THE ROLE OF VASOACTIVE AGENTS IN THE TREATMENT AND DEVELOPMENT OF COLORECTAL LIVER METASTASES

A thesis submitted to the University of London for the Degree of Doctor of Medicine

By

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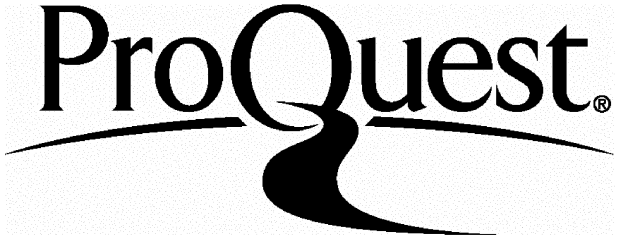
November 1998



Abstract

The role of vasoactive agents in the development and treatment of colorectal liver metastases.

The blood vessels supplying colorectal liver metastases lack both a smooth muscle layer and neuronal innervation. Co-administration of vasoconstricting agents with hepatic arterial therapy should therefore cause blood to be shunted from the liver into the tumour and hence improve drug delivery. One such vasoconstricting agent, endothelin-1 (ET-1), in addition to its actions as a vasomodulator may also play a role in the regulation of the growth of both primary and secondary colorectal cancers.

The aims of this thesis were to study: firstly, the effects of hepatic arterial infusion of vasoconstricting agents on liver and tumour blood flow and drug delivery in a animal model of colorectal liver metastases and secondly to determine whether ET-1 is produced by cell types within colorectal liver metastases using immuno-electronmicroscopy and also measure whether such production translates into elevated plasma levels in patients with colorectal cancer with and without liver metastases.

Although the vasoconstricting agents caused a transitory elevation in the tumour/normal blood flow ratio only noradrenaline produced improvements in drug uptake. A variety of cell types within the tumour were found to produce ET-1 and the plasma levels of ET-1 were elevated in both groups of cancer patients. Use of vasopressor agents may allow improvements in hepatic arterial chemotherapy, whilst application of antagonists to ET-1 may play a role in the treatment of colorectal cancer.

Statement of originality

The studies described and presented in this thesis are the original work of the author. All animal and human studies were performed by the author with the following exceptions:

1) All immuno-electronmicroscopy was performed in conjunction with Dr G Aliev in the Department of Anatomy, University College London.

2) The radio-immunoassay for endothelin-1 and the enzyme immunoassay (ELISA) were performed in conjunction with Mr S Fredericks, at The Analytical Unit, St Georges Hospital Medical School, London.

3) All specimens of liver and colorectal liver metastases were obtained by Professor I Taylor at surgery.

No part of this work has been submitted to any other university for consideration for a higher degree.

All the clinical studies in this thesis were performed in accordance with protocols approved by the Ethical Committee and with patients informed consent.
All animal studies were performed under appropriate Home Office project and personal licences.

Acknowledgements

I would first like to thank Prof I Taylor and Prof G Burnstock for permitting me to undertake this research project and also for their constant support and advice. My thanks also go to Dr M Loizidou for being not only an excellent supervisor but also an invaluable friend, without whom I am sure this research could not have been completed.

My thanks also go to all in the department of surgery especially Dr F Savage and Prof PB Boulos.

I am also indebted to Mr S Fredericks at St Georges Hospital Medical School for his help in completing this work and for his humour with regard to sample collection and analysis.

Thanks also to Prof TG Allen-Mersh for his generous loan of the laser doppler machine and for the advice from his department regarding the animal model.

Finally I would like to thank my parents and my wife for their unending support during my research which I am sure they thought would never end.

List of abbreviations

Ach - acetylcholine
Ag-II - angiotensin-II
ALP - alkaline phosphatase
A-P - abdomino-perineal
Ant Res - anterior resection
AST - aspartate transaminase
ATP - adenosine triphosphate
BP - blood pressure
Bx - biopsy
CEA - carcinoembryonic antigen
CGRP - calcitonin gene related peptide
CT- computerised tomography
CTAP - computerised tomography during arterial portography
DMEM - Dulbeccos modified eagles medium
ELISA - enzyme linked immuno-absorbent assay
ET-1 - endothelin-1
5FU - 5-fluorouracil
FUDR - fluorodeoxyuridine
GGT - gamma glutaryl transferase
HABR - hepatic arterial buffer response
HAI - hepatic arterial infusion
HA - hepatic artery
Hemi - hemicolectomy
Hep - hepatectomy
HPI - hepatic perfusion index
5-HT - 5-hydroxytryptymine
IOUS - intraoperative ultrasound
ILT - interstitial laser therapy
IV - intravenous
L-NAME - l-*N* --L-arginine methyl ester
Lt - left
mcg - microgram(s)
Metac - metachronous
Mets - metastases
mg - milligram(s)
MRI-magnetic resonance imaging

MRS - magnetic resonance spectroscopy
NA - noradrenaline
NGS - normal goat serum
NO - nitric oxide
NOS - nitric oxide synthase
NPY - neuropeptide Y
NS - not significant
PAP - peroxidase -antiperoxidase
PET - positron emmission tomography
RIA - radioimmuno-assay
Rt - right
SD - standard deviation
Sync - synchronous
TM - thrombomodulin
T/N - tumour/normal ratio
U/S - ultrasound
Vas - vasopressin
VIP - vasoactive intestinal peptide

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Chapter I

Introduction and historical review

Colorectal carcinoma continues to be one of the commonest cancers in the western world coming second only to lung cancer as a cause of death from malignant disease(Seventh Kings fund forum 1990).

Given this incidence it is not surprising that colorectal liver metastases remain a serious problem in the management of colorectal cancer. Up to 15 % of patients presenting with a primary colorectal cancer will have synchronous liver metastases. 50% of the remaining patients who initially underwent a ' curative' resection develop recurrent disease (Goligher 1981, Gill and Morris 1978), which in approximately 40% will be limited to the liver (Cedermark et al 1977). The presence of metastases is invariably associated with poor prognosis and ultimately death. Therefore research in the field has focused on early detection and improving current treatments. This thesis investigates the nature and manipulation of molecules which affect primarily blood flow within liver metastases.

Section 1.1 of the introductory chapter describes the detection of liver metastases section 1.2 deals with the development of liver metastases, section 1.3 summarises current treatments, section 1.4 looks at treatments based on the blood supply of liver metastases and section 1.5 summarises the attempts at improving these therapies.

Section 1.1 - The detection of colorectal liver metastases

With liver resection offering a selected group of patients improved survival and the possibility of an advantage gained from chemotherapy (whether systemic or regional), or other forms of adjuvant therapy, early identification of liver metastases may improve treatment and hence survival.

Most patients with liver metastases are asymptomatic at the time of presentation, with small lesions especially difficult to detect using conventional imaging. The late features of liver failure due to hepatic metastases represent gross hepatic replacement and as such are a terminal feature of the disease process (Foster et al 1981).

1.1.1 Blood tests

Blood tests associated with the presence of liver metastases are unfortunately often unchanged despite hepatic involvement. These include plasma levels of the liver enzymes aspartate transaminase (AST), alkaline phosphatase (ALP), gamma glutaryl transferase (GGT) and carcinoembryonic antigen (CEA). The most reliable of these in terms of sensitivity and specificity are ALP and GGT. The combination of elevated ALP, lactate dehydrogenase and GGT are believed to be the most accurrate predictors of the presence of liver metastases (Huguier et al 1981).

