular weight standards* (Santa Cruz Biotech) (23K-132K) were used for gel electrophoresis.

4.9.2.2.b Secondary Antibodies

A peroxidase-tagged *anti-mouse IgG* (Santa Cruz Biotech) secondary was used.

4.10 Experimental Protocol

Six conditioned-medium incubations were examined, with HDMEC grown for 24hrs in (1) MEM + serum; (2) MEM – serum; (3) TCM + serum; (4) TCM – serum; (5) PCM + serum; (6) PCM – serum. Since HDMEC necessarily underwent the incubation with an unfamiliar culture medium (MEM), conditions 1 and 2 acted as controls. Application of the test medium at 80% confluence allowed the cells to become fully-confluent at the time of harvest, ruling out any possible cell density-related variability in protein expression. All incubations were repeated in triplicate.

4.11 Results

4.11.1 Tumour-Conditioned Medium

TCM (both with and without serum) caused HDMEC to take on a ‘spiky’ morphology. This was not seen with control incubations, suggesting that it was a direct effect produced by factors within the TCM. In a 72hr ‘test’ incubation of cells with TCM, cells retained their spiky morphology throughout, only returning to their more characteristic polygonal shape after being returned to control culture medium.

4.11.1.1 iNOS

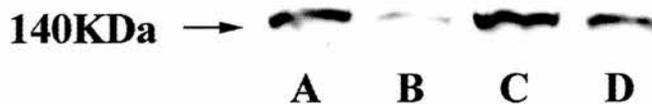
As anticipated from the findings of section 4.6.1, anti-iNOS antibodies failed to react with either control or TCM-exposed extracts of HDMEC on nitrocellulose membranes.

4.11.1.2 eNOS

Detection of eNOS in samples was optimal with a loading of 15µl of 400µg/ml protein lysates. Nitrocellulose membranes were incubated overnight (4°C) with primary antibody (1:1000) and for 2 hrs (RT) with secondary (1:1000).

The results for control and TCM-exposed lysates are shown in plate 4.12.

Plate 4.12 Control versus TCM-exposed HDMEC lysates probed for eNOS (A-D)



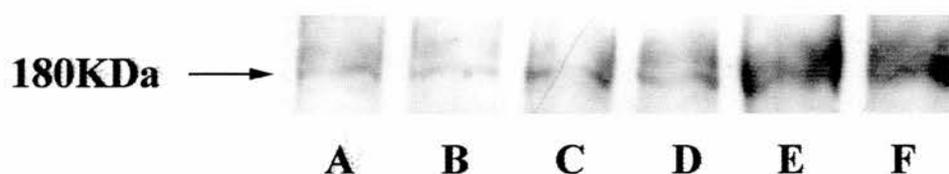
Key - Control + serum (lane A); Control – serum (lane B); TCM + serum (lane C); TCM – serum (lane D).

Clearly, cells grown in control medium with serum expressed considerably more eNOS than those grown in the absence of serum. The addition of TCM both with and without serum, caused a marked increase in eNOS expression relative to controls.

4.11.1.3 KDR/Flk-1

Detection of KDR/Flk-1 in cell samples required the maximum sample loading of 20µl of 400µg/ml protein. Nitrocellulose membranes required overnight incubation (4°C) with primary antibody at 1:200 and 2hr (RT) incubation with secondary antibody (1:600). Even then, protein banding was weak, reinforcing the findings of section 4.3.6 which indicated a low expression of KDR/Flk-1 on these cells at confluence. For ease of comparison, in the light of this weak detection, Plate 4.13 illustrates KDR/Flk-1 expression in control and both TCM- and PCM-treated cells.

Plate 4.13 Control versus TCM- and PCM-exposed HDMEC lysates probed for KDR/Flk-1 (A-F)



Key - Control + serum (lane A); Control – serum (lane B); TCM + serum (lane C); TCM – serum (lane D); PCM + serum (lane E); PCM – serum (lane F).

Cells grown in control media either with or without serum demonstrated low expression of the KDR/Flk-1 receptor. Incubation of cells in the presence of TCM caused a slight increase in receptor expression, relative to controls. On the other hand, the addition of PCM induced a marked increase in KDR/Flk-1 expression. Although difficult to conclude with certainty, the presence or absence of serum in culture medium did not appear to be a major contributory factor to the level of receptor expression, with approximately equivalent staining occurring in both lanes of each of the three sets of conditions.

4.11.1.4 PECAM-1/CD31

Detection of PECAM-1/CD31 in samples was optimal with a loading of 15µl of 400µg/ml protein lysates. Nitrocellulose membranes were incubated overnight (4°C) with primary antibody (1:800) and for 2 hrs (RT) with secondary (1:1000). The results for control and TCM-exposed lysates are shown in plate 4.14.

Plate 4.14 Control versus TCM-exposed HDMEC lysates probed for PECAM-1/CD31 (A-D)



Key - Control + serum (lane A); Control – serum (lane B); TCM + serum (lane C); TCM – serum (lane D).

The removal of serum from control cultures induced an increase in PECAM-1/CD31 expression, relative to the serum-containing equivalent. Serum-free TCM medium increased the expression of PECAM-1/CD31 relative to serum-free control cultures.

4.11.2 Pericyte-Conditioned Medium

A 24 hour incubation with PCM induced a similar ‘spiky’ cell morphology to that induced by TCM. In a 72hr ‘test’ incubation with PCM, cells retained their spiky morphology, only returning to a more characteristic polygonal shape after being returned to control cell medium.

4.11.2.1 iNOS

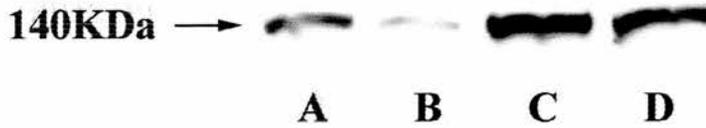
Anti-iNOS antibodies failed to react with either control or PCM-exposed extracts of HDMEC on nitrocellulose membranes.

4.11.2.2 eNOS

Detection of eNOS in samples was optimal with a loading of 15µl of 400µg/ml protein lysates. Nitrocellulose membranes were incubated overnight (4°C) with primary antibody (1:1000) and for 2 hrs (RT) with secondary (1:1000).

The results for control and PCM-exposed lysates are shown in plate 4.15.

Plate 4.15 Control versus PCM-exposed HDMEC lysates probed for eNOS (A-D)



Key - Control + serum (lane A); Control – serum (lane B); PCM + serum (lane C); PCM – serum (lane D).

Compared with the incubation of cells with TCM (Plate 4.11), incubation of cells with PCM caused an even more marked increase in eNOS expression. The same pattern of higher eNOS expression in the presence (compared with the absence) of serum was observed.

4.11.2.3 KDR/Flk-1

See section 4.11.1.3 for the results of KDR/Flk-1 expression in the presence of PCM.

4.11.2.4 PECAM-1/CD31

Detection of PECAM-1/CD31 in samples was optimal with a loading of 15µl of 400µg/ml protein lysates. Nitrocellulose membranes were incubated overnight (4°C) with primary antibody (1:800) and for 2 hrs (RT) with secondary (1:1000).

The results for control and PCM-exposed lysates are shown in plate 4.16.

Plate 4.16 Control versus PCM-exposed HDMEC lysates probed for PECAM-1/CD31 (A-D)



Key - Control + serum (lane A); Control – serum (lane B); PCM + serum (lane C); PCM – serum (lane D).

The addition of PCM with serum had a slight positive effect on PECAM-1 expression relative to the equivalent control culture. In contrast to TCM however,

there was a marked reduction in expression observed with the addition of serum-free PCM, with expression barely detectable in lane D.

4.12 Discussion

For the purposes of discussion, the results presented here are based on the assumption that, broadly speaking, medium conditioned by contact with cell cultures will contain similar ‘paracrine’ growth-modulatory substances to those produced by the cells *in vivo*. However, this will depend upon the *in vitro* stability/half life of growth factors. Other investigators (see sections 1.9.3 and 1.14.4) have reported that both TCM and PCM contain pro-angiogenic paracrine factors which stimulate the proliferation and migration of ECs *in vitro*.

Variations in protein expression occurring between serum-containing and serum-free cultures are difficult to interpret on account of the complex composition of serum. Previous investigators have reported a failed induction of gene expression in the absence of serum, which is restorable by the re-addition of serum (Szabo *et al.*, 1994b). Essential factors in serum include adhesion factors, peptide growth factors, minerals, vitamins, fatty acids and intermediary metabolites and hormones. Clearly the presence or absence of these constituents has a significant bearing on the biochemical environment to which cells are exposed.

Both tumour- and pericyte-conditioned media induced distinct morphological changes in HDMEC. After a 24hr incubation, cells changed from their polygonal shape and became ‘spiky’. This is unlikely to be due to any nutritive deficiency, as control cells which were cultured in the same minimal essential medium retained their normal morphology. Neither is it the result of serum-starvation, as it was observed with both serum-containing and serum-free cultures. It is therefore tempting to suggest that this altered cell morphology may reflect a shape change along the pathway towards tubulogenesis. Although it was not the characteristic ‘elongation’ of ECs about to become mobile, and a test incubation of cells in both

TCM and PCM for 72hrs failed to achieve a more advanced stage of tube formation, it may reflect the transition to an 'angiogenic' phenotype.

4.12.1 iNOS

The failure of anti-iNOS antibodies to react with any of the HDMEC lysates used here is in keeping with the absence of immunoreactivity observed in whole HDMEC cells in section 4.6.1. TCM would be expected to contain both a number of pro-inflammatory cytokines (e.g. $\text{TNF}\alpha$, IL-1, $\text{IFN-}\gamma$) with the potential to induce iNOS expression and a number of anti-inflammatory cytokines (e.g. IL-4, IL-10, IL-13) which would downregulate iNOS induction (Drapier *et al.*, 1988; Schneemann *et al.*, 1993).

Additionally, both polyamines (e.g. spermine) which inhibits iNOS induction (Szabo *et al.*, 1994b) and pro-angiogenic $\text{TGF-}\beta$ which decreases the translation of iNOS and interferes with protein stability (Vodovotz *et al.*, 1993) may also be present. The use of polymyxin B sulfate to block potentially-contaminating endotoxin, further reduces the potential iNOS-inducing stimuli.

4.12.2 eNOS

The expression of eNOS was dramatically reduced in cells cultured in the absence of serum. Clearly the presence of a serum cofactor(s) is necessary to permit and /or mediate the induction of eNOS expression. The application of TCM induced a moderate increase in eNOS expression, and the application of PCM induced an even greater increase. This suggests that paracrine factor(s) present within these media are responsible for up-regulating eNOS expression. The promoter of the eNOS gene contains a consensus sequence involved in the transcriptional response to $\text{TGF-}\beta$, which may be involved in the regulation of eNOS gene expression (Wang & Marsden, 1995). If pro-angiogenic $\text{TGF-}\beta$ is the factor responsible, then increased eNOS expression can be positively correlated with angiogenesis.

4.12.3 KDR/Flk-1

The application of TCM to HDMEC induced a modest increase in KDR/Flk-1 expression. PCM induced an even greater increase. This suggests that VEGF may be present amongst the pro-angiogenic factors within the media, and that this up-

regulates VEGF receptor expression. It is interesting that the greatest receptor upregulation was observed with PCM, suggesting that a VEGF-like molecule may represent the currently unidentified pro-angiogenic paracrine factor known to be released by pericytes.

4.12.4 PECAM-1/CD31

If the HDMEC were in the initial stages of tubulogenesis, PECAM-1 expression would be expected to be elevated (Llan *et al.*, 1998) (see section 1.15.5.2). This was observed with the application of serum-free TCM, which induced a marked increase in PECAM-1 expression. However, the application of serum-free PCM produced the opposite effect, dramatically-reducing PECAM-1 expression. This is clearly in contradiction to a pro-angiogenic effect of PCM, and a possible explanation is elusive.

Chapter Five

Fluid Shear Stress and the Tumour Endothelium

5.1 Introduction

The abnormal architecture of the vasculature within a tumour generates distinctive characteristics of blood flow and consequently fluid shear stress (FSS), as reviewed in section 1.15.3. This has significant implications for both tumour angiogenesis and metastasis (see section 1.15.4). Therefore, the research presented here examines the potential of FSS for modifying *in vitro* endothelial cell expression of proteins involved in angiogenesis.

Accordingly, experiments were designed to do the following:

1. Expose both micro- and macrovascular endothelial cells (HDMEC and HCAEC) to varying time periods of acute fluid shear stress using a parallel-plate flow chamber.
2. Examine the effect of FSS on the expression of iNOS, eNOS, KDR/Flk-1 and PECAM-1/CD31.