The most commonly measured marker is the CEA level which is elevated in up to 90% of patients with liver metastases (Sugarbaker 1990). It has also been found that the level of CEA is an independent predictor of survival after liver resection (Nordlinger et al 1992 and Cady et al 1991).

1.1.2 Radiology

1.1.2.1 Ultrasound

This is the most widely used investigation for the detection of liver metastases, although it becomes less useful when applied to lesions under 2cm in size where its sensitivity drops to approximately 56% (Sheu et al 1984). In a recent study ultrasound (U/S) detected only 20% of lesions <1cm while computerised tomography(CT) detected 50% (Wendricke et al 1991). In larger tumours its improved sensitivity of up to 94% coupled with it being non-invasive and relatively inexpensive makes it a commonly utilised modality.

Problems are encountered when differentiating colorectal liver metastases from benign lesions especially haemangiomas (Metha et al 1994). Colorectal liver metastases are hyperechoeic except when associated with a degree of widespread necrosis (Tubiana et al 1992).

Other problems making percutaneous ultrasound less reliable include obesity, overlying bowel gas, a high lying liver and inter-observer variation.

1.1.2.2 Computerised tomography

Traditional computerised tomography (CT) with and without contrast has now been superceded to a large extent by other forms of CT. These new techniques have a higher pick up rate particularly for small lesions and include dynamic CT scanning during the arterial phase of portography (CTAP), delayed CT scanning, bolus dynamic CT and ethiodised oil emulsion-13 enhanced CT.

CTAP involves injecting a bolus of contrast into the superior mesenteric artery (SMA) via a cannula and then scanning the liver during the portal phase. One of the drawbacks of this technique is the necessity of obtaining a pre-cannulation angiogram, an invasive technique, which has led to restricted use. Another problem of CTAP is the number of false positives findings due to perfusion defects from flow artefacts. CTAP does however allow localisation of lesions as small as 5mm within any of the hepatic segments (Tubiana et al 1992). When compared to hepatic angiography, U/S or contrast CT, CTAP was found to be better at detecting lesions <10mm (Yamaguchi et al 1991). Similarly when compared to other modalities of CT, CTAP was more sensitive than both delayed and contrast enhanced CT (Karl et al 1993).

Bolus dynamic CT has a detection rate of tumours >1cm in size of between 87-93%, dropping to 68% for smaller tumours (Tubiana et al 1992). Delayed CT scanning has similar levels of sensitivity and specificity to bolus dynamic CT but requires higher doses of contrast (Sugarbaker 1990).

Colorectal liver metastases are hypovascular when compared to surrounding hepatic tissue. When contrast is injected they become surrounded by a hyperdense ring as the contrast is taken up by the adjacent parenchyma. It is this hypovascular appearance which allows their differentiation from lesions such as hepatocellular carcinomas, hepatomas and haemangiomas which are hypervascular.

1.1.2.3 Magnetic resonance imaging

Magnetic resonance imaging (MRI) is proving to be increasingly useful in liver imaging. As already stated colorectal liver metastases are often difficult to differentiate from haemangiomas especially using U/S, whilst MRI has upto 90% specificity in distinguishing haemangiomas from metastases (Stark et al 1985). The reason is that haemangiomas have a high signal due to slow blood flow on T2 weighted images.

Overall studies comparing CT and MRI show similar levels of detection for both modalities, but MRI is better at characterisation of the lesion (Chezmar et al 1988). CT does however remain the imaging modality of choice, given its greater ability to pick up extra hepatic lesions (Chezmar et al 1988).

1.1.2.4 Intra-operative ultrasound

The central drawback to this technique is the special training required for its usage. It is however of particular use in routine screening for undetected metastases at primary surgery and in delineation of tumour deposits with regard to other structures at liver resection.

Studies have shown that intraoperatve ultrasound (IOUS) is both specific and sensitive at detecting liver metastases when compared with preoperative percutaneous U/S, CT and intraoperative palpation (Hagspiel et al 1995 and Rafaelsen et al 1995). IOUS has been shown to pick up undetected tumours in 5% of patients with negative preoperative scans (Soyer et al 1993) and identifies further tumours in an additonal 33% of patients with known metastases (Paul et al 1994).

This technique may also be performed laparoscopically with the added bonus that biopsies may be obtained. This has altered management in some patients to whom it has been applied (John et al 1994).

1.1.2.5 Hepatic perfusion index

Both animal and human studies demonstrate that hepatic blood flow is altered in the presence of liver metastases regardless of tumour size (Hemmingway et al 1991, Nott et al 1991 and Hemmingway et al 1993). Portal blood flow is reduced secondary to raised splanchnic resistance and hepatic arterial flow is elevated, presumably due to the predominantly arterial supply of tumours. Therefore the hepatic perfusion index, which is the ratio of hepatic arterial blood flow to total liver flow, is elevated.

Such a change in blood flow has been illustrated in patients with colorectal liver metastases using dynamic hepatic scintigraphy (Leveson et al 1983). Similar changes can be recognised at the micrometastatic stage of development (Hemmingway et al 1993).

Accordingly, these changes represent a means of identifying undetectable occult metastases. Recent use of colour coded duplex ultrasound to calculate flow in the hepatic artery and portal vein and hence the HPI (appropriately renamed the doppler perfusion index DPI) has proved effective in this regard (Leen et al 1991, Leen et al 1993). In one study DPI when compared to conventional ultrasound and CT scanning, was more effective in identifying occult liver metastases at initial presentation (Leen et al 1995). The presence of these occult lesions has been shown to markedly affect survival (Finlay et al 1986).

Increased splanchnic resistance which causes the majority of the elevated HPI, appears to be due to an unidentified, circulating vasoconstrictor (Hemmingway et al 1993 and Carter et al 1994). This has been demonstrated in animal models where plasma from animals with colorectal liver metastases caused vasoconstriction in the splanchnic bed of normal animals. Moreover application of antagonists to known vasoconstrictors, including the monoamines and angiotensin, did not abolish this effect (Warren et al 1993). As yet the agent responsible for this effect has not been identified (see later).

However given the operator dependent nature of such investigations the results originally stated have been difficult to reproduce (Shuman 1995).

Section 1.2 - The development of colorectal liver metastases

1.2.1 The vascular dissemination of tumour cells

The first step in the formation of liver metastases necessitates the shedding of tumour emboli from the primary into the systemic circulation. This involves a combination of processes: separation from the primary, degradation of the extracellular matrix and basement membrane, and intravasation into the portal venous tributaries draining the primary, which occurs predominantly at the periphery of the tumour(Effert and Stromeyer 1995).

Studies have attempted to correlate vascular invasion with the metastatic potential of a colorectal tumour. Unfortunately the histological techniques applied in the various studies are not consistent, and as such the inferences made are difficult to compare. It is observed however, that as tumours become less differentiated or their penetration through the bowel wall increases, there is a corresponding increase in venous invasion(Minsky and Meis 1989). In addition, as venous invasion increases it is known that the incidence of metachronous liver metastases rises (Shirouzu et al 1991). The degree of venous invasion has also been correlated to the Dukes stage of the primary and appears to influence survival in stages B and C (Krasna et al 1988). It has been shown that patients with Dukes B and vascular invasion have a worse survival than those with Dukes C without vascular involvement.

Shedding of tumour microemboli into the portal system, apart from being related to vascular invasion, also appears to be influenced by the operative techniques applied. It has been suggested that reduced handling of the primary and early ligation of the vascular pedicle ("no touch" technique), reduces the incidence of liver metastases (Fisher and Turnbull 1955). Subsequent studies applying this so called 'no touch' technique have shown a reduction in the number of liver metastases, although this has not translated into a survival advantage (Wiggers et al 1988).

1.2.2 The metastatic process

For successful liver metastases to occur after intravasation, tumour microemboli must survive blood borne delivery in the portal venous system, impaction in distant vessels and extravasation (Effert and Stromeyer 1995). Growth at a new site is achieved by considerably less than 0.1% of cells originally detached from the primary.

The predilection of these cells for the liver is a consequence of the venous drainage of the colon and rectum with cells arriving via the portal vein (Murphy et al 1988). Hepatic derived chemoattractants may also be involved. In the development of bone metastases the tumour cells respond to growth stimulating factors (Manishen et al 1986) and chemotactic agents (Orr et al 1995) derived from bone. A similar system may exist in the development of liver metastases.

The expression of receptors and cell adhesion molecules on the endothelial surface of the hepatic vessels, which encourage tumour adhesion and hence extravasation (Nicolson 1988) may also contribute to organ specificity. This interrelation of cells with one another is the key to understanding the evolution of metastases both with regard to tumour-to-adjacent cells and tumour- to-matrix interactions. Manipulation of such processes might offer the opportunity of inhibiting the establishment of early micrometastases.

In order for tumour emboli to gain access to the liver they must breach the basement membrane of the vascular endothelium which is achieved by secreting appropriate metalloproteinase enzymes eg Gelatoproteinase (Steeg 1992, Goldfarb and Brunson 1992). In a rat model manipulation of matrix metalloproteinase inhibitors led to a reduced incidence of liver metastases (Watson et al 1995).

1.2.3 Tumour angiogenesis

The development of metastases greater than 200micrometers is dependent on the concurrent development of a supportive vasculature (Folkman 1990). This formation

of new blood vessels from the existing vascular bed (angiogenesis) is a complex multistep process controlled by angiogenic peptides leading to extension of the existing vasculature into and around the tumour.

Much of our understanding of angiogenesis and its control comes from work on normal tissue healing and associated benign angiogenic conditions. These studies have shown that angiogenesis in tumours and normal tissue share many common steps but are differentiated by incomplete regulation and an altered vascular structure in the tumour. Angiogenesis commences with retraction of the capillary endothelial cells and surrounding pericytes. This is followed by breaking down of the basement membrane and extracellular matrix which facilitates the movement of the endothelial cells towards the tumour. The enzymes concerned with this step include the matrix metalloproteinases (eg gelatoproteinase) and plasminogen activators, which are secreted by the tumour, surrounding endothelial cells and fibroblasts in response to stimuli such as bFGF (Paweletz and Knierim 1989, Blood and Zetter 1990). The endothelial cells now form a solid cord which becomes cannalised and provides an infrastructure of interconnecting microvascular vessels.

As tumour development is angiogenesis dependent, intervention at this stage seems a logical manoeuvre.

Surprisingly angiogenic inhibitors have recently been isolated from tumours. This suggests a degree of previously unsuspected intrinsic autoregulation (Prehn 1991) and may partly explain why the rate of tumour growth declines as the tumour enlarges, subsequent to an equivalent rise in inhibitor production. The systemic release of such tumour products also suggests that the primary tumour might inhibit neovascular development and hence growth of distant metastases (O'Reilly 1994). A potent anti angiogenic peptide -Angiostatin -has been identified in the plasma of mice with subcutaneously implanted tumours (Lewis lung carcinoma). Once the primary is removed then the levels of angiostatin fall rapidly and growth of distant metastases rises(O'Reilly 1994).

Identification of these inhibitors represents a major advance in understanding tumour growth modulation. Direct application of angiogenesis inhibitors to animal models of liver metastases have been attempted at various stages following tumour cell inoculation and have a maximal effect if given during the micrometastatic phase. Similar experiments on established tumours have provided disappointing results.
In a rabbit model utilising VX2 carcinoma cells, and in immunosupressed mice models using human colorectal cancer, the angiogenesis inhibitor TNP-470 (which is an extract of the fungus fumagillin) produces a significant inhibitory effect on both tumour size and neovasculature development (Koishi et al 1994, Hiroyuki et al 1995, Tanaka et al 1995).
Other mechanisms include blocking the receptor for vascular endothelial growth factor (VEGF), which is frequently expressed in human colorectal liver metastases. Inhibition with monoclonal antibodies to VEGF in immunosuppressed mice inoculated with human colorectal cancers led to a marked reduction in metastatic growth (Warren et al 1995).
Finally the analogue of the hormone somatostatin, octreotide, when given intraportally causes a reduction in hepatic micro metastatic growth of intraportally inoculated Walker Cells in rats (Nott et al 1989). Suggested mechanisms for this action vary from direct angiogenesis and growth inhibition to splanchnic vasoconstriction and a subsequent reduced portal flow .

1.2.4 Occult liver metastases

There is no clear association between vascular invasion, the presence of tumour cells in the systemic circulation and the subsequent development of hepatic metastases. This may represent inefficiency in the metastastic process or may reflect the inability of current radiological techniques to pick up micrometastatic occult lesions.
The existence of such microscopic lesions has been demonstrated in a number of studies and their presence markedly reduces survival (Finlay and McArdle1986).

Tumour cells succesfully deposited in the liver parenchyma may succumb to host defenses, such as attack by monocytes, remain as undetectable but viable micrometastases or develop into obvious macrometastases. What triggers the development of occult tumours into macrometastases is incompletely understood, but almost certainly involves the interaction of a number of local and systemic factors. Some groups have shown that positive growth stimuli to occult tumours are released as a result of liver trauma and subsequent regeneration, as occurs after hepatic resection of liver metastases(Loizidou et al 1991). This may explain the observed disease recurrence in adjacent liver after an apparent curative procedure(Hughes et al 1986, Steele et al 1989). Panis et al (1992) injected rats with tumour cells, and divided them into three groups, one group was given cyclosporin, another had a partial hepatectomy and the final group acted as controls. The cyclosporin and hepatectomised rats had a higher incidence of hepatic tumour development compared to the controls. Presumably cyclosporin acted as a direct growth stimulator, whilst in the resected group it is proposed local positive stimuli released during liver regeneration encouraged the development of tumour growth. These results may help to explain the recurrences seen in human liver resection studies.

On a more subtle scale growth factors have been implicated in the microinjury hypothesis of metastatic development (Kawaguchi and Nakamura 1977, Warren 1980). Here it is suggested that an initial tumour emboli fails to develop but causes a degree of local trauma followed by healing. A subsequent tumour microembolus would then receive positive stimuli generated by the local healing microenviroment which encourages its development.

Recent studies in rats , which had undergone intraportal inoculation of tumour cells and subsequent development of liver metastases, have indicated that administration of tumour necrosis factor alpha at the time of resection of the metastases reduces these stimulatory signals and post-operative tumour regrowth (Slooter et al 1995). It is therefore suggested that TNF alpha might be given as "adjuvant" perioperative

therapy. However other groups, using different models, have demonstrated promotion of growth of liver metastases in animals by TNF alpha (Orosz et al 1985).