5.2 Methods and Materials

5.2.1 Methods

5.2.1.1 Cell Culture

HDMEC (< passage 6) were seeded onto collagen-coated standard 19cm² glass microscope slides at a density of 10,000 cells/cm². HCAEC (< passage 8) were seeded onto uncoated slides at a density of 20,000 cells/cm² (see appendix 8.6). Each slide was placed into a 90mm petri dish, and received 25ml of culture medium. Slides were incubated in 5% CO₂/air, with fresh medium added on alternate days, until fully confluent (~6 days).

5.2.1.2 The Apparatus – the Parallel-Plate Flow System

The test system used here was based on that originally described by Viggers *et al.* (1986), as a closed circulating loop filled with culture medium. It was designed to

challenge cells with defined shear stresses. The system consisted of 4 main components as illustrated in figure 5.1 - an upstream flask, with a parallel-plate flow chamber between them. A roller pump circulated medium from the upstream flask, through the flow chamber to the downstream flask, through gas impermeable tubing. The flow rate was under the control of the pump speed.

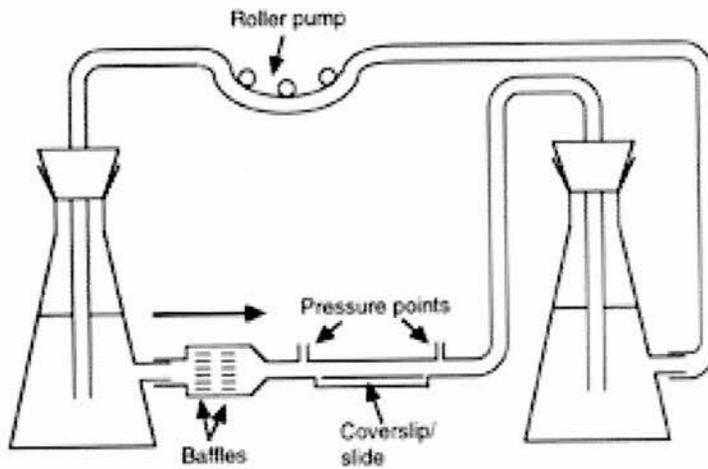


Fig. 5.1 Parallel-plate flow system used to subject cells to FSS

The flow chamber was designed to generate uniform laminar flow and was engineered of polycarbonate with an upper and lower plate, and a flow channel between them. In cross-section it was rectangular with a height that was much less than its length and width. The combination of 2 baffles with equally spaced holes, and a tapering chamber entrance acted to establish a nearly constant fluid velocity at the beginning of the laminar flow section. After diffusion by the baffles, all fluid elements have essentially equal energy content and equal velocities. Converging flow through the entrance further reduces velocity variation. Flow is therefore evenly spread over the entire cross section of the flow channel.

During an experiment, the slide grown with ECs was positioned into a recess on the bottom of the flow channel so that its cell-lined surface made an uninterrupted smooth covering. It was kept from vibrating or deflecting into the flow stream by a

thin layer of grease holding it in place. The two flasks served as reservoirs for the medium, absorbed pulsations from the roller pump, kept system pressure near to atmospheric, and acted as traps for air bubbles. The pressure difference across the chamber was measured via two pressure ports.

The performance characteristics of the chamber were examined by C Beers. Briefly, theoretical wall shear stress (τ) (dyn.cm^{-2}) within a uniform, rigid cylinder can be derived from Poiseuille's law. The derivation of this 'Newtonian system' for a non-cylindrical channel has been calculated as:

$$\tau = 3Q \eta / 2a^2w$$

where:

Q = fluid flow rate (ml/s)

η = fluid viscosity (poise)

a = half the channel height (cm)

w = channel width (cm)

This theoretical FSS was calculated for the flow chamber. The flow rate was determined by measuring the volume of medium delivered by the pump within a specified time period. The medium viscosity at 37°C was measured using a Cannon-Fenske viscometer. The channel height was measured with a light microscope, by focusing on the base and surface perspex of the chamber and using the microscope focus calibrations to determine the distance between the two. The channel width was measured directly with a ruler.

The 'actual' FSS generated within the chamber was determined from the following equation:

$$\text{FSS (dyn.cm}^{-2}\text{)} = \rho g h a / I$$

where:

ρ = density of the culture medium (g/cm^3)

g = acceleration due to gravity

h = pressure difference across the flow chamber

a = 1/2 height of the flow channel (cm)

I = distance between the above two pressure points (cm)

The density of the medium was measured at 37°C using a density bottle and comparisons with the known density of water at various temperatures allowed the density to be determined. Acceleration due to gravity (g) was taken to be 980cm.s^{-2} .

The wall shear stress was directly related to the flow rate and the viscosity of the medium, and could be calculated accurately from the roller pump speed, as the medium viscosity was a known constant. The theoretical and actual FSS values measured within the chamber were compared, and found to be closely allied. The system could therefore be calibrated to a number of fixed shear forces by varying the flow rate (altering the roller pump speed).

5.2.1.3 Fluid Shearing of Endothelial Cells

Before a cell shearing run, the apparatus components were autoclaved and assembled within a sterile flow hood. The loop was filled with 500ml of warmed culture medium. A sizeable amount of gas (200ml) within the flasks acted as a pH control.

The slide of confluent ECs was positioned carefully onto the bottom of the flow chamber and the upper chamber half secured. The chamber was perfused immediately with medium to keep the cells moist and the perfusion rate was gradually increased until the required shearing force was achieved. A shearing run was carried out for the required time period (see section 5.3). The two flasks were kept at the same level to avoid a drop in hydrostatic pressure over the flow chamber. The medium was equilibrated with 5%CO₂/air to maintain the pH at 7.4. The large volume of circulating medium ensured optimal cell maintenance, and the entire apparatus was kept in a thermostatically-controlled 37°C room.

Vacuum grease held a removable standard coverslip in place on the chamber base. This served as a port for fast and easy removal of the cell slide following the completion of a shear run.

5.2.1.4 Cell Harvesting

Following a cell shearing run, the flow of fluid in the apparatus was halted and the microscope slide carefully removed through the basal port of the flow chamber. The cells were examined under an inverted microscope for monolayer integrity and any changes in morphology resulting from shearing. The cell monolayer was then rinsed

with PBSc to remove any residual medium, and an ice-cold cell lysate was made (appendix 8.4.2) for subsequent protein gel electrophoresis.

5.2.1.5 Biochemical Analysis

The protein content of each cell lysate was estimated (appendix 8.4.3) and samples were subsequently diluted to give a standard protein content (HDMEC = 400µg/ml; HCAEC = 200µg/ml). Samples were run on 6% gels (see appendix 8.4.4) alongside MW standards and the expression of iNOS, eNOS, KDR/Fik-1, PECAM-1/CD31 was studied. Coomassie brilliant blue gel staining was performed to confirm equal protein loading across all lanes. All gels analysed demonstrated equivalent banding intensities with this stain.

5.2.2 Materials

5.2.2.4 Cell Shearing Apparatus

The polycarbonate *chamber* was manufactured by Mr George Wallace, University of St Andrews. A Masterflex console drive *roller pump* was used. Gas impermeable *tubing* was purchased from PharMed. The Cannon-Feske *viscometer* was purchased from BDH.

5.2.2.5 Cell Culture

Suppliers of *cells*, *culture medium* and *supplements* were as for section 4.5.21.

5.2.2.6 Immunological Reagents

All primary and secondary *immunological reagents* were as for section 4.9.2.2.

5.3 Experimental Protocol

Cells were exposed to a shear stress of 15dyn.cm⁻² for 5, 10, 30, 60, 120 and 240 mins. There were two static control slides. Two complete runs were performed with each cell type to ensure reproducibility of results.

5.4 Results

Both HDMEC and HCAEC remained adherent to their substratum after shearing. Microscopic examination of slides revealed only minor areas of cell denudation following shearing. There were no gross morphological changes observed in any of the sheared slides, even following the maximum shearing time (4 hrs). Cells remained polygonal in shape, with random orientations.

5.4.1 iNOS

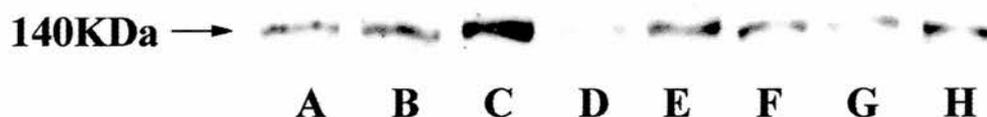
As observed under basal culture conditions with immunofluorescent cell staining (section 4.6.1) and following stimulation with tumour- and pericyte-conditioned media (section 4.11.1.1), no iNOS immunoreactivity was detected in either HDMEC or HCAEC before or after shearing.

5.4.2 eNOS

5.4.2.1 Human Coronary Artery Endothelial Cells

Detection of eNOS in samples was optimal with a loading of 10 μ l of 200 μ g/ml protein lysates. Nitrocellulose membranes were incubated overnight (4°C) with primary antibody (1:600) and for 2hrs (RT) with secondary (1:800). The results for increasing cell shearing time are shown in plate 5.1.

Plate 5.1 *Effect of Increasing time periods of FSS on eNOS protein expression in HCAEC (A-H)*



Key – *Static controls (lanes A and B); 5 min (lane C); 10 min (lane D); 30 min (lane E); 60 min (lane F); 120 min (lane G); 240 min (lane H).*

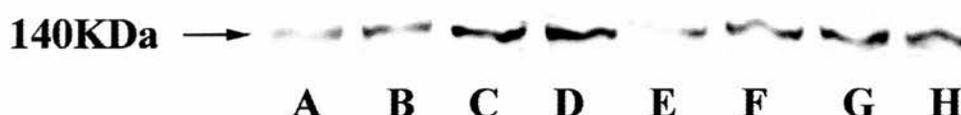
The initiation of shear stress (5 mins) stimulated a rapid upregulation of eNOS, followed by a sharp downregulation (10 mins). For longer shear periods (30 mins

and upwards), eNOS expression in sheared cells was restored to the level of static controls.

5.4.2.2 Human Dermal Microvascular Endothelial Cells

Detection of eNOS in samples was optimal with a loading of 20µl of 400µg/ml protein lysates. Nitrocellulose membranes were incubated overnight (4°C) with primary antibody (1:800) and for 2hrs (RT) with secondary (1:800). The results for increasing cell shearing time are shown in plate 5.2.

Plate 5.2 *Effect of Increasing time periods of FSS on eNOS protein expression in HDMEC (A-H)*



Key – *Static controls (lanes A and B); 5 min (lane C); 10 min (lane D); 30 min (lane E); 60 min (lane F); 120 min (lane G); 240 min (lane H).*

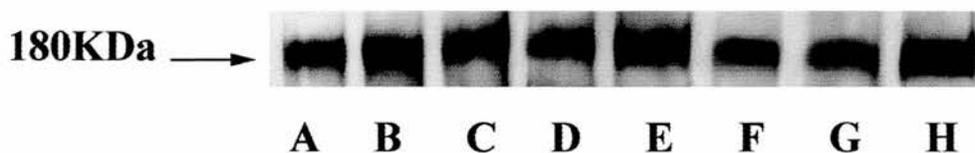
As for HCAEC, the initiation of shear (5–10mins) stimulated an upregulation of eNOS in HDMEC. This was followed by a down-regulation at 30mins. For longer shear periods (1hr and upwards), the level of eNOS expression in sheared cells was similar to that of static controls.

5.4.3 KDR/Flk-1

5.4.3.1 Human Coronary Artery Endothelial Cells

Detection of KDR/Flk-1 in samples was optimal with a loading of 20µl of 200µg/ml protein lysates. Nitrocellulose membranes were incubated overnight (4°C) with primary antibody (1:200) and for 2hrs (RT) with secondary (1:800). The results for increasing cell shearing time are shown in plate 5.3 overleaf.

Plate 5.3 Effect of Increasing time periods of FSS on KDR/Flk-1 protein expression in HCAEC (A-H)



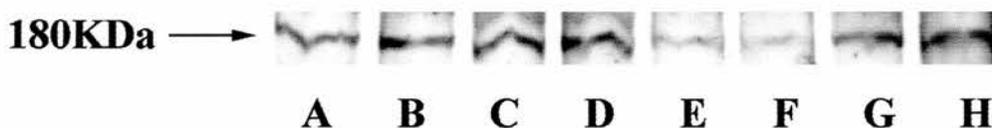
Key – Static controls (lanes A and B); 5 min (lane C); 10 min (lane D); 30 min (lane E); 60 min (lane F); 120 min (lane G); 240 min (lane H).

The expression of KDR/Flk-1 receptors was unaffected by shearing times of less than one hour. Receptor expression was reduced over shearing times of 1-2 hours, but returned to control levels by 4 hours.

5.4.3.2 Human Dermal Microvascular Endothelial Cells

Detection of KDR/Flk-1 in samples was optimal with a loading of 15 μ l of 400 μ g/ml protein lysates. Nitrocellulose membranes were incubated overnight (4°C) with primary antibody (1:200) and for 2hrs (RT) with secondary (1:800). The results for increasing cell shearing time are shown in plate 5.4.