1.2.5 The blood supply of micrometastases

Micro and macro metastases have a completely different blood supply. When less than 2mm in size colorectal liver metastases have a predominantly portal input (Haugheberg et al 1988)which is not surprising since they originate from portal venous radicles. Whilst larger tumours are supplied by the hepatic artery (see later), . What causes this switch in vascular supply from portal to hepatic arterial is unclear, but probably results from the release of angiogenic factors from the tumour. Presumably these agents then act preferentially on the arterial circulation to encourage vessel growth. This development of a surrounding vascular network is a prerequisite for tumour growth (Folkman et al 1990)-the so called angiogenesis dependent effect- and it is at this point, when neovascular development is underway, that the switch of blood supply occurs.

Interestingly it has been shown that if the hepatic artery is ligated in patients with established metastases collaterals develop from the portal venous system to compensate, again reversing the source of their blood supply (Taylor et al 1979 and 1981).

1.2.6 The blood supply of macrometastases

The switch from a micro to a macro metastasis in the liver involves a change not only in the size but also in the nature of its blood supply. As angiogenesis proceeds and a primitive neovasculature is formed, the emphasis shifts from the portal vein to the hepatic artery which subsequently accounts for approximately 95 percent of the blood supply to the tumour (Breedis and Young 1954). Accordingly, locoregional treatment

of established metastases is via the hepatic artery rather than the portal vein, in an effort to improve drug delivery.

This identification of a dual blood supply and the switch from one system to another has been demonstrated by different techniques. The initial work was performed on postmortem specimens obtained from patients with colorectal liver metastases, and involved injecting silicone rubber into the hepatic vascular tree (Lien and Ackerman 1970). This demonstrated that the predominant blood supply of established metastases was from the hepatic artery. Subsequent studies using coloured silicon injected into both the arterial and portal systems confirmed the earlier findings but also indicated a greater degree of portal venous input than was previously believed (Lin et al 1984). These studies also demonstrated multiple porto-arterial shunts which could explain why agents administered into one system gain access to the other, and also the maintenance of a tumour blood flow when one or other vessel is interrupted. However given that these investigations involved the use of post mortem specimens their relevance to functional anatomy maybe questionable.

Other early groups injected indian ink into the arterial and portal systems of human liver tumours and observed the relative uptake of dye in tumours compared to normal liver (Breedis and Young 1954). Such studies are not as accurate as silicone injections and are limited by their inability to differentiate between micro and macrometastatic lesions, but nevertheless produce results similar to the microfil experiments.

More recently, intravenous radioactive tracer compounds such as xenon 133 have been infused to monitor hepatic flow and showed differences between macro and micro metastases. Using this technique Archer and Gray (1989) suggested that all tumours (even those less than 2mm) possessed a predominantly arterial supply and that the portal circulation contributed relatively little to both micro and macrometastases.

Xenon clearance can also be used to demonstrate liver blood flow. Gelin et al (1968) used this technique to compare the relative changes in blood flow in human liver

metastases on occlusion of the hepatic artery and the portal vein. Only occlusion of the hepatic artery resulted in a marked reduction of xenon clearance from the tumours compared to the normal liver. This was later confirmed by Taylor and co-workers (1979) who employed xenon washout in a similar fashion. They also showed that if the hepatic artery is occluded eventually a portal venous flow increases via porto-arterial communications.

Ridge et al (1986) infused radiolabelled albumin into both the hepatic artery and portal vein in patients with colorectal liver metastases and noted that more than twice as much of the blood supply came via the artery compared to the portal vein. They also indicated that the greater arterial input translated into an increased substrate uptake , as demonstrated by a greater concentration of radiolabelled albumin within the tumour.

1.2.7 The interstitial pressure of tumours

The passage of substances through the tumour microcirculation is influenced not only by the blood vessels but also by surrounding interstitial pressure. Studies using a wick-in-needle technique in both human breast and colorectal liver metastases have shown that the pressure within them is markedly elevated relative to the normal surrounding tissue, in some cases upto ten times greater (Jain 1994, Less et al 1992). This phenomenon affects diffusion of molecules, including chemotherapeutic agents, into the tumour by causing outward convection at the tumour periphery, leading to reduced drug delivery. The actual cause of this elevation is uncertain but could be related to a change in the composition of the extracellular matrix or to extrinsic tumour cell compression on the tumour circulation.

Manipulation of this pressure phenomenon might allow improvements in delivery of chemotherapeutic agents to such tumours.

The presence of this pressure phenomenon casts doubt on the functional significance of the previous microfil studies. This is of particular importance when considering

the venous findings, as it is unlikely that the low intravascular pressure in the portal venous system would be able to keep the vessels patent in the face of such a steep pressure gradient.

Section 1.3 - The treatment of colorectal liver metastases

1.3.1 The natural history of untreated liver metastases

Before embarking upon treatment of any condition it is first necessary to determine what the outcome would have been without treatment. All the following modalities of treatment are subject to complications and as such need to be justified in terms of survival advantage and quality of life for the patients involved. Many practitioners still believe that treatment offers these patients, with disease is limited to the liver, no added advantage over a no treatment policy.

Most colorectal liver metastases are slow growing tumours, with the time predicted for a occult tumour to develop into a overt 2cm focus estimated to be approximately 3 years (Allen-Mersh 1991). Given this long natural history it is estimated that the metastasis is present for approximately 4 years before it kills the patient (Finlay and McArdle 1986), with extrahepatic spread developing as a result of tumour cells shed from the hepatic metastasis. The only way to truly determine any possible advantage a treatment may have is to include a non-treatment arm into a randomised study. Most patients and many clinicians, however, would not accept 'no treatment' as an option and therefore the results against which potential treatments are compared are historical controls.

Author and year	Tumour load	Mean survival (months)	Median survival (months)	Longest survivors (months)
Pestana et al 1969	*	9	*	11
Swinton et al 1967	*	13	*	46
Cady et al 1970	*	*	13	84
Nielson et al 1973	Few Several multiple	*	18 9 5	144
Wood et al 1976	Solitary unilobar Bilateral	7	16.7 10.5 3.1	36
Goslin et al 1972	<4 >4	*	24 12	59
Lahr et al 1983	Unilobar Bilobar	*	12 4.5	67
Hughes et al 1989	*	*	12	50

Table 1. The results of no treatment for colorectal liver metastases.

In addition to the absence of a no treatment arm, such studies are potentially flawed by a number of other factors. These include;inclusion of both synchronous and metachronous tumours in the same group, which may lead to an element of lead time bias (Pestana et al 1964) and comparison of tumours with different natural histories and inclusion of patients with liver metastases other than from colorectal cancer .
A major determinant of survival in these patients is the percentage hepatic replacement (PHR). Those patients with less than 25% involvement live a median of 6.2 months, whilst those with 25-75% live 5.5 months and above this level 3.4 months (Hughes et al 1989, Stehlin et al 1988).
Other factors shown to have an adverse relationship to patient survival include abnormal liver function tests (especially albumin and serum lactate dehydrogenase), primary tumours that are not resected, histological grade of the primary tumour and spread to extrahepatic sites (Rougier et al 1995).

1.3.2 Hepatic resection

Background

As already stated in many patients reccurrent disease will be limited to the liver alone, which in the USA approaches 18,000 per annum(Steele et al 1994). Data from the USA if extrapolated to the UK suggest that up to 1000 patients each year might benefit from liver resection (Steele et al 1991).
The two central criticisms of data pertaining to liver resection are firstly the absence of prospective randomised control trials (the no treatment arms usually being historical, as stated above) and the increasingly selective nature of patients included in such studies. Selected groups of untreated patients exist in whom long term survivors have been identified(Wagner et al 1984). Perhaps this group should be used as the gold standard against which results of resection should be compared .

Complications of resection

Given the small numbers of patients that benefit from resection, the incidence of complications becomes more important. In the larger series published mortality rates vary between 0 to 8% with morbidity rates of 10-39% (Jatzko et al 1995, Gayowski et al 1994, Adson et al 1984, Wilson et al 1976, Steele et al 1991, Hughes et al 1988, Van Oigen et al 1992, Nordlinger et al 1992, Scheele et al 1995, Doci et al 1995). Improvements in post operative mortality and morbidity are due to a number of factors which include improved post operative intensive care, greater understanding of liver anatomy, intraoperative use of ultrasonic dissectors and other blood loss reducing devices and improved use of hepatic vascular inflow and outflow control. Such results improve with increasing experience and access to appropriate facilities to manage any complications that may ensue. As such, most would agree that such procedures should be performed in specialist units (Scheele et al 1995).

Results of resection

Hepatic resection offers the only chance of long term survival in patients with disease limited to the liver. It appears that the pattern of tumour spread is altered by liver resection, with recurrence shifting from hepatic to extrahepatic sites such as the lung. So that while the patients remain alive they are not disease free, hence skewing the survival data (Hughes et al 1989, Steele et al 1989).

Early studies from the USA suggested 5 year survival rates of between 20-28% (Wilson et al 1976) in selected groups of patients.

Since then more recent studies show 5 year survival rates vary from 16-48% (Jatzko et al 1995, Gayowski et al 1994, Adson et al 1984, Steele et al 1991, Hughes et al 1988, Nordlinger et al 1992, Scheele et al 1995, Fortner et al 1984, Ekberg et al 1986, Cobourn et al 1987, Holm et al 1989, Doci et al 1991, Rosen et al 1992, Sugihara et al 1993, Hugh et al 1997). Once most patients reach 5 years they pocess similar survival curves to an age and sex matched non-cancer cohort.

Determinants of survival

When analysed separately a number of independent variables have been identified which predict the outcome of resection in these patients.

Firstly the size of the metastases, which is directly related to its age. Some studies suggest a tumour>5cm is associated with poorer survival than smaller lesions (Hughes et al 1988, Nordlinger et al 1992, Scheele et al 1991) whilst others suggest it is of no importance(Ekberg et al 1986). It has been suggested that synchronous lesions carry a poorer prognosis than metachronous lesions although a number of studies suggest that prognosis is not altered by the time of detection (Ekberg et al 1986, Doci et al 1991, Lise et al 1990).

The number of metastases resected only become significant when more than three are attempted. Above this number long term survival is much reduced (Ekberg et al 1986, Hughes et al 1988, Van Ooijen 1992, Nordlinger et al 1992, Ekberg et al 1986). Gayowski et al(1994) have shown that in their large unit in the USA patients with >3 metastases who were resected did worse than those with fewer tumours. However this group still had a 5 year survival rate of 20%. Scheele et al(1995) resected patients with upto 5 tumours if they had sufficient residual liver volume and had a number of long term survivors.

The resection margin obtained is also significant with studies advocating a clearance of at least 1cm. These groups have demonstrated a poorer 5 year survival and disease free interval when smaller margins were obtained (Nordlinger et al 1992, Ekberg et al 1986). Scheele et al (1995) state that obtaining a margin of >1cm is not important.

The presence of extrahepatic disease is generally accepted as a contraindication to liver resection . The exception to this rule is found when a superficial metastases erodes into an adjacent structure, usually the diaphragm, and is resected enbloc. If the clearance is adequate then the patient has similar survival rates to those without involvement .

Age does not appear to influence survival and the biological rather than chronological age should be the determinant, since they have similar postoperative complication

rates (Fong et al 1995). It has been observed however that the elderly posses reduced reserves of hepatic regeneration.

Some studies have suggested that the position of the primary tumour affects the outcome of treatment for the secondary. Younes et al (1991) and Rougier et al (1995) suggested that metastases from right sided tumours did worse than others.

The majority of reports however show no significant difference between site of primary tumour.

Both increasing grade (Jatzko et al 1995, Scheele et al 1995) and stage (Scheele et al 1995, Fortner et al 1984, Doci et al 1991) of the primary tumour appear to be associated with poorer prognosis of the liver metastases. These findings are hardly surprising since these two factors predict more aggresive tumour biology and hence greater metastatic potential.

The subject of repeat liver resection for those patients who develop recurrence limited to the liver has been the subject of a number of studies dealing with small numbers of patients. Upto 65% of patients undergoing primary liver resection will develop recurrence with 20% of these having disease limited to the liver (Nordlinger et al 1992, Wanebo et al 1996). Some of the larger series of repeat hepatic resections show similar survival rates to primary resection (Wanebo et al 1996).

1.3.3 Physical methods of destruction

Laser therapy

Interstitial laser therapy (ILT) was first described by Bown in 1983. A fibre optic tipped probe is placed in the centre of the area to be ablated under U/S guidance. This allows accurate delivery of energy with minimal overspill to adjacent tissue.

Most studies looked at small numbers of patients with hepatocellular carcinomas. Masters et al (1992) in a pilot study treated liver metastases with ILT. 10 patients with 18 liver metastases were treated with a response rate of 44% as measured by CT and biopsies. Amin et al (1993) treated 26 patients with 70 liver metastases. All the

tumours showed evidence of necrosis and 61% of the patients showed greater than 50% reduction in tumour volume . In this study the follow up period was short post treatment and was further complicated by many of the patients receiving other forms of adjuvant treatment. Of the 21 treated 6 died, giving a median survival of 28 months, range 4-40 months, with 15 still being alive with a follow up of 3-30 months, median 6 months. ILT may become part of the therapeutic arsenal but results of further follow up are awaited.

Cryotherapy

This technique involves placing probes containing liquid nitrogen directly onto the tumour surface, using U/S as a guide, with subsequent tissue destruction. However as it involves a laparotomy and hence a general anaesthesia it tends to be used in patients with unresectable disease limited to the liver, although it has also been used to 'sterilise' resection margins in those areas which for technical reasons cannot be removed at primary metastectomy. Side effects include fatal multi organ failure and coagulopathies (Weaver et al 1995), whilst others have reported no deaths (Morris et al 1993). CEA levels appear to fall after treatment (Preketes et al 1994) and a few long term survivors have been reported (Ravikumar et al 1991).
More recently a probe suitable for laparascopic placement has become available which may increase the use of this technique.

Radiotherapy

External beam radiotherapy has very little to offer in treating colorectal liver metastases. Although Gray et al (1980) showed tumour regression no study has proven that this translates into a survival advantage. Some groups using radioactive implants to treat residual disease have attempted to sterilise positive resection margins. Unfortunately both local and distant reccurrence often occur at an early stage (Armstrong et al 1994).

Selective internal radiotherapy can be achieved by infusing radioctive particles into the hepatic arterial circulation which provides the majority of colorectal liver metastases blood supply and hence deliver high doses directly to the tumour. A recent study looked at this technique combined with hepatic arterial chemotherapy and demonstrated significant tumour regression (Gray et al 1992).