Plate 5.4 Effect of Increasing time periods of FSS on KDR/Flk-1 protein expression in HDMEC (A-H)



Key – Static controls (lanes A and B); 5 min (lane C); 10 min (lane D); 30 min (lane E); 60 min (lane F); 120 min (lane G); 240 min (lane H).

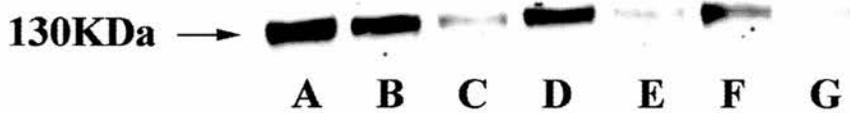
KDR/Flk-1 receptor down-regulation was apparent after 30mins of cell shearing and returned to control levels by 2 hours.

5.4.4 PECAM-1/CD31

5.4.4.1 Human Coronary Artery Endothelial Cells

Detection of PECAM-1/CD31 in samples was optimal with a loading of 5 μ l of 200 μ g/ml protein lysates. Nitrocellulose membranes were incubated for 1.5hr (RT) with primary antibody (1:2000) and for 1hr (RT) with secondary (1:1500). The results for increasing cell shearing time are shown in plate 5.5.

Plate 5.5 *Effect of Increasing time periods of FSS on PECAM-1/CD31 protein expression in HCAEC (A-G)*



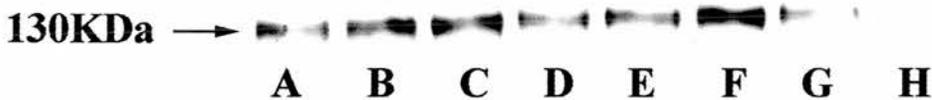
Key – Static controls (lanes A and B); 5 min (lane C); 30 min (lane D); 60 min (lane E); 120 min (lane F); 240 min (lane G).

PECAM-1/CD31 expression demonstrated considerable modulation with the FSS stimulus. Receptor expression appeared to be down and up-regulated in cycles with increasing cell shearing times.

5.4.4.2 Human Dermal Microvascular Endothelial Cells

Detection of PECAM-1/CD31 in samples was optimal with a loading of 5 μ l of 500 μ g/ml protein lysates. Nitrocellulose membranes were incubated for 2hr (RT) with primary antibody (1:500) and for 2hrs (RT) with secondary (1:1000). The results for increasing cell shearing time are shown in plate 5.6.

Plate 5.6 *Effect of Increasing time periods of FSS on PECAM-1/CD31 protein expression in HDMEC (A-H)*



Key – Static controls (lanes A and B); 5 min (lane C); 10 min (lane D); 30 min (lane E); 60 min (lane F); 120 min (lane G); 240 min (lane H).

As in HCAEC (plate 5.5), PECAM-1 expression in HDMEC appeared to fluctuate. Relative to static controls, expression was reduced slightly by 10 – 30mins of FSS, but then appeared to be up-regulated by a shear period of an hour. For longer periods of FSS exposure, expression was markedly reduced, with PECAM-1/CD31 almost undetectable after 4 hours exposure to the shear stimulus.

5.5 Discussion

The acute FSS to which ECs were exposed in the present study mimics one aspect of blood flow within a tumour. It most closely resembles what might happen on the resumption of flow following a period of stasis (see section 1.7.2). The results presented here show that shear stress has the potential to modulate the expression of a number of angiogenically-important proteins in endothelial cells. This implies that the patterns of blood flow within a solid tumour could have some influence on the development of tumour neovasculature.

Novel protein biosynthesis could involve the induction of gene expression, the transcription of mRNA and the biosynthesis of new protein. Together, these processes would generally be expected to require a time-frame in the range of hours. Modulation of expression at the level of regulating mRNA stability however, could conceivably occur on a shorter time scale.

In the experiments performed here, modified levels of protein expression were detectable after extremely short time-periods of shear exposure, in some instances (i.e. 5-10mins). In view of the above considerations, such rapid decreases and recoveries in expression are surprising, and despite experiments being repeated, may possibly represent experimental variability. It may be that during Western blotting, an uneven transfer of protein to the nitrocellulose membrane occurred in a particular region of a gel. This would clearly generate misleading results. It would therefore be fruitful, in future work, to repeat these experiments using an internal (housekeeping) ‘marker’ such as glucose 6-phosphate dehydrogenase (G6PDH). The level of expression of such a marker is not affected by the FSS stimulus.

No endothelial cell shape changes or alterations in alignment were detected in either cell type following the application of up to 4 hours of 15 dyn.cm^{-2} laminar flow. This contrasts with the findings of other investigators using large vessel endothelial cells (Dewey *et al.*, 1981; Flaherty *et al.*, 1972). It may be that chronic FSS or shearing forces $>15 \text{ dyn.cm}^{-2}$ are required to induce cell elongation and realignment in HCAEC and HDMEC.

5.5.1 iNOS

The expression of iNOS in HCAEC and HDMEC could not be detected in ECs stimulated by FSS. Fluid shearing is therefore unlikely to contribute, significantly, to the release of iNOS-derived NO in ECs.

5.5.2 eNOS

The patterns of eNOS expression in FSS-exposed HCAEC and HDMEC were similar. Both cell types displayed an upregulation of expression shortly after exposure of static cultures to flow. This is unlikely to be a result of experimental error, as similar observations have been reported by a number of other investigators using other EC types (Uematsu *et al.*, 1995; Ziegler *et al.*, 1998). As part of the heterogeneity of tumour blood flow, tumour vessels experiencing temporary occlusion/re-opening may therefore be expected to show an upregulation of eNOS expression, as a result of experiencing short ‘pulses’ of FSS. Interestingly, more prolonged periods of FSS failed to affect eNOS expression here. This contrasts with the results of a number of other investigators who have reported that longer shearing times (>24 hrs) are able to generate significant eNOS induction (Ziegler *et al.*, 1998). It has been reported that neither turbulent (Noris *et al.*, 1995) nor oscillatory (Helmlinger *et al.*, 1995) flow is able to induce eNOS expression. Given that tumour blood flow is expected to display a significant oscillatory component, it can be postulated that the contribution made to intertumour eNOS expression by FSS may not be great.

5.5.3 KDR/Flk-1

The expression of KDR/Flk-1 receptors in both cell types was unaffected by short periods of shear stress (<30 mins). Recent investigations have demonstrated

activation of the receptor in BAECs following very short exposure (~5mins) to 12dyn.cm^{-2} FSS (Chen *et al.*, 1999). Clearly this timeframe was not associated with changes in receptor expression in HCAEC or HDMEC here. Intermediate time periods of shearing (30min – 2hr) down-regulated receptor expression in both macro- and microvascular ECs. Expression was restored to control (unsheared) levels during more prolonged shearing times. Clearly then, heterogeneous blood flow through the tumour vasculature has the potential to modulate KDR/Flk-1 receptor expression.

5.5.4 PECAM-1/CD31

The patterns of PECAM-1/CD31 expression were similar in shear-exposed HCAEC and HDMEC. HCAEC demonstrated marked fluctuations in expression over the range of increasing shear times. HDMEC also showed fluctuating expression, although this was less striking. These fluctuations in expression may indicate a stabilisation of the monolayer during the stress stimulus, since the formation and re-formation of cell contacts and the strengthening of cell adhesion between cells would be expected to involve PECAM-1.

Chapter Six

Discussion

6.1 Background to the Present Study

NO entered the scientific limelight twenty years ago as endothelium-derived relaxing factor (Furchgott & Zawadzki, 1980). From these humble beginnings, it has advanced increasingly into the arena of clinical medicine, and our understanding of its implications in health and disease has expanded accordingly. Nevertheless, the involvement of NO in many areas of research remains poorly-understood, with often paradoxical or 'double-edged sword' aspects to its roles. The involvement of NO in the growth of solid cancers is no exception.

The research undertaken in this study aimed to contribute some information on the role of NO in the growth of an experimental solid tumour. Currently, the precise role(s) of NO in tumour progression are controversial, and a variety of apparently opposing functions have been both proposed and consolidated with convincing experimental evidence. One particular aspect of tumour growth in which NO is unequivocally involved in some way, is the *angiogenic response*. Without the induction of angiogenesis and the development of new vasculature, tumour growth is stunted (Folkman, 1972). Tumours are unable to grow beyond ~2-3mm in diameter and are similarly unable to metastasise. As a result, anti-angiogenic therapy represents a very promising avenue of cancer treatment, and as such, the involvement of NO in the process is clearly important. Its position however is far from clear.

Numerous observations of both pro- and anti-angiogenic roles for NO in solid tumours have been documented (see tables 1.7 – 1.8). Some opposing observations can be explained in terms of the quantities of NO being produced. Whilst low levels of NO act both to stimulate cell proliferation and protect against toxicity and apoptosis (Dimmeler *et al.*, 1997; Wink *et al.*, 1995), higher levels can mediate cytostatis, cytotoxicity and immunosuppression (Lejeune *et al.*, 1994; Nicotera *et al.*, 1995). Clearly then, NO will be expected to affect tumour growth in a dose-dependent manner. The situation however is more complex than this.

In this research, the potential of the L-arginine:NO pathway as an intervention point in the angiogenic process was considered, alongside a number of other aspects, as

part of a framework. These included the corresponding effects of NO-reducing drugs on tumour growth rate and the NOS and KDR/Flk-1 status of tumours. The potential therapeutic window of drugs was also considered in terms of their observed tolerance and toxicity.

In addition to these *in vivo* studies, *in vitro* EC experiments were also performed to examine aspects of the L-arginine:NO pathway in terms of three major tumour-associated factors – tumour- and pericyte-derived paracrine factors and the FSS imposed by bloodflow.

There were some inherent limitations of the animal tumour model used here, which merit consideration. Clearly the experimental ‘induced’ nature of the model somewhat limits the accuracy of extrapolating observations to a real, spontaneously-arising cancer. Nevertheless the model served as an efficacious ‘second-best’. The necessity of isogeneity of both the tumour and the host animals also had a particularly relevant bearing on this study. This is discussed more fully in section 6.4.

6.2 NO and Tumour Growth

The results from sections 2.4.7 and 3.4.7 unequivocally demonstrate that reducing the [NO], by two mechanistically-distinct methods – reducing its synthesis or removing it following synthesis – reduces the growth rate of solid tumours. NO is therefore functioning in a *pro-tumour* capacity in this model. The situation is also clearly more complex than the ‘low [NO], pro-growth’ theory discussed in section 6.1, as reducing NO to low levels is clearly also able to reduce tumour growth.

The use of NOS inhibitors with different isoform selectivity yielded strikingly different results. The iNOS-selectivity of AG and 1400W was reinforced by the observation that animals receiving these drugs remained normotensive. Both of these compounds were similarly ineffective at retarding tumour growth. The non-specific inhibitor L-NAME however, was highly effective. It is tempting to speculate, therefore, that NO derived from the eNOS isoform is of more significance in promoting tumour growth than that derived from inducible NOS. However, the

possibility of insufficient dosage and possible oral-ineffectiveness should be taken into account with results from 1400W use.

There was a marked time-dependency of the growth-retarding effect of L-NAME in tumours. An attempt to pre-empt the angiogenic response through drug administration prior to tumour implantation was unsuccessful. Maximum growth-retardation was only seen with later drug-administration times – 12 - 17 days after animals were implanted with tumours. The use of NO scavengers post-implantation (day 10) was similarly effective. This suggests that the stage of tumour development is critical in the efficacy of drugs, and implies that NO may potentiate tumour growth in the later stages.

6.3 NO and the Angiogenic Response

The results from sections 2.4.8 and 3.4.8 clearly demonstrate that both the inhibition of NOS (using L-NAME) and the scavenging of NO, limit the angiogenic response of this solid tumour. These drugs can therefore be considered as highly-effective anti-angiogenic compounds in this model, and NO can accordingly be considered to play a *pro-angiogenic* role. Inhibitors that were more selective for iNOS failed to limit the vascularity of tumours. This once again implies that NO derived from the eNOS isoform is perhaps more significant in the establishment of the angiogenic phenotype.