Treatments of labelling anti-CEA antibodies with radioactive materials have been disappointing. In most cases the radioactive delivery was insufficient to cause an appreciable effect (Order et al 1986). Lipiodol, an agent known to sludge in the vessels of both primary and secondary tumours in the liver when injected via the hepatic artery, has been assessed in colorectal metastases. When combined with radioactive agents (I^{131}) it is effective in treating HCC but not colorectal liver metastases (Bretagne et al 1988).

Percutaneous alcohol injection

This technique has long been used to treat HCC with some success but not to any effect in colorectal liver metastases (Amin et al 1993). Part of the problem is that colorectal liver metastases have a harder consistency than HCC and hence the alcohol tends to spread back down the injection tract rather than into the tumour.

1.3.4 Immunotherapy

This technique of immune modulation in the perioperative period at the time of liver resection is aimed at reducing post operative recurrence. If the immune system could be upregulated in this period then theoretically the development of microemboli shed at surgery would be reduced. Interleukin II when given preoperatively in a phase II trial increased the post operative lymphocyte count, with theoretical immune stimulation (Elias et al 1995).

Immunisation has also been used perioperatively to reduce tumour reccurrence after resection. Schlag et al (1992) immunised patients with metastasis derived deactivated

tumour cells. Those vaccinated had a significantly reduced incidence of tumour recurrence.

1.3.5 Systemic chemotherapy

Background

Systemic chemotherapy has been used in the treatment of colorectal liver metastases for the last 30 years. Unfortunately it has had little influence on survival. There are many suggested reasons for this failure but central to the problem are the cell kinetics of the tumour. Colorectal cancer cells have a small growth fraction and as chemotherapy is most effective against rapidly growing cells this partly explains the poor results obtained.

5-flurouracil(5-FU]) as a single agent

This agent still forms the backbone of most treatment regimes for colorectal liver metastases. It remains inactive until it is metabolised to one of a number of nucleotides.

It is cleared mostly via the liver by the enzyme dihydrofluorouracil dehydrogenase, with a small amount cleared by the lungs. The actions of 5-FU upon the cell are; interactions with the cell membrane, incorporation into RNA and DNA and inhibition of thymidylate synthetase. As 5-FU has a high first pass extraction ratio by the liver it is not usually given orally. When given as bolus therapy it produces unimpressive response rates with no improvement in survival. In an attempt to improve on these disappointing results adjusted bolus regimens were devised. These produced higher response rates but only slight improvements in survival (Seifert et al 1975, Barbounis et al 1989, Hansen et al 1989). Overall 5-FU has little effect on its own and is usually used in combination.

5-FU and folinic acid

Folinic acid interacts with 5-FU inside the cell, with 5-FU activation dependent on the concentration of reduced folate. This interaction results in the formation of a tertiary complex between 5-FU and folinic acid with a resultant improvement in 5-FU cytotoxicity.

Both continuous infusions and bolus infusions of the two agents appear to produce improved response rates (Kohnewompner et al 1992) and two studies demonstrated improved survival: Poon et al (1989) demonstrated a median survival of 7.5 months with 5-FU alone, 12.3 months with 5-FU/high dose folinic acid and 13.8 months with 5-FU/low dose folinic acid. Erlichman et al (1988) showed a median survival of 9.6 months with 5-FU alone vs 12.6 months with 5-FU/folinic acid. Other studies using weekly regimens of 5-FU/folinic acid have demonstrated median survivals varying from 11.5-12.75 months with response rates of 19-30% (Petrelli et al 1989, Sobrero et al 1989). The advanced colorectal cancer research project performed a meta-analysis on 10 studies which compared 5-FU vs 5-FU/folinic acid including a total of 1500 patients. The combination group showed an overall improved response rate (23% vs 11%) but only a few studies demonstrated improved survival which when taken together were not significant. The combination of 5-FU and folinic acid in the treatment of patients with liver metastases and extrahepatic disease is currently being assessed within the confines of the CRO6 trial.

5-FU combination therapy

5-FU in combination with methylnitrosurea+vincristine, carmustine, methyl-carmustine methyl-carmustine+vincristine and cis-platinum have all been compared to 5-FU alone but have failed to demonstrate an improvement in survival (Moertal et al 1975, Einhorn et al 1975, Baker et al 1975, Lokich et al 1977, Kemeney et al 1990, Loehrer et al 1988).

Interferon has also been co-administered with 5-FU, initially with marked improvements in response rates but a large degree of side effects necessitating

reduction in dose both of interferon and 5-FU. This ultimately concluded in an insignificant improvement in survival (Kemeny et al 1990, Pazdur et al 1990, Wadler et al 1989). The more recent MRC study studied a similar combination and showed no improvement in survival.

Other single agent chemotherapy

A variety of other agents have been used apart from 5-FU in treating colorectal liver metastases, producing response rates varying from 7-33% but no improvements in survival over 5-FU have been demonstrated (Davis 1982, Ogawa et al 1981).

1.3.6 Isolated liver perfusion

The principle of this technique is to isolate the organ/area to be treated from the systemic circulation and hence allow high doses of chemotherapeutic therapy to be administered. The first use of this technique was in the treatment of disseminated malignant melanoma of a limb and was first applied to the liver for the treatment of colorectal liver metastases in 1981 by Aigner et al. Since then a number of small series have been reported by the same group suggesting improvements in survival. The highly interventional nature of this procedure precludes widespread application of this technique.

1.3.7 Liver transplantation

Apart from neuroendocrine tumours (Pichlmayr et al 1995) liver transplantation has no role to play in the management of colorectal liver metastases (Penn 1991) given the high rates of early recurrence demonstrated.

Section 1.4 - Treatments using the blood supply of colorectal liver metastases

1.4.1 Portal vein based therapies

There is now a greater understanding of the mechanisms underlying the development of these metastases, especially with regard to the portal circulation. This section will demonstrate how this knowledge has been applied to both prevention and treatment of established colorectal liver metastases using the portal venous system.

1.4.1.1 Adjuvant prophylactic therapy

Tumour emboli are released from colorectal carcinomas and may later develop into liver metastases. The risk of tumour cell release is greatest both at the time of resection of colorectal cancers and perioperatively. Taking into account these factors there are two types of treatment available which have been applied in an attempt to reduce the incidence of perioperative hepatic tumour seeding ; improved operative techniques, foremost of which is early ligation of the vascular pedicle and adjuvant chemotherapy.

Operative techniques

In order to reduce the number of tumour cells released by intraoperative handling of the primary colonic lesion a no touch technique was devised by Turnbull(1970). Before the tumour is resected the vascular pedicle containing the feeding vessels and lymphatics is ligated. Of the 471 patients included in the study, the age corrected 5 year survival was 81.6%, which is almost double the expected survival rate of patients without this technique.

A subsequent randomised trial conducted by Wiggers et al (1988) applying this technique demonstrated a reduction in the incidence of liver metastases in those patients treated in this way, although the survival was only minimally improved.

Adjuvant systemic therapy

Multiple agents have been given systemically in an attempt to reduce the incidence of colorectal liver metastases. Most early studies using 5FU alone failed to produce an overall survival advantage. Recently groups have combined 5FU with other agents- the most notable being levamisole(Moertal et al 1990)- and have produced some encouraging results.

Perioperative portal vein therapy

Given that the route of delivery and initial blood supply of colorectal hepatic micrometastases is from the portal vein, prophylactic chemotherapy via this route seems an attractive option.

As early as 1956 Cruz et al demonstrated in an animal model that nitrogen mustard, if given via the portal route after a systemic injection of tumour cells, reduced the incidence of subsequent hepatic metastases. Both the route of administration and timing of treatment appear to be crucial when attempting this form of treatment. A more recent animal model emphasised this (Suranto-Ward et al 1992): if portal vein chemotherapy is given immediately following tumour cell infusion, the disease occurrence shifts from hepatic to extrahepatic, with an associated improvement in survival. If however the chemotherapy is delayed by 72 hours, this improvement did not occur. A possible explanation for this observation is that within 24 hours of inoculation, tumour cells were trapped and adhering to the vessel endothelium, or may have even been in the process of extravasation. Therefore therapeutic agents given via the portal route, which forms the initial blood supply of such emboli, reached the tumour cells when they were most vulnerable. However, after 72 hours the tumour cells may have extravasated deep into the hepatic parenchyma and perhaps triggered changes in the local microenvironment which provide protection against blood born cytotoxics.

Perioperative portal vein infusion therapy has been applied in a number of prospective trials in an attempt to reduce the formation of post operative colorectal liver

metastases, and so hopefully improve survival. Such a trial was initiated in 1975 by Taylor et al, whereby patients undergoing primary resection of colorectal cancers without detectable liver metastases were randomised into receiving either no adjuvant therapy or a 7 day course of continuous portal vein infusion of 5-Fluorouracil. Subsequently 5 treated patients and 22 control patients developed liver metastases and although the local recurrence rate was unaffected a survival advantage was conferred on patients with Dukes stage B tumours.

As a result of this initial study several further prospective studies were performed. One trial carried out by Gray et al(1987) in Australia and New Zealand included only patients with colonic cancer. 372 patients were randomised to receive either no adjuvant therapy, a portal vein infusion of 5FU, or systemic 5FU. A statistically significant survival advantage was demonstrated for patients with Dukes stage C carcinomas who received portal vein 5FU. The Mayo clinic reported a trial involving 224 patients with Dukes stage B2 or C colorectal cancers. Patients were randomised to either receive no therapy, or to 7 days of 5FU via the portal vein (Beart et al 1990). It should be noted that in this study the treatment was delayed for 5 days post operatively and failed to demonstrate any survival advantage after 5.5 years follow up, with an equal distribution of liver metastases in both arms of the study.

Wereldsma et al in 1990 randomised patients to either no treatment, portal vein infusion of 5FU and heparin, or portal vein infused urokinase. The only group showing any improvement was the 5FU group, with the number of liver metastases reduced to one third that of the control group. Although the death rate was 75% that in the control group the figures failed to reach statistical significance.

Wolmark et al in 1990 in the NSAPB study, randomised 1158 patients to receive either portal vein infusion of 5FU and heparin or observation alone. A survival and disease free survival advantage were both demonstrated, but failed to show a statistical improvement in the incidence of liver metastases.

The Swiss group for clinical cancer research carried out a randomised trial (1995) where patients received either no adjuvant therapy or postoperative portal vein 5FU and mitomycin C .
Of the 469 patients included at a median follow up of 4 years, there was a survival rate of 57% and 70% in the no treatment and treatment arms respectively.

Although a meta-analysis of all the trials performed so far demonstrates both a survival advantage and a reduction in the incidence of liver metastases, the relatively small numbers involved in each study prevent a overall survival advantage being demonstrated. It is for this reason that the AXIS trial has been devised, where 4000 patients will be randomised to portal vein infusion of 5FU or no adjuvant therapy, with rectal cancer patients being randomised to radiotherapy also. Initial results of which suggest upto 5% improvements in survival.
Cannulation of the portal vein is normally performed via one of its tributaries as direct access is technically difficult. The various options available include;
-Cannulating the umbilical vein which is obliterated in the non foetal state -and is accessed and reopened by dissecting it out of the falciform ligament.
-Using the gastro-epiploic or gastro-duodenal veins lying on the greater curvature of the stomach.
-cannulation of either the inferior or small bowel mesenteric veins.

1.4.1.2 Portal vein chemotherapy for established colorectal liver metastases
There are two main situations in which this route of treatment is used; as an adjunct to colorectal liver metastases resection, and in conjunction with hepatic arterial ligation.
As already mentioned, although a macroscopically curative hepatic resection may have been performed, occult metastases, which are currently undetectable, may still persist and cause delayed intrahepatic disease recurrence. Therefore as these

micrometastases have a predominantly portal blood supply adjuvant therapy via this route should theoretically impair their development.

Twelve patients undergoing hepatic resection for colorectal liver metastases were incorporated into a phase 2 study where they received postoperative portal vein infusions of 5FU (Elias et al 1987). Unfortunately only 40% of the patients tolerated 5 courses of 14 days 5FU infusion, because of problems related to mechanical failure; in the patients completing the course no haematological disturbances were demonstrated. This study could of course draw no clinical conclusions. It does however illustrate the technical feasibility of such a therapy but requires a randomised control trial to confirm its usefulness in preventing disease recurrence.

Established tumours derive the majority of their blood supply from the hepatic artery and therefore portal vein therapy alone is unable to deliver sufficient quantities of a given chemotherapeutic agent. However it is known that if the hepatic artery is ligated the portal blood flow increases to compensate(Taylor et al 1979, Ackerman et al 1986). Therefore a theoretical advantage might be gained by combining hepatic arterial ligation with portal vein chemotherapeutic infusion.

Murray-Lyon et al in 1970 performed this technique in 5 patients all of whom derived symptomatic benefit and whose survival varied between 10 and 20 months. In 1984 Laufman and his co-workers treated 19 patients with a combination of hepatic artery ligation and portal vein infusion of 5FU and mitomycin C. This achieved a 63% response rate with a median survival of 13 months from commencing treatment and 14 months from diagnosis.

Taylor et al in 1981 performed a study involving 35 patients with multiple liver metastases who were categorised using strict selection criteria depending on the degree of hepatic replacement. Again the patients underwent hepatic arterial ligation combined with postoperative portal vein 5FU. Those patients with hepatic

replacement of less than 20% achieved the greatest median survival of 15 months with those with greater than 70% surviving only a median of 5.5 months with an overall median survival of 10 months.

In 1991 Gerard et al in a prospective trial compared the combination of hepatic arterial ligation with post operative portal vein infusion of 5FU, with hepatic arterial ligation alone, in patients with colorectal liver metastases. Unfortunately the complication rate for hepatic arterial ligation was high with 4 patients developing hepatic failure. The combination treatment arm achieved a partial response in 5 patients with only 1 in the hepatic arterial ligation alone group. Only one patient developed a complete response in the combination group with none in the other, with a median survival of 12 months in both groups. These results are hardly surprising given the predominantly arterial supply of such tumours.

1.4.2 Hepatic arterial chemotherapy

1.4.2.1 Background

Appreciation of the importance of the hepatic artery in the blood supply of colorectal liver metastases, has led to the evolution of hepatic arterial chemotherapy (HAI). Given the steep dose response curves seen with systemic chemotherapy, hepatic arterial chemotherapy allows larger doses to be given and hence achieve greater tumour exposure. Also, considering the high first pass ratio of 5-FU and fluorodeoxyuridine (FUDR) through the liver, this technique leads to high hepatic drug concentrations whilst minimising systemic exposure.