As reviewed in section 1.8.3.2, tumour-supply vessels exist in a state of exaggerated vasodilation as a result of expressing a high level of iNOS. Accordingly, previous research both from this group (Bisland, 1996; Kennovin *et al.*, 1994b) and elsewhere (Fukumura *et al.*, 1997; Gallo *et al.*, 1998; Meyer *et al.*, 1995; Swaroop *et al.*, 1998; Tozer *et al.*, 1997b; Whittle *et al.*, 1996) has demonstrated that NOS inhibitors have a potent constrictor effect on these vessels. Similarly, NO scavengers have vasoconstrictor action *in vivo* and *ex vivo* (Fricker *et al.*, 1997). It cannot therefore be excluded, that part of the tumour-retarding effect of the compounds examined here may result from an anti-vascular mechanism, effectively ‘starving’ the tumour

of blood. It is pertinent to note however, that a similar study involving the *i.v.* infusion of an NO scavenger to rats, an imidazolineoxyl *N*-oxide, C-PTIO, had no effect on tumour or normal tissue bloodflow (Tozer *et al.*, 1997), indicating an absence of vasoconstrictor action. In addition, the ineffectiveness of *iNOS*-selective inhibitors in reducing either tumour growth or tumour angiogenesis here, stands more in favour of an anti-angiogenic mechanism in the main. Ultimately however, the reduced vascularisation of tumours speaks for itself in presenting the anti-angiogenic weapon with which these drugs may potentially attack tumours. Of note, as discussed in section 1.9.4.1.a, vasodilation of tumour vessels represents the initial step in the angiogenic process. It could therefore be claimed that to an extent, an anti-vascular effect is in fact, also potentially anti-angiogenic anyway.

A final consideration of possible anti-vascular effects of these drugs is the presence of pericytes in the tumour microcirculation. In terms of the contractile (and hence vasoconstrictor) action of these cells, the findings that pericytes contain GC, relax in response to NO donors (Haefliger *et al.*, 1994,1997; Kelley *et al.*, 1987) and express NOS (Chakravarthy *et al.*, 1995) suggests the potential for the operation of the L-arginine: NO pathway. It could be hypothesised therefore, that in areas of the tumour microvasculature where the EC-pericyte contact is intact, NOS inhibitors and NO scavengers may induce pericyte-mediated vasoconstriction. However, the apparent disorganised nature of the EC-pericyte contact within large areas of tumour vasculature (see section 1.14.3), suggests that the contribution of this effect to the observed drug-action is unlikely to be major.

Although no information is available regarding the mechanism by which NO was able to promote angiogenesis in this model, it is interesting to consider briefly some possibilities. It has been suggested that NO may stimulate tissue degradation prior to EC migration (Murrell *et al.*, 1995), sustain EC proliferation (Morbidelli *et al.*, 1996; Ziche *et al.*, 1997b) and induce EC migration (Noiri *et al.*, 1998) and tubulogenesis (Papapetropoulos *et al.*, 1997). The direct associations between NO and VEGF for each stage of the angiogenic process (reviewed in table 1.9) present a host of possibilities. Unfortunately, since the expression of KDR/Flk-1 receptors in tumour sections was not localised to ECs but was widespread across both tumour and

vascular compartments, no information could be gathered with respect to a possible interaction between NO and VEGF here.

6.4 NOS Localisation and Expression

In agreement with most studies, both i- and eNOS were expressed by this tumour. Although iNOS expression was weak, this may result in part from the absence of an immune response to the implanted tumour (as a result of isogeneity). A low level of pro-inflammatory stimuli would be expected to limit the opportunity for inducing iNOS expression. In addition, the absence of sensitised lymphocytes would be expected to limit macrophage recruitment and activation. The contribution of macrophage tumouricidal activity will clearly then be reduced, and this potential additional source of NO will be absent.

Localisation of NOS isoforms was confined entirely to the vasculature of tumours. It may be hypothesised that restriction of the enzymes to this location could render the tumours more susceptible to NOS inhibition/NO scavenging regimes.

Western Blot analysis demonstrated that both NOS inhibitors and NO scavengers were able to modulate intratumoural NOS at the level of expression. However, no information is available as to whether or not changes in enzyme expression were translated into changes in activity i.e. greater or lesser production of NO. Both of the NO scavengers used here reversibly-reduced the expression of both e- and iNOS within tumours. This may indicate that, in addition to scavenging NO, part of the mechanism of these drugs is modulation of its production. However, other findings are in opposition to this, including the observation that L-NAME did not affect eNOS expression, yet induced mild hypertension and potently reduced tumour growth and angiogenesis. Similarly, 1400W did reduce eNOS expression, yet had no effect on blood pressure or tumour growth/angiogenic response.

Although biochemical analysis of KDR/Flk-1 was not successful, the relevance of this to the 'angiogenic response' of the tumour is unclear in view of widespread,

non-endothelial (i.e. non-functional) expression of this receptor across the tumour cell population.

6.5 Paracrine Factors and Angiogenesis

Pericytes inhibit EC growth in a contact-dependent manner (Orlidge & D'Amore, 1987). However, their effect on ECs via the release of paracrine factors appears to be growth-promotory, as they release an angiogenic factor into their culture medium (Murata *et al.*, 1994; Watanabe *et al.*, 1997b) which stimulates EC growth and angiogenesis *in vitro* (Sato *et al.*, 1987; Watanabe *et al.*, 1997b; Wong *et al.*, 1997). Tumour-conditioned medium similarly contains pro-angiogenic factors which promote *in vitro* angiogenesis (Folkman *et al.*, 1979; Folkman & Haudenschild, 1980; Klagsbrun *et al.*, 1976; Zetter, 1980).

In this study, TCM caused a marked increase in the EC-expression of both the eNOS enzyme and the KDR/Flk-1 receptor. PCM induced an even greater upregulation of these. This indicates a number of interesting possibilities. It highlights the potential of paracrine factors in both tumour- and pericyte-mediated angiogenesis. It also appears to reinforce the findings of the *in vivo* studies presented here, in suggesting that the enhancement of eNOS expression is an important pro-angiogenic event.

6.6 Fluid Shear Stress

As discussed in section 1.15.3, flow and consequently FSS in the tumour vasculature will be different from that within the normal vasculature. Both will lack uniformity and on average will be lower than that of normal tissues. Some areas of the vasculature will experience acute FSS, and flow will often be more turbulent than laminar. Clearly the flow/FSS characteristics across the entire tumour network will be a highly complex assortment, and the uniform, laminar, experimental conditions examined here can necessarily only represent one aspect of it. Nevertheless, the results seen in this study indicate that FSS has the potential to modulate the

expression of angiogenically-relevant proteins, and thus to impact on the process of intratumour angiogenesis.

Clearly a highly complex experimental set-up would be required in order to mimic, accurately, all the various flow conditions across tumour vasculature. Such an arrangement would allow a direct extrapolation of FSS effects to the angiogenic process. This is clearly beyond the scope of the present study.

6.7 Conclusions

- In this rat subcutaneous solid tumour model, NO appears overwhelmingly to *potentiate* tumour growth and progression, particularly in the later stages of tumour development.
- The L-arginine:NO pathway plays a key role in intratumoural *angiogenesis* in this model.
- Retardation of tumour growth achieved by reducing [NO] is likely, in part, the result of an attenuated angiogenic response.
- Of the synthetic enzymes responsible for NO production, the action of *endothelial* NOS appears to be the most relevant in terms of intratumour NO.
- NO-reducing drugs are able to modulate the NOS enzymes at the level of gene expression.
- The *pro*-angiogenic activity of pericytes is a paracrine effect and involves the L-arginine: NO pathway.
- Fluid shear stress arising from tumour bloodflow has the potential to modulate the intratumour angiogenic response.

6.8 Future Work

The intratumoural [L-arginine] is an important consideration in the effect of NOS inhibitors on tumour growth, and accordingly, the lack of effect of a NOS inhibitor has in one instance been put down to high intratumour [L-arginine] levels (Thomsen *et al.*, 1997). High levels would be expected to generate enhanced competition between L-arginine and the drug for the NOS binding site, thereby reducing the potential drug efficacy. It would be pertinent to measure the basal intratumoural [L-arginine] of the *p22* carcinosarcoma to determine the possibility for such competition in this model.

The angiogenic effects of VEGF may be mediated by increased NO synthesis. Conversely, NO is capable of both suppressing (Liu *et al.*, 1998) and enhancing (Ambs *et al.*, 1998; Chin *et al.*, 1997; Frank *et al.*, 1999) the induction of VEGF. Based on the inadequacy demonstrated here, of using KDR/Flk-1 expression as a gauge of angiogenic activity, it would be interesting to examine the relationship between NO and VEGF protein itself in the *in vivo* rat solid tumour model.

Since the effect of NO on tumour growth appears to relate to the *p53* status of a tumour, an examination of the *p53* status of the experimental carcinosarcoma used here would be pertinent.

Having established that the pro-angiogenic actions of pericytes involve NO and are mediated by a paracrine effect, it would be interesting to establish the pericyte status of the experimental carcinosarcoma used here, to determine the possible contribution made to intratumoural angiogenesis by these cells.

The injection of tumour vasculature with a polymerising material enables vascular casts of tumours to be produced. It would be valuable to examine the vascular architecture of tumour casts in animals treated with NO-reducing drugs, to confirm the reduction in vascularisation of these tumours.

Chapter Seven

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Chapter Eight

Appendices

Appendix 8.1

Rat Subcutaneous Solid Tumour Implantation

1. Third generation cryopreserved *p22* carcinosarcoma was rapidly defrosted in a excess volume of MEM to remove all traces of DMSO.
2. Recipient BDIX rats were administered a general *i.p.* anaesthetic (90mg.Kg⁻¹ Ketamine; 10mg.Kg⁻¹ Xylazine) and the fur from the dorsal surface was shaved. Skin around the injection sites was swabbed with antibacterial hibitane.
3. Defrosted tumour pieces were finely minced using sterile scissors/scalpel and injected *s/c* into 2 sites on the back of the BDIXs. Post-operative analgesia was administered and animals monitored until conscious.
4. After ~ 2-3 weeks growth (max 5% body weight), 'passaged' tumours were of sufficient size to dissect out. Animals were killed by a schedule one method, and their tumours dissected out using sterile technique. The outer tumour cortex was removed, minced into 1mm³ pieces and maintained over ice.
5. Experimental male BDIX rats were anaesthetised (as in step 2), their dorsal fur shaved, and the skin swabbed with hibitane.
6. The 1mm³ pieces of freshly-dissected 4th generation tumour were implanted into 2-4 *s/c* sites on the dorsal surface of the animals, using a sterile 16G trochar. Post-operative analgesia was administered and animals monitored until consciousness. Tumours growth was apparent within ~ 7 days.

Appendix 8.2

Histological Preparation of Rat Tissue

I. Paraffin Wax Embedding and Histological Staining

8.2.1 Tissue Preparation

1. Whole tumours and small pieces of liver and lung tissue were isolated from freshly-sacrificed experimental animals.
2. Tumours were sectioned in half and fixed by submersion in 4% neutral-buffered paraformaldehyde (Fisons lab reagent) for 1-2 nights. Pieces of liver and lung tissue were fixed by immersion in 1% neutral-buffered paraformaldehyde overnight.
3. Fixed tissue was processed for paraffin wax embedding – dehydration by immersion in 96% alcohol (2 changes), absolute alcohol (2 changes), absolute alcohol/chloroform (1 change), chloroform (2 changes); 1-2 hours in each solvent.
4. Dehydrated samples were infiltrated with wax by immersion in molten paraffin wax (3 changes, with 1.5hr per change). Care was taken to minimise required wax infiltration time to avoid unnecessary loss of sample antigenicity.
5. Wax-impregnated samples were ‘blocked out’ by embedding in a mould of molten paraffin wax and being allowed to set at 4°C.
6. Tissue sections (~7µm) were cut from the wax blocks with a microtome, floated out onto 45°C water to remove folds and picked up onto poly-L-lysine-coated microscope slides (BDH).

8.2.2 Haematoxylin and Eosin (H+E) Staining

1. Sections were de-waxed in xylene (2 changes, 5 mins in each) and hydrated through graded alcohols (absolute, 96%, 75%) to water.
2. Immersion in Mayer’s haematoxylin was for ~12 mins.
3. Sections were washed well in running, alkaline tap water until ‘blue’ (~5mins).
4. Differentiation was in 1% acid alcohol for ~20 secs.
5. Sections were again washed until ‘blue’.
6. Immersion in 1% eosin yellow for ~5 mins.
7. Sections were washed in running tap water for ~5mins.
8. Sections were dehydrated back through graded alcohols, ‘cleared’ in xylene and mounted in DEPX permanent mountant (BDH).

8.2.3 Masson's Trichrome Staining

1. Sections were de-waxed in xylene (2 changes, 5 mins in each) and hydrated through graded alcohols (absolute, 96%, 75%) to water.
2. Immersion in Celestine blue was for ~10 mins.
3. Sections were rinsed in DH₂O and immersed in Mayer's haemalum for ~10mins.
4. Sections were washed well in running, alkaline tap water for ~5mins.
5. Immersion in Yellow mordant (Orange G and Lissamine fast yellow) was for ~3mins.
6. Sections were washed well in running, alkaline tap water for 2-10mins.
7. Immersion in Ponceau acid fuchsin was for ~5mins.
8. Sections were briefly rinsed in tap water and immersed in 1% phosphomolybdic acid until connective tissue was free of red dye.
9. Immersion in Light green was for 2-3mins.
10. Sections were rinsed in 1% acetic acid.
11. Sections were dehydrated back through graded alcohols, 'cleared' in xylene and mounted in DEPX permanent mountant (BDH).

Appendix 8.3

Histological Preparation of Rat Tissue

II. Snap-Freezing and Immunohistochemical Staining

8.3.1 Tissue Preparation

1. Whole tumours were isolated from freshly-sacrificed experimental animals.
2. Tumours were sectioned in half and immediately submerged into pre-cooled isopentane in liquid nitrogen.
3. When frozen (~5 secs), sections were removed, sealed into airtight containers, and stored frozen at -70°C until use.
4. At the time of processing, frozen tumour pieces were mounted in O.C.T-Tissue-Tek (BDH) and were maintained at -20°C in a cryostat, whilst $\sim 7\mu\text{m}$ sections were cut from the blocks.
5. Sections were picked up and thaw-mounted on poly-L-lysine or gold-coated microscope slides (BDH) and were air-dried overnight at RT.
6. Fixation was by a 10 min immersion in acetone, followed by a further overnight period of air-drying. (Adequate air drying at stages 5 and 6 prevented changes to tissue morphology. Drying at 37°C appeared to disrupt morphology and was therefore avoided).

After this stage of processing, sections were stored at 4°C and immunostained within 48 hrs of fixation.

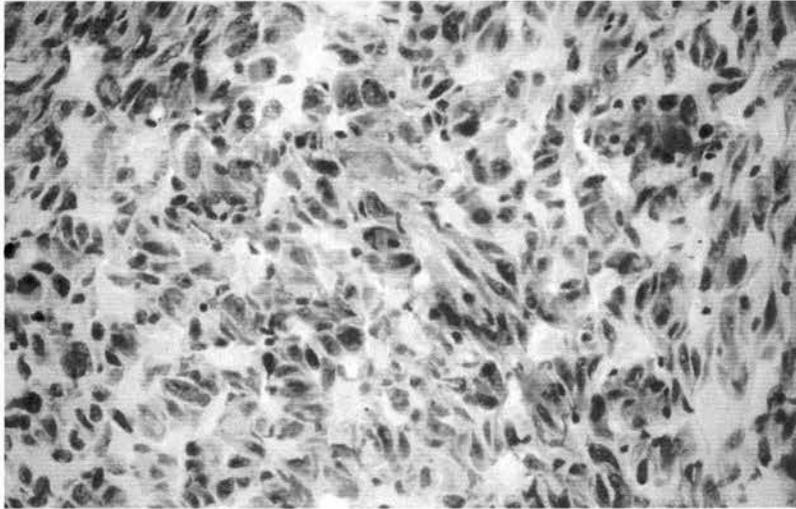


Plate 8.1 *Frozen Tumour Section Stained with Trypan Blue.*

It can be seen from plate 8.1 that tumour sections retained good morphology when processed according to the above schedule.

8.3.2 Labelled Streptavidin-Biotin Immunoperoxidase Staining of Sections

All immunohistochemical labelling of frozen tumour sections was performed with a highly-sensitive LSAB-2 kit (Vector). This was pre-absorbed against rat tissue and consequently non-specific cross-reactivity, and the need for a blocking (serum) step was eliminated. All antibody incubations were performed at RT to minimise background staining.

1. Frozen sections were re-hydrated in PBS for 5-10mins.
2. Slides were drained thoroughly, and excess PBS was removed from around tissue sections with filter paper. Each section was encircled with a water-repellent immunohistochemical pen (DAKO) in order that solutions would be contained around sections.
3. Endogenous peroxidase blocking was found to be necessary* and a mild blocking method was used. Sections were incubated in 0.3% hydrogen peroxide (H₂O₂) in 70% methanol for 30min at RT, after which they were rinsed in PBS.
4. Sections were incubated with diluted (in 1% bovine serum albumin) primary antibody** at RT for 15-30mins. Control slides*** were prepared alongside test slides.
5. Sections were rinsed in DH₂O and *immediately* incubated with the biotinylated link antibody for 15-30mins at RT.
6. Sections were rinsed in DH₂O and incubated with streptavidin for 15-30mins at RT.
7. Sections were rinsed in DH₂O and incubated in the peroxidase-chromogen solution (diaminobenzadine (DAB) tablets (Sigma) in PBS, activated with 30% H₂O₂) for 10 to 45mins at RT.
8. Sections were rinsed in DH₂O, dehydrated through graded alcohols (20%, 75%, 96%, 100%), cleared (2X changes of xylene) and mounted in permanent DPX mounting medium (BDH).

* Blocking endogenous peroxidase activity is not routinely necessary in frozen sections and the effect of H₂O₂ may destroy or alter antigenic sites. However, the 'milder' block employed here (0.3% H₂O₂ rather than the standard 3% H₂O₂ used for paraffin sections) was found to be beneficial in reducing non-specific binding and had no detrimental effect on section antigenicity.

** On account of the sensitivity of the streptavidin detection system, primary antibody, biotinylated antibody and streptavidin incubations are recommended for 10mins at RT. However, for labelling most proteins in this research, longer incubations times were found to be beneficial (20-60min, RT). Although this necessarily increased non-specific background staining, it was found to improve specific staining considerably.

*** Two negative control slides were routinely produced. (1) Incubation with only the avidin-biotin-peroxidase complex to test for non-specific binding of avidin. (2) Incubation with only 1% BSA in place of the primary antibody as a secondary antibody control.

8.3.2.1 eNOS Staining Protocol

Incubation with primary antibody (1:100), secondary antibody and streptavidin for 15mins each at RT. Incubation with chromogen solution for 30mins at RT.

8.3.2.2 iNOS Staining Protocol

Incubation with primary antibody (1:20), secondary antibody and streptavidin for 30mins each at RT. Incubation with chromogen solution for 45mins at RT.

8.3.2.3 KDR/Flk-1 Staining Protocol

Incubation with primary antibody (1:50), secondary antibody and streptavidin for 15mins each at RT. Incubation with chromogen solution for 15mins at RT.

8.3.2.4 PECAM-1 Staining for MVD Determination

Incubation with primary antibody (1:100), secondary antibody and streptavidin for 15mins each at RT. Incubation with chromogen solution for 10mins at RT.

Appendix 8.4

Detecting Protein Expression

8.4.1 Tissue Preparation (tumour)

8.4.1.1 Tissue Homogenisation

Tumour homogenisation was performed ice-cold, with RIPA lysis buffer.

RIPA buffer– 1 X PBS, 1% Nonidet P-40 (NP40) (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% SDS. This was made up as a 100ml stock solution and stored for up to 6 months at 4°C. At the time of use, the following protease inhibitors (all from Sigma) were added:

- (i) 10mg/ml Phenylmethylsulphonyl fluoride (PMSF). This is active against chymotrypsin and trypsin. A stock solution was made up in isopropanol and stored at –20°C. Used at 10µl/ml buffer.
- (ii) Aprotinin. This is active against kallikrein, trypsin, chymotrypsin and plasmin. Used at 30µl/ml buffer.
- (iii) Sodium orthovanadate (Na₃Va₄). This is active against protein phosphotyrosyl-phosphatases. A 100mM stock solution (0.18g into 10ml PBS) was made up. For maximum protease inhibition, the solution was ‘activated’- the pH was adjusted to 10 (using 1N NaOH or 1N HCL) at which the solution was yellow. It was boiled until clear and cooled to RT. The pH was readjusted to 10 once again, and the boiling /cooling process repeated until the solution remained clear at pH10. Activated solution was stored frozen at –20C until use. Used at 10µl/ml buffer.

Aprotinin and sodium orthovanadate were stable within the buffer solution for 24hours. PMSF has a $t_{1/2}$ of just 30mins in aqueous solution. It was therefore discarded after this time and fresh was made up.

1. Tumour pieces were removed from their cryovials, weighed and placed into ready-cooled universals with 3ml ice-cold RIPA buffer per gram of tissue. The tissue was then diced up with scissors and homogenised over ice with an Ultraturrax T75 (Janke & Kunkel; IKA) for 5 mins at a speed of 24,000RPM. An additional 30µl of 10mg/ml PMSF stock was added per gram of tissue, and the homogenate was incubated on ice for 30mins.
2. Samples were pipetted into ice-cold microcentrifuge tubes and immediately centrifuged at 10,000G for 20mins at 4°C. The supernatant was removed and stored at –20°C until use.

8.4.1.2 Electrophoresis Sample Preparation

Following protein estimation of homogenates (see section 8.4.3), tissue samples were prepared for loading onto gels.

Samples were diluted to the required [protein] using (2X) electrophoresis sample buffer (loading buffer). This was necessary as the density of glycerol was required in order for samples to be accepted into gel lanes.

A stock loading buffer was made up - 1ml glycerol (BDH), 0.5ml β -mercaptoethanol (Sigma), 3ml 10% SDS, 1.25ml 1M tris-HCL pH 6.7 (Sigma), bromophenol blue (BDH) (1 μ l/100 μ l solution). This stock was stored at -20°C . After defrosting and addition to samples, they were boiled for 5mins to denature proteins and destroy disulphide bonds. Complete electrophoresis samples were stored at -20°C thereafter.

8.4.2 Cell Lysate Preparation

1. The cell culture medium was removed from a 90mm petri dish and the monolayer carefully rinsed with PBS at RT. After this point, petri dishes were kept over ice.
2. 600 μ l of ice-cold RIPA (with freshly-added inhibitors) was added to the petri dish (see section 8.4.1.1).
3. The barrel of a syringe was scraped over the monolayer to dislodge the cells and the cells and buffer were transferred to ice-cold microcentrifuge tubes through a 21G needle.
4. The petri dish was rinsed with a further 300 μ l RIPA buffer and any remaining cells and buffer were combined with the first lysate. The complete lysate was passed through a 21G needle several times to shear the cell DNA.
5. 10 μ l of 10mg/ml PMSF was added to each tube and samples were incubated on ice for 30mins.
6. After incubation, lysates were centrifuged at 10,000G for 10mins at 4°C . The supernatant (all solubilised proteins) was removed and stored at -20°C until use.

Following protein estimation (see section 8.4.3), cell lysates were prepared for gel electrophoresis as described in section 8.4.1.2.

8.4.3 Protein Estimation of Tissue/Cell Extracts

The protein content of tumour homogenates and cell lysates was estimated using the *Detergent Compatible Protein Assay* (Biorad) which measures [protein] in the range of 0.1 to 2mg/ml. This is a colourimetric assay for protein concentration following detergent solubilisation. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. There are 2 steps to the assay which lead to colour development: The reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. Proteins effect a reduction of the Folin agent, thereby producing one or more of several possible reduced species which have a characteristic blue colour with maximum absorbance at 750nm and a minimum absorbance at 405nm.

Tumour homogenates (high protein) were diluted to 1:20; cell lysates (low protein) were diluted to 1:2. This brought the protein content of both sample sets into the

range which was measurable accurately by the kit. Each standard and sample was tested in triplicate.

1. A stock solution of BSA (1.44mg/ml protein) was made up from lyophilised powder in 1:20 or 1:2 RIPA buffer:PBS. The stock was diluted to give a range of 4 standard solutions of increasing [protein] – 0.2, 0.4, 0.8 and 1.2mg/ml.
2. 250ul of REAGENT A (alkaline copper tartrate solution) per sample to be tested was pipetted into a universal. 20ul of REAGENT S was added per ml of A.
3. Samples were prepared in spectrophotometric cuvettes (BDH):
To *blanks* (spectrophotometric calibration) : 50µl of 1:2 or 1:20 RIPA was added.
To each of the 4 *standards*: 50µl of the appropriate dilution was added.
To *test samples*: 25µl PBS + 25µl sample (for cell lysates) OR
47.5µl PBS + 2.5µl sample (for tumour homogenate) was added.
4. 250µl of REAGENT A+S was added to each cuvette. Each was vortexed immediately.
5. 2ml of REAGENT B (dilute Folin reagent) was added to each cuvette. Each was vortexed immediately.
6. All samples were left to stand at RT for 30mins to allow the colourimetric reaction to take place (absorbances remain stable for ~1hr).
7. The UV/Vis spectrophotometer was 'air zeroed' and the 2 blank cuvettes read against each other to calibrate the machine to zero.
8. Each sample was read at 720nm and a calibration curve was derived from the *standard* readings (absorbance at 720nm against standard [protein] (mg/ml). A linear curve fit was applied to the graph and the gradient equation ($Y = mX + c$) and r^2 value was noted. The Y value was replaced with the absorbance readings obtained from the test samples to calculate the X value. The [protein] of samples were therefore estimated, taking into account the initial X2 or X20 dilution factor.

8.4.4 SDS-Polyacrylamide Gel Electrophoresis, Western Blotting and Electro-chemiluminescence (ECL) Detection

8.4.4.1 SDS-Polyacrylamide Gel Electrophoresis

An Anachem MV2-DC gel rig was used for all gel electrophoresis. Polyacrylamide gels are composed of chains of polymerised acrylamide which are cross linked. The separation of proteins onto the gel depends on the concentration of acrylamide and the amount of cross-linking. The lower the molecular weight of a protein, the further the distance it will migrate through an acrylamide gel during the application of an electrical current.

1. The glass gel plates were thoroughly cleaned prior to each use. They were washed in detergent (Decon), DH₂O, 70% alcohol and finally DH₂O – drying between each wash.
2. The plates were assembled vertically in the gel setting rig with side spacers placed between them and a rubber base mat placed beneath them. Plates were tightened together with side clamps, ensuring sufficient space was available between them for the insertion of the sample comb.
3. Each gel consisted of 2 regions – a 'stacking' gel (4%) into which samples were loaded; and a 'running' gel, through which protein separation occurred. Since all

proteins examined in this research had MW >130KDa, running gels were routinely prepared to 6%.

Constituents for *one* gel:

6% Running Gel – Milli-Q H₂O 8.8ml; 40% acrylamide (Scotlab) 2.2ml; 1.5M Tris buffer pH 8.8 3.8ml; 10% SDS 150μl. To polymerise: 10% ammonium persulphate* (APS) (Sigma)150μl; TEMED (Sigma) 12μl.

4% Stacking - Milli-Q H₂O 3.6ml; 40% acrylamide 623μl; 1M Tris buffer pH 6.8 630μl; 10% SDS 50μl. To polymerise:10% APS 50μl; TEMED 5μl.

* 10% APS (in DH₂O) remains active in solution for ~1 week at 4°C.

All gel constituents (except for the polymerising agents) were made up in 25ml universals.

4. The base of the rig was sealed by removing 1ml from the running buffer and adding 40μl 10% APS + 4μl TEMED. This mixture was quickly pipetted between the gel plates, tilting the rig to ensure the base was covered. This sealing gel polymerised within 1min. Any surface bubbles were removed from the polymerised sealing gel with a sheet of filter paper.
5. The APS and TEMED were added to the running gel, which was then pipetted between the gel plates to ~5mm below the base of the sample comb. Any air bubbles were removed from the surface of the running gel and a little 0.1% SDS was pipetted onto the gel surface to exclude air during polymerisation. The gel was left to polymerise for ~20 mins.
6. After polymerisation, the SDS sealant was poured away and the gel surface rinsed with DH₂O. The exposed glass above the running gel was carefully dried with filter paper.
7. The APS and TEMED are added to the stacking gel, which was then pipetted onto the running gel, up to the top of the gel plate. The sample comb was carefully added, avoiding the introduction of any bubbles. The running gel was left to polymerise for ~15 mins.
8. When polymerised, the gel was removed from the setting rig and assembled in the MV2-DC rig. The apparatus was filled with electrode buffer (400ml DH₂O + 100ml 5x Tris/glycine buffer) to above the level of the sample comb.
9. The sample comb was carefully removed and any air bubbles/unpolmerised acrylamide were rinsed out of the sample wells with electrode buffer.
10. Samples and MW markers were pipetted into the wells (maximum of 20μl/well).
11. Cold water was circulated through the apparatus to remove the heat generated by the electrical current. The rig was connected to a power pack. An output voltage of 70V was applied as the samples progressed through the stacking gel. Once within the running gel, the voltage was increased to 100 –150V. Samples were separated through the running gel until the dye front reached the base of the gel plates (~1.5hr). Power was switched off.
12. The gel was disassembled from the rig and the upper gel plate and side spacers were removed. The bottom left corner of the gel was cut to identify the gel orientation. The stacking gel was removed with a sharp razor and the remaining gel measured.

13. The gel was carefully removed from the remaining glass plate and immersed in Tris /glycine /SDS transfer buffer for ~20 mins (3g tris, 14.4g glycine, 200ml methanol, 3.75ml of 10% SDS. DH₂O up to 1litre). Nitrocellulose membrane (Amersham) and two thick filter paper pieces (Biorad) were cut to the size of the gel and also submerged into transfer buffer for ~20mins.

8.4.4.2 Western Blotting

A Biorad Western Blotting transfer unit was used for protein transfer from a gel to a nitrocellulose membrane.

14. The anode and cathode of the transfer unit were moistened with water and one sheet of soaked filter paper was rolled flat onto the anode with a wet glass rod, to expel air bubbles. The nitrocellulose membrane was carefully positioned onto the filter paper and rolled. The gel was then added to the stack, and once again rolled. Finally the second soaked filter paper was placed on top of the gel stack, and rolled. The cathode was then placed on top of the stack and the unit lid added.
15. The transfer unit was connected to a powerpack and set to 15 volts. Transfer was for 1-2hrs, depending on the MW of the protein under detection (eNOS and iNOS = 1hr; KDR/Flk-1 = 1.5hr).
20. Following transfer, the gel stack was dismantled and the filter paper discarded. The bottom left corner of the membrane and the gel front were marked.
21. The nitrocellulose membrane was placed into ponceau red non-permanent stain (Sigma) to confirm the presence of protein banding. The gel was placed into coomassie brilliant blue protein stain (Sigma) at 4°C o/n to detect any residual, non-transferred proteins. The membrane was rinsed in DH₂O to remove ponceau red stain.

8.4.4.3 Labelling of Protein Bands

22. Non-specific binding sites on the nitrocellulose membrane were blocked by an overnight incubation of the membrane (4°C) in 5% non-milk fat (Boots) in PBS with 0.05% Tween (PBS-T) (Sigma).
26. After blocking, the membrane was removed from the milk mixture and rinsed with 2 x large volumes of PBS-T.
27. The primary antibody was diluted to the required concentration in 5% blocking milk solution. 0.1ml antibody/milk mix was required per cm² of the membrane.
28. The membrane was placed into a plastic packet and incubated in the antibody mix for the required time.
29. The coomassie stained gel was placed into de-stain solution, and the amount of residual protein present was noted. This gave a gauge of the efficacy of protein transfer.
30. Following primary antibody incubation, the nitrocellulose membrane was rinsed in PBS-T x 2, leaving in the last rinse for 15 mins. A further 2 x 5mins rinses in fresh PBS-T were performed.
31. The secondary antibody was diluted in 5% marvel to the required concentration and the rinsed membrane was incubated in the secondary solution for the required time.

33. Following secondary incubation, the membrane was once again rinsed x 2 in PBST, leaving in last rinse for 15 mins. A further 2 x 5 mins rinses in fresh PBST were performed.

8.4.4.4 *Enhanced Chemiluminescence (ECL) – Detection of Protein Banding*

The ECL Western Blotting detection system (Amersham) was used for the detection of protein bands on nitrocellulose membranes. This is a light-emitting, non-radioactive method for the detection of immobilised specific antigens, conjugated with HRP-labelled antibodies. The principles behind this detection system are that HRP/hydrogen peroxide catalyse the oxidation of luminol. The luminol is thereafter in an excited state which decays to ground state via a light-emitting pathway. Enhanced chemiluminescence is achieved by performing the oxidation of luminol by the HRP in the presence of chemical enhancers. This increases both the intensity and emission time of light output. The maximum light emission is at a wavelength of 428nm which can be detected by a short exposure to blue-light sensitive autoradiography film.

34. Sufficient ECL reagents (A+B) to cover the membrane (surface area in $\text{cm}^2 \times 0.125\text{ml} / 2$) were mixed in a universal and immediately pipetted onto the protein side of the rinsed and drained nitrocellulose membrane. Reagents were left for precisely 1 min.
35. The membrane was then drained and placed protein side down onto cling-film wrap (Lakeland) and a smooth, bubble-free packet was made around the membrane.
36. The membrane was placed into a film developing case in a dark room, and all white lighting was switched off. A single piece of ECL hyperfilm (Amersham) was placed over the membrane, and the developing case lid was tightly closed.
37. Film development was allowed from 5-60 mins depending on the intensity of the signal.
38. Hyperfilm was placed into photographic developer (Kodak) for 3 mins, rinsed in H_2O , then placed into photographic fixative (Kodak) until 'clear'. The pattern of primary and secondary-tagged protein banding and the Santa Cruz MW markers on the membrane, was reproduced on the film.

Appendix 8.5

The Griess test - Quantitative determination of NO in blood samples

The transient nature of NO makes it unsuitable for most conventional detection methods. However, since most NO is oxidised to nitrite (NO_2^-) and nitrate (NO_3^-), the concentration of these anions has become a standard assay for the indirect measure of total NOx. This is the rationale behind the Griess test.

The most commonly used method for the determination of nitrite is an assay based on the Griess reaction. The procedure consists of the conversion of all nitrate (NO_3^-) to nitrite (NO_2^-) using nitrate reductase enzyme, followed by the spectrophotometric determination of total NO_x^- at 540nm. The assay can be performed in duplicate (i.e. with and without reduction) to determine nitrate and nitrite separately.

The chemical reaction is divided into 2 steps - initially a two stage diazotization reaction in which acidified NO_2^- produces a nitrosating agent which reacts with sulfanilic acid to produce the diazonium ion. Secondly, this ion is coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative (purple) which absorbs light at 540nm.

SOLUTIONS

Milli-Q water was used throughout to avoid contamination of reagents with nitrate or nitrite. All reagents were warmed to RT before use.

Phosphate buffer solution pH 7.3

- | | |
|---|-------------|
| 1 Potassium Dihydrogen Orthophosphate (Analar) | MW = 136.09 |
| 2 DiPotassium Hydrogen Orthophosphate trihydrate (Analar) | MW = 228.23 |

A 400mM stock solution of each of 1 and 2 was made and solution 1 was mixed with solution 2 in a ratio of 2:8. The pH was checked to be 7.3 and the solution stored at 4°C.

By adding 22.5ul of phosphate buffer to each tube a final concentration of 40mM phosphate (pH 7.3) was present.

Diazochromophore agents

- A 1% sulfanilamide (Sigma) in 5% phosphoric acid in milli-Q water
B 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in milli-Q water

Stock solutions of A and B were made and stored at 4°C for up to 2 months.

β -Nicotinamide Adenine Dinucleotide Phosphate, reduced form (β -NADPH)

MW = 833

NADPH (Sigma) was stored frozen at -20°C for a maximum of 45 days. Solution was made up fresh at the time of use.

By adding 4 mg NADPH into 1 ml of milli-Q water a solution of 4.8mM was produced. Adding 22.5ul of this to each tube gave a final concentration of 480uM

NADPH. (This is below the level requiring oxidation to avoid interference with detection). Reconstituted NADPH was kept on ice during the assay.

Flavin Adenine Dinucleotide (FAD) MW = 829.5

FAD (Sigma) was stored frozen at -20°C . Solution was made up fresh at the time of use.

By adding 4mg FAD into 10mls milli-Q water, a 482uM solution is made.

Adding 22.5ul of this to each tube gives a final concentration of 48.2uM.

Nitrate Reductase enzyme (NR)

Nitrate reductase purified from the *Aspergillus* species (Boehringer Mannheim) in powder form was used. That from other species or suppliers has been found to give incomplete recovery (Schmidt and Kelm, 1996).

This was stored dry, protected from light at -20°C for a maximum of 3 months.

Batch size is 20mU and 70mU of NR per sample is required. By diluting the 20mU into 2mls of milli-Q water at the time of use, 7ul of this solution contains the required 70mU. The solution was vortexed to ensure complete dissolution before use. Reconstituted NR was kept on ice during the assay.

Nitrate (NO_3^-) and Nitrite (NO_2^-) Standards

2mM stock solutions of Analar sodium nitrite (Sigma) (MW = 69) (138mg/1000ml) and sodium nitrate (Sigma) (MW = 84.99) (170mg/1000ml) were prepared in milli-Q water.

PROTOCOL

Serial dilutions of NO_2^- and NO_3^- internal standards (controls) were prepared from the stock solutions. Test (serum) solutions were measured against these.

22.5ul of $\text{NO}_2^-/\text{NO}_3^-$ stock:

			<u>Amount present</u>
1.	2mM	20ml stock solution	45 nmoles
2.	1mM	10ml stock + 10ml milli-Q	22.5 nmoles
3.	0.5mM	5ml stock + 15ml milli-Q	11.25 nmoles
4.	0.25 mM	2.5ml stock + 17.5ml milli-Q	5.625 nmoles
5.	0.125mM	1.25ml stock + 18.75ml milli-Q	2.8125nmoles

Two sets of 5 tubes each of test (plasma) and standard (water) solutions were made up in spectrophotometric cuvettes:

Set 1 = 5 tubes containing 127ul reconstituted *plasma* spiked with 22.5ul NO_3^- standard dilutions respectively.

Set 2 = 5 tubes containing 127ul reconstituted *plasma* spiked with 22.5ul NO_2^- standard dilutions respectively.

Set 3 = 5 tubes containing 127ul *milli-Q water* spiked with 22.5ul NO_3^- standard dilutions respectively.

Set 4 = 5 tubes containing 127ul *milli-Q water* spiked with 22.5ul NO_2^- standard dilutions respectively.

Into each tube the following were added:

22.5ul mixed phosphate buffer
22.5ul NADPH solution
22.5ul FAD solution
7ul NR

After the addition of NR, cuvettes were incubated for 2 hours at 37°C (to allow complete conversion of nitrate to nitrite).

Diazochromophore agents were then added: 224ul solution A and 224ul solution B were added to each tube and incubated for a further 15 mins at 37C.

Following this the absorbances were read on a UV/Vis spectrophotometer at a wavelength of 540nm. When a linear graph is plotted of absorbance (Y axis) against $[\text{NO}_3^-/\text{NO}_2^-]$ (X axis), the negative X axis intercept represents nanomoles of NOx in the plasma sample.

REFERENCE CELL

The reference cell for NOx levels in serum should contain: 127ul serum sample, 22.5ul NADPH solution, 22.5ul phosphate buffer solution, 224ul solution A, 22.5ul of 2mM NO_3^- internal standard and 253.5ul milli-Q water (which takes into account the 22.5ul of FAD solution, 224ul of solution B and 7ul of NR).

Appendix 8.6

Endothelial Cell Culture

8.6.1 Cell Derivation from Cryopreservation

Cells obtained from a commercial supplier as a frozen stock were established as a growing culture in the following way:

1. The cryovial of frozen cells was defrosted rapidly by immersion in a 37°C waterbath. Immediately the cells were defrosted, the cryovial seal was wiped with 70% alcohol and, in a sterile cabinet, the contents were carefully pipetted into a sterile universal containing a small volume of pre-warmed culture medium. Delay at this point can cause cell damage due to dimethyl sulphoxide (DMSO) in the freezing medium. The cryovial was gently rinsed out with a small volume of culture medium to ensure that all cells were removed.
2. In the universal, the cell suspension was gently pipetted up and down to break up any cell clumps, taking great care not to damage the cells.
3. The contents were transferred to a T-25 cell culture flask together with a total of 15ml of culture medium.
4. Cells were incubated overnight in 5%CO₂/air, and undergo a medium change after 24 hours to remove any remaining traces of DMSO.

8.6.2 Routine Subculture of Cells (HCAEC and HDMEC)

Having derived cells from frozen stock, they were routinely 'passaged' to provide cells for experimentation.

1. Once a T-25 culture flask contains an ~90% confluent culture of cells, the cell medium was removed from the flask and the monolayer rinsed with warmed PBSa.
2. 2.5ml of cold 0.025% trypsin in 0.01% EDTA (Gibco) was pipetted onto the cell monolayer, the flask was tilted to coat all cells with a fine layer of the enzyme, and 1.5ml of the solution was immediately removed and discarded.
3. The progress of trypsinisation was monitored using an inverted microscope and as soon as >80% of the cells had rounded up and detached, the process was halted by the addition of 5ml of culture medium (serum stops the action of the enzyme).
4. The trypsinised cells were pipetted into a sterile universal, and the flask was rinsed with a further 5ml of culture medium, which was then also added to the cell universal.
5. The universal was gently inverted to distribute the cells throughout the culture medium, and 1ml of the cell suspension was removed and the cell content counted using a Coulter particle counter. The complete count of cells derived from the flask was calculated.
6. The universal of cells was centrifuged (1000 RPM for 10mins), the supernatant medium was removed and discarded, and the cell pellet was re-suspended in the required volume of culture medium.
7. HCAEC were seeded into T-75 culture flasks at a density of 20,000 cell/cm²; HDMEC* at a density of 10,000 cells/cm². A total of 30ml of cell culture medium was added to each T-75 flask.

8. Cells were once again incubated in 5%CO₂/air until ~90% confluent, undergoing a change of medium on alternate days. The cell splitting procedure was repeated.

*All cell culture ware in which HDMEC were grown was first pre-coated with a collagen solution (Cell Applications Inc.) to ensure optimal cell attachment.

1. 1ml of attachment factor solution (AFS) was added per 10cm² of surface area.
2. Flasks were rocked to distribute the solution evenly and to cover the whole surface.
3. The culture ware was incubated at 37°C for 30mins.
4. AFS was pipetted off and discarded. The flask was then ready to receive HDMEC.

8.6.3 Cryopreservation of Cells

A culture of confluent cells was returned to a frozen stock in the following way:

1. A freezing solution of 60% culture medium; 20% serum and 20% DMSO was prepared.
2. The cells were harvested from culture flasks and centrifuged as in section 8.6.2.
3. The cells were re-suspended in freezing solution at 500,000 cells per ml.
4. 1ml of the solution was pipetted into each cryovial for freezing.
5. Cryovials were placed into a Nalgene cryo 1°C freezing container, containing isopropanol. This was stored at -70°C for 24 hours and served to freeze the cells at a slow, controlled rate (1°C/hour) preventing the shock of immediate immersion in liquid nitrogen.
6. After 24hours cryovials were placed into liquid nitrogen for long-term storage.

Appendix 8.7

Two-Step Indirect Immunofluorescence

1. Confluent coverslips of cells were checked for cell integrity and the absence of bacterial contamination.
2. The coverslips were transferred to a Coplin jar (BDH) and the cell layers rinsed for 5 mins X 2 in PBSc.
3. Coverslips were placed into 1% formaldehyde* for 10 mins to fix.
4. Coverslips were rinsed for 5 mins X 2 with PBSc.
5. Cells were permeabilised with 0.1% NP-40 for 20 mins.
6. Coverslips were rinsed 5 mins X 2 with PBSc.
7. Cells were placed in a blocking solution for 20 mins (5% serum from the species in which the *secondary*** antibody was raised, diluted in PBSc; or 1% BSA).
8. Excess liquid was drained from the coverslips and they were placed into a humid chamber.
9. 100µl of primary antibody (diluted in 5% blocking serum/BSA) was carefully pipetted onto the monolayer. (5% blocking serum/BSA was used in place of the primary antibody for a secondary control coverslip).
10. This was incubated for 2 hr at RT (incubation at 37°C shortens the required incubation time, but also increases non-specific background staining).
11. Coverslips were returned to the coplin jar and rinsed 3 mins X 3 with 5% blocking serum/BSA.
12. Coverslips were drained and returned to the incubation chamber, and 100µl of secondary antibody (diluted in 5% blocking serum/BSA) was added.
13. Incubation was at RT for 1 hour.
14. Coverslips were returned to the coplin jar and rinsed 5 mins X 2 with PBSc.
15. Coverslips were mounted onto standard microscope slides with Gelvatol mountant (Sigma) (containing 100mg/ml DABCO (also Sigma) as an anti-fade agent).
16. They were stored in the dark at 4°C until the mountant was set, and photographed immediately if applicable.

* An alternate method for both fixing and permeabilising cells is immersion in ice-cold methanol for 5mins. This is particularly recommended for cell membrane receptors e.g. KDR/ Flk-1. However, this method was found to be less successful than 1% paraformaldehyde/NP-40.

** Blocking non-specific binding sites with serum derived from the *primary* antibody species can interfere with specific binding.

Appendix Table 8.6 to Fig. 2.10

Average systolic, mean and diastolic blood pressures of AG-fed animals (Chapter 2, section 2.4.3).

DAY NO.	BLOOD PRESSURE (mmHg)								
	AV. SYSTOL.	S.E.M.	n	AV. MEAN	S.E.M.	n	AV. DIASTOL.	S.E.M.	n
1	161	2	2	147	0	1	139	0	1
2	175.7	0.75	4	133.2	6.98	4	112.2	10.74	4
3	166	1.73	3	119	0	3	95.66	0.88	3
6	169	0	1	106	0	1	75	0	1
7	163.3	9.20	3	113.6	9.36	5	97.6	6.88	3
8	141	3	2	93.5	12.5	2	70	20	2
9	159.4	5.94	7	121.4	3.27	7	102.5	2.91	7
10	179	4.52	8	135.6	4.41	8	114.1	4.75	8
13	186.4	6.62	5	134.6	5.23	5	108.8	5.82	5
15	185.8	3.56	7	142.8	4.57	7	121.4	5.67	7
17	168.6	7.75	6	125.1	5.02	6	103.6	6.60	6
18	170.6	3.04	8	122.1	4.02	8	96.6	5.86	8
20	173.8	1.2	5	125.2	6.06	5	101.2	8.65	5
22	165.6	4.49	6	126	7.06	6	106.5	9.47	6
24	178	1.73	4	123.5	2.87	4	96.2	4.13	4
29	175	0	1	100	0	1	63	0	1
33	175	0	2	128	3	2	104.5	4.5	2
35	131	0	1	94	0	1	76	0	1
36	169	0	1	106	0	1	75	0	1

Appendix Table 8.7 to Fig. 2.11

Average systolic, mean and diastolic blood pressures of 1400W-fed animals (Chapter 2, section 2.4.3).

DAY NO.	BLOOD PRESSURE (mmHg)								
	AV. SYSTOL.	S.E.M.	n	AV. MEAN	S.E.M.	n	AV. DIASTOL.	S.E.M.	n
1	150.6	6.22	3	106	0	1	87	0	1
2	162.2	7.57	7	113.7	3.03	7	89.8	3.93	7
3	172	9	2	131	0	2	110.5	4.5	2
7	155.3	9.27	6	121.4	9.12	5	103.2	10.70	5
8	159.5	9.5	2	120.5	1.5	2	101	6.42	2
9	163	0	1	131	0	1	116	0	1
10	176.8	4.21	7	130.8	2.79	7	108	3.09	7
13	169	5.64	5	117.8	5.45	5	92.4	6.45	5
15	176.8	6.19	7	140.71	4.46	7	123	5.88	7
17	169	2.19	6	123	2.96	6	100	5.13	6
18	171.4	3.00	7	122.1	3.62	7	97.7	6.22	7
22	156.4	4.31	7	111.2	2.60	7	89	4.85	7
24	168.7	12.86	4	128	3	4	107.7	2.13	4
29	197	0	1	128	3	1	93.5	4.5	1

Appendix Tables 8.8 and 8.9 to Figs. 2.12 – 2.14

Mean tumour volumes for Control, L-NAME-, AG- and 1400W-treated animals (Chapter 2; study one, section 2.4.7.1).

DAY NO.	TUMOUR VOLUME (mm ³)					
	CONT.			L-NAME		
	MEAN	S.E.M.	n	MEAN	S.E.M.	n
13	33.1	37.48	4	67.3	20.49	5
15	146.6	28.94	5	331.4	70.53	3
16	278.0	64.69	7	244.6	30.35	5
17	391.5	93.60	7	269.5	73.50	8
18	451.0	128.88	7	309.1	54.23	8
19	587.2	155.25	7	330.4	76.54	8
20	718.4	168.66	7	461.2	90.75	8
21	965.0	198.71	7	572.3	125.33	8
22	1202.9	276.54	7	538.0	151.33	8
23	922.6	203.45	8	730.4	160.05	8
24	1196.3	331.59	8	575.3	158.59	8
25	1757.3	381.32	8	788.8	189.49	8
26	1697.0	388.63	8	1006.9	232.34	8
27	1632.5	465.37	8	1086.3	264.07	8
28	2240.3	496.1	8	1297.2	374.65	8
29	2342.6	471.18	8	1060.7	280.78	8
30	2341.6	498.75	8	1319.2	318.55	8
32	3041.6	682.24	8	1386.4	433.38	8
33	3025.0	682.26	8	1578.4	369.14	8
35	3739.6	812.06	8	1746.2	435.66	8
36	4646.7	978.51	8	2119.0	552.89	8
37	4936.2	1155.39	8	2800	761.20	8
38	6336.9	1290.11	8	2887.3	646.05	8

(Appendix Table 8.9)

DAY NO.	TUMOUR VOLUME (mm ³)					
	AG			1400W		
	MEAN	S.E.M.	n	MEAN	S.E.M.	n
13	35.7	11.66	6	72.6	16.47	4
15	175.8	72.37	7	233.9	55.79	6
16	218.0	72.1	6	238.4	46.10	8
17	290.2	90.06	8	291.7	63.88	11
18	327.4	96.26	8	394.9	88.03	11
19	410.7	125.37	8	612.7	148.43	11
20	512.9	113.68	8	869.0	235.52	11
21	945.8	281.94	8	1081.7	247.19	11
22	898.7	300.53	8	921.4	227.45	12
23	1249.8	361.06	8	1349.4	337.48	12
24	1130.9	385.76	8	1244.8	308.56	12
25	1502.8	451.05	8	1578.2	388.73	12
26	1937.0	577.56	8	1989.9	460.60	12
27	1894.4	633.96	8	2232.7	550.27	12
28	1952.2	598.27	8	2201.5	544.41	12
29	2143.3	724.04	8	2614.4	680.55	12
30	2475.6	916.36	8			
32	2919.3	946.96	8			
33	3644.9	1261.14	8			
35	3930.2	1500.58	8			
36	4389.8	1529.31	8			
37	4480.4	1592.88	8			
38	5640.4	2133.56	8			

Appendix Table 8.10 to Fig. 2.15

Mean tumour volumes for Control, L-NAME (from day-7) and L-NAME (from day +12) –treated animals. (Chapter 2; study two, section 2.4.7.1).

DAY NO.	TUMOUR VOLUME (mm ³)								
	CONT. MEAN	S.E.M.	n	L-NAME -7 MEAN	S.E.M.	n	L-NAME +12 MEAN	S.E.M.	n
12	157.7	23.96	13	142.3	18.45	11	165.9	19.49	12
13	164.7	16.85	14	165.0	20.72	12	197.4	19.38	12
14	279.6	32.16	14	277.6	41.99	13	299.6	40.55	12
15	456.8	58.95	15	460.8	61.91	15	465.9	80.56	12
16	590.2	85.82	15	584.7	83.92	15	656.9	121.40	12
17	548.5	79.07	15	567.5	83.48	15	522.7	76.56	12
18	815	102.14	15	640.1	114.42	15	635.2	74.42	12
19	930.2	143.15	15	830.2	150.88	15	651.1	97.33	12
20	1127.3	160.15	15	934.1	157.07	15	609.5	114.93	12
21	996.6	141.65	15	931.8	160.96	15	728.3	119.67	12
22	1250.6	221.92	15	1211.1	238.49	15	795.0	151.62	12
23	1559.0	269.05	15	1171.2	206.30	15	850.8	181.14	12
24	1765.1	302.54	15	1350.1	230.20	15	942.2	177.57	12
25	1956.9	338.84	15	1438.9	255.20	15	1052.4	207.98	12
26	2205.5	415.15	15	1705.6	282.64	15	1129	285.65	12
27	2639.1	490.51	15	2164.0	338.94	15	1480.1	322.09	12
28	3346.1	705.93	15	2564.5	409.38	15	1812.3	389.20	12
29	3851.9	724.25	15	3104.2	560.98	15	2029.9	489.00	12
30	4084.5	736.28	15	3535.6	658.99	15	2442.8	598.60	12
31	4359.3	835.14	15	3681.8	603.81	15	2432.3	557.28	12
32	4474.9	942.31	15	4367.9	798.58	15	2713.6	662.75	12

Appendix Table 8.11 to Fig. 2.16

Mean tumour volumes for Control, AG (from day-7) and AG (from day +12) – treated animals (Chapter 2; study two, section 2.4.7.1).

DAY NO.	TUMOUR VOLUME (mm ³)								
	CONT. MEAN	S.E.M.	n	AG -7 MEAN	S.E.M.	n	AG +12 MEAN	S.E.M.	n
12	157.7	23.96	13	169.9	33.71	10	207.4	24.91	17
13	164.7	16.85	14	250.3	80.09	10	272.4	30.89	17
14	279.6	32.16	14	319.9	107.92	11	381.7	32.60	17
15	456.8	58.95	15	424.7	126.89	12	614.3	72.64	17
16	590.2	85.82	15	575.7	176.20	12	702.0	95.23	18
17	548.5	79.07	15	517.6	164.62	12	947.0	129.33	18
18	815	102.14	15	640.1	242.15	12	940.1	142.25	18
19	930.2	143.15	15	960.1	366.28	12	1282.2	201.20	18
20	1127.3	160.15	15	1169.3	385.52	12	1306.2	213.85	18
21	996.6	141.67	15	1246.8	375.20	12	1546.2	254.61	18
22	1250.6	221.92	15	1347.0	399.59	12	1574.4	242.58	18
23	1559.0	269.05	15	1571	451.25	12			
24	1765.1	302.54	15	1850.2	486.75	12			
25	1956.8	338.84	15	1972.4	523.47	12			
26	2205.5	415.15	15	2402.1	741.26	12			
27	2639.1	490.51	15	3046.0	906.41	12			
28	3346.1	705.93	15	3409.9	1044.83	12			
29	3851.9	724.25	15	3529.9	970.94	12			
30	4084.5	736.28	15	3984.9	1097.89	12			
31	4359.3	835.14	15	4289.7	1182.66	12			
32	4474.9	942.31	15	4816.0	1353.43	12			

Appendix Table 8.12 to Fig. 3.1

Mean body weights for Control, AMD6245- and AMD6221-treated animals. n=5 animals/group. (Chapter 3, section 3.4.1).

DAY NO.	BODY WEIGHT (g)					
	CONT. MEAN	S.E.M.	AMD6245 MEAN	S.E.M.	AMD6221 MEAN	S.E.M.
-10	325	5.35	315	6.12	315	5.28
-9	324	5.56	315	5.85	316	5.70
-8	327	5.6	317	6.78	320	5.37
-7	332	6.20	320	6.87	323	5.55
-6	331	6.74	322	7.18	324	5.91
-5	334	5.94	320	7.24	324	5.68
-4	333	6.87	320	7.41	324	5.83
-3	332	6.78	321	7.748	322	6.23
-2	334	7.10	322	7.90	325	6.80
-1	334	7.81	322	7.58	327	6.42
0	337	6.82	327	7.82	331	6.06
1	327	6.80	314	7.69	318	5.95
2	330	6.83	317	6.69	320	6.31
3	329	6.96	317	7.41	320	5.84
4	333	6.98	320	7.37	321	5.96
5	331	6.95	319	7.63	322	5.00
6	330	7.297	319	8.51	323	4.93
7	336	6.56	324	8.82	327	4.72
8	336	7.25	322	9.31	327	4.36
9	338	7.56	324	9.06	329	4.24
10	342	7.20	329	9.55	330	4.27
11	342	7.43	327	9.40	335	3.87
12	340	7.46	325	8.99	334	3.48
13	343	7.82	327	8.88	335	3.08
14	345	7.88	330	8.24	338	4.29
15	349	7.07	331	9.00	339	4.69
16	352	7.43	334	9.08	340	4.27
17	353	7.02	332	9.28	339	4.37
18	355	7.73	332	8.49	342	3.93
19	360	8.30	337	8.46	347	4.86
20	361	7.27	337	8.92	346	4.64
21	360	8.35	331	5.18	347	5.16
22	362	7.15	329	4.90	348	4.28
23	365	8.19	331	5.03	349	4.15
24	368	8.32	330	5.50	348	4.99
25	366	9.25	332	5.02	348	5.24
26	368	8.94	332	3.21	349	5.00
27	371	9.31	335	4.06	351	4.82
28	372	9.24	337	4.38	354	5.77
29	369	9.58	333	4.75	347	4.58
30	374	9.48	339	5.67	352	5.01
31	368	10.22	338	5.70	350	4.89
32	375	9.54	342	6.6	353	5.34
33	379	9.79	344	6.18	359	5.52
34	382	10.12	346	6.53	359	5.12

Appendix Table 8.13 to Figs. 3.2 and 3.3

Mean drinking rates of Control, AMD6245- and AMD6221-treated animals.
n = 5 animals/group (Chapter 3, section 3.4.2).

DAY NO.	WATER DRUNK (ml)					
	CONT. MEAN	S.E.M.	AMD6245 MEAN	S.E.M.	AMD6221 MEAN	S.E.M.
-9	29.4	1.02	31.2	1.01	30.2	1.35
-8	26.6	1.20	25.6	1.16	26.4	1.43
-7	28.2	1.06	28.2	0.73	27	1.30
-6	28	1.04	28.4	1.72	27.6	1.32
-5	27.6	1.6	23.2	1.11	26.2	1.24
-4	25.8	0.96	27.8	0.86	27.4	2.29
-3	19.4	1.02	21.2	0.66	20.4	1.16
-2	22.8	0.8	21	0.54	22.6	1.53
-1	23.8	1.95	22	0.94	23.8	0.86
0	23.2	0.73	23.2	0.48	23.4	1.07
2	18.4	0.67	19.4	0.87	18.4	1.32
3	24.2	0.96	24.8	0.86	23.8	2.13
4	24	0.70	24.8	0.8	25.8	0.96
5	23.8	0.58	23.6	0.50	26.2	2.24
6	26.6	0.81	29.8	1.28	30.2	1.95
7	25	0.70	23	0.44	24.4	1.20
8	25.2	0.73	25.6	1.02	26.6	1.32
9	25.8	0.96	24.6	0.4	25	1.41
10	28	0.54	25.8	0.73	25.8	0.86
11	24	0.44	23	1.92	26.6	1.93
12	25.4	0.50	23	1.26	25.2	0.8
13	25.6	0.97	25.8	2.10	26.4	1.72
14	25.2	0.37	23.4	0.67	26.2	2.13
15	25.6	0.67	23.2	1.93	22.6	1.93
16	25.4	0.67	24.6	1.83	23.8	1.28
17	23.8	1.06	21.6	1.28	23	1.14
18	24	0.63	23.8	2.47	25	1.70
19	29.8	1.42	28.2	2.31	28.4	1.66
20	31.4	1.32	27.6	1.16	29	0.94
21	27.4	1.56	25.25	0.47	27.2	1.52
22	27.4	1.02	25.5	0.86	26.4	1.28
23	29	2.07	24.8	0.73	26.8	1.15
24	26.8	1.31	21.6	1.12	26.2	0.8
25	24.8	1.46	25	1.09	27.4	1.43
26	26.8	2.37	25.2	1.93	24.6	1.83
27	28.6	1.6	27.2	1.39	27.4	1.02
28	26.4	1.56	25.6	2.56	26.4	1.80
29	31	1.87	29.4	2.71	25.6	1.02
30	27.75	0.94	26	2.54	28.2	1.15
31	24.6	1.63	24.6	0.74	25	0.94
32	28	1.30	25	0.89	27	1.30
34	25.8	1.82	26.6	0.97	27.6	1.16
35	24.2	1.01	24.4	0.74	25.2	1.46

Appendix Table 8.14

Plasma nitrite [NO₃⁻] of Control, AMD6245- and AMD6221-treated animals. Day +28 groups are animals administered drugs continuously; day +35 groups are animals which underwent a drug-withdrawal period. No data is available for the AMD221-treated drug-withdrawal group (Chapter 3, section 3.4.3).

PLASMA [NITRITE] (μ mol / l)					
CONT. (day +28)	CONT (day +35)	AMD6245 (day +28)	AMD6245 (day +35)	AMD6221 (day +28)	AMD6221 (day +35)
7.31	5.36	3.44	8.21	5.21	
9.78	8	2.9	6.79	3.68	
7.68	8.1	4.4	5.63	7.84	
5.42		3.2		3.6	
8.57		5.48			

Appendix Table 8.15 to Fig. 3.5

Mean tumour volumes for Control, AMD6245- and AMD6221-treated animals (Chapter 3, section 3.4.7).

DAY NO.	TUMOUR VOLUME (mm ³)								
	CONT. MEAN	S.E.M.	n	AMD6245 MEAN	S.E.M.	n	AMD6221 MEAN	S.E.M.	n
10	197.3	46.62	10	156.4	20.20	10	98.6	11.58	8
11	249.5	61.36	10	171.7	24.92	10	139.6	22.27	8
12	378.5	62.26	10	314.5	49.32	10	264.3	32.98	8
13	498.4	93.22	10	330.7	44.09	10	264.3	37.48	8
14	480.4	96.52	10	276.7	49.64	10	200	34.11	8
15	571.2	121.41	10	332.1	61.32	10	316.2	57.62	8
16	789.9	161.57	10	432.8	73.41	10	382.1	40.99	9
17	913.5	212.89	10	482.1	97.81	10	446.9	63.56	9
18	983.7	167.09	10	544.4	108.16	10	515.7	86.99	9
19	1225.9	252.78	10	599.6	133.49	10	511.4	70.12	9
20	1581	374.43	10	748.3	163.02	10	648.8	126.09	9
21	1905.3	447.02	10	935.7	298.76	10	753.5	135.68	9
22	2325.2	522.17	10	949.4	219.54	10	891.1	168.40	9
23	2811.4	671.07	10	993.8	263.39	10	1170.5	244.57	9
24	3256.3	808.05	10	1188	287.55	10	1328.1	264.93	9
25	3441.7	830.93	10	1300	336.09	10	1556.8	298.68	9
26	4368.4	1165.06	10	1612.4	402.22	10	1841.4	349.98	9
27	5168.8	1512.10	10	1639.8	484.37	10	1922.7	382.12	9
28	5490.2	1680.59	10	1694.6	492.60	10	2361.1	501.46	9
29	6163.8	1890.67	10	1955.6	616.89	10	2525.8	622.99	9
30	6691.4	2088.53	10	2145.2	620.80	10	3124.6	650.55	9
31	7812.4	2520.52	10	2448	737.49	10	3733.1	803.01	9
32	8790.3	2689.35	10	2898.1	846.94	10	4297.5	825.93	9
33	9992.1	3187.74	10	3164.9	981.85	10	5082.2	1090.35	9
34	10718.9	3434.06	10	3193.8	975.59	10	5395.8	1106.57	9
35	11411.2	3672.18	10	3615.6	1088.72	10	6460.74	1360.30	9