Access to the hepatic arterial circulation is gained via the gastroduodenal artery, utilising a incision silmilar to that for open cholecystectomy. All patients require a preoperative angiogram to ensure there are no vascular anomalies.

1.4.2.2 The pharmacokinetics of hepatic arterial chemotherapy

Since a chemotherapeutic agent will enter the systemic circulation once it has passed through and been partly metabolised in the liver, all its advantages must be obtained on its first pass.

The pharmacokinetic advantages of HAI are those factors which are stated in Ficks principles of diffusion, namely; the first pass extraction rate through the liver, hepatic arterial flow rate and rate of drug elimination through the body.

Expressed as an ideal, the perfect agent should be rapidly extracted from both the body and the liver, whilst having a slow hepatic transit.

Chemo-embolisation using degradable microspheres either containing or in conjunction with chemotherapeutic agents has been attempted. The rationale being that this technique should lead to acute episodes of tumour ishaemia whilst also slowing drug transit and hence improving drug delivery. Studies have suggested that this may improve blood flow to underperfused metastases while blocking blood flow to the healthy liver, although there does not seem to be any survival advantage (Lang et al 1993, Rougier et al 1992). Hunt et al (1990) did however demonstrate that patients with low volume disease did better with this treatment over HAI chemotherapy alone.

An understanding of how these agents are extracted is necessary prior to devising a clinical trial. Ensminger et al (1978) determined the extraction ratios of both FUDR and 5-FU. On first pass 50-87% of 5-FU is extracted with 94-99% for FUDR. However the pharmacokinetics of 5-FU are non-linear suggesting that the mechanisms involved are saturable. Therefore high dose intravenous therapy leads to a relatively reduced hepatic uptake whilst causing higher systemic levels (Goldberg et al 1988). Another reason for using prolonged infusions over bolus therapy is that 5-FU appears to be cytotoxic in the S phase of the cell cycle. Longer infusions would 'catch' more susceptible cells. Lokich et al (1989) demonstrated improved results with infusions compared with bolus treatment.

In order to assess the uptake of these agents by the liver in vivo a number of techniques have been devised. Initial studies involved biopsying the liver of patients with colorectal liver metastases. Peters et al (1993) found intrahepatic levels of 5-FU upto ten times the plasma level extending to 48 hours after bolus injection.

Non invasive techniques for assessing drug uptake include magnetic resonance spectroscopy (MRS) and positron emission tomography (PET). MRS studies looking at compounds labelled with ^{19}fluorine are able to quantify in vivo drug concentrations including 5-FU and its metabolites. Although high drug concentrations are required in order to be detected.

Clinical studies have demonstrated long half lives of 0.3-1.3 hours for 5-FU within tumours leading to increased intra-tumour drug retention (Wolf et al 1990, Present et al 1990). An intra-tumoural half life of greater than 20mins is associated with clinical tumour response (Schlemmer et al 1991, Findlay et al 1993).

The uptake of 5-FU labelled with ^{19}fluorine into colorectal liver metastases in patients can be measured using PET. Using this technique the tumour perfusion was found to be upto four times greater for HAI vs IV therapy. However this only translated into a 1.7 times greater uptake by the tumour (Strauss et al 1991, Hohenberger et al 1993).

1.4.2.3 Results of HAI chemotherapy

Initial studies were hampered by a variety of technical problems related to HA catheter placement and maintenance. Now with implantable pumps and subcutaneous ports coupled with improved surgical technique many of these initial problems have been negated.

Studies using FUDR in HAI chemotherapy compared to intravenous therapy revealed consistently improved response rates although this did not translate into improved survival (Kemeny et al 1987, Hohn et al 1987, Chang et al 1987, Martin et al 1990). Response rates varied from 42-62% (all being significant $p<0.05$) although all the studies contained small numbers of patients (mean=83). In addition some of these studies also incorporated a degree of patient crossover between the groups studied.

In the study by Kemeny et al (1987) 60% of the patients who received systemic chemotherapy later received HAI chemotherapy having initially failed to respond. Those patients who crossed over had a mean survival of 18 months compared to 8 months in those who only received systemic therapy. Rougier et al (1992) using FUDR demonstated improved 1 year survival and response rates of upto 43% in the HAI group, although the control group was composed of untreated and inadequately treated individuals. More recently Allen-Mersh et al (1994) demonstrated a survival advantage of 405 vs 226 days (median) using HAI FUDR compared with a control arm composed of either IVtreated (21% of patients) or observation only.

In many studies although intrahepatic disease appears to be reduced by HAC the pattern of reccurrence shifts from hepatic to extrahepatic, usually involving the lungs.

Safi et al (1989) demonstrated that if FUDR is given both by HAI and IV this led to fewer extrahepatic relapses. Another option is to give doses via the hepatic artery which lead to sufficient systemic as well as locoregional treatment. This design has been incorporated into the CRO5 trial currently underway where patients, with colorectal liver metastases but no extrahepatic disease, are randomised to receive either HAC or systemic 5FU and folinic acid.

Given that intravenous systemic therapy of 5-FU is improved by co-administration with folinic acid it would appear logical that the same would apply to HAC. However the addition of folinic acid via this route leads to an unacceptable incidence of catheter related problems (Anderson et al 1991). Therefore co-administration of IV systemic folinic with HAI 5-FU should overcome this problem. In trials Combination

Author and year	Nos of patients	Drug combination	Response rate (%)	Median survival (months)
Ansfield et al 1975	381	5-FU	55	5
Johnson et al 1985	40	FUDR	44	12
Chang et al 1987	32	FUDR	62	15
Kemeny et al 1987	48	FUDR	50	17
Kemeny et al 1992	25 25	FUDR FUDR+DEX	40 71	15 23
Rougier et al 1992	81	FUDR	43	15
Allen-Mersh et al 1994	51	FUDR	40	13.5

Table 2. The results of studies using intrahepatic arterial chemotherapy to treat colorectal liver metastases.

5-FU=5 fluorouracil, FUDR=5-fluorodeoxyuridine, DEX=dexamethasone

of HAI 5-FU with IV systemic folinic acid does seem to improve survival (Anderson et al 1992).

Further improvements on these results using HAI chemotherapy may be improved by greater drug targeting (see below).

Section 1.5 - The manipulation of the blood flow to colorectal liver metastases

1.5.1 Background

As already demonstrated HAI appears to produce the greatest improvement in response and survival for patients with irresectable liver metastases. The question remains as to whether or not further improvements in drug targetting will lead to better results. The intention of this section is to illustrate the differences that exist between tumour blood vessels and those within the normal liver and how this knowledge might be used to improve drug delivery.

The first section deals with the structure and control of vasomotor tone in normal blood vessels. The next section contrasts these features with those found in the blood vessels of tumours and the final section describes experiments which have attempted to manipulate these differences.

1.5.2 The structure of blood vessels and control of blood flow in health

1.5.2.1 The Vessel Wall

Normal vessels are composed of an inner endothelial intimal layer, a media in which the contractile smooth muscle is contained and an outer connective tissue adventitia. Blood vessels have two types of nerves; the paravascular nerves which run in the adventitia and innervate structures more distally, and the perivascular nerves which terminate in the media and are concerned with local vasomotor control. The vessels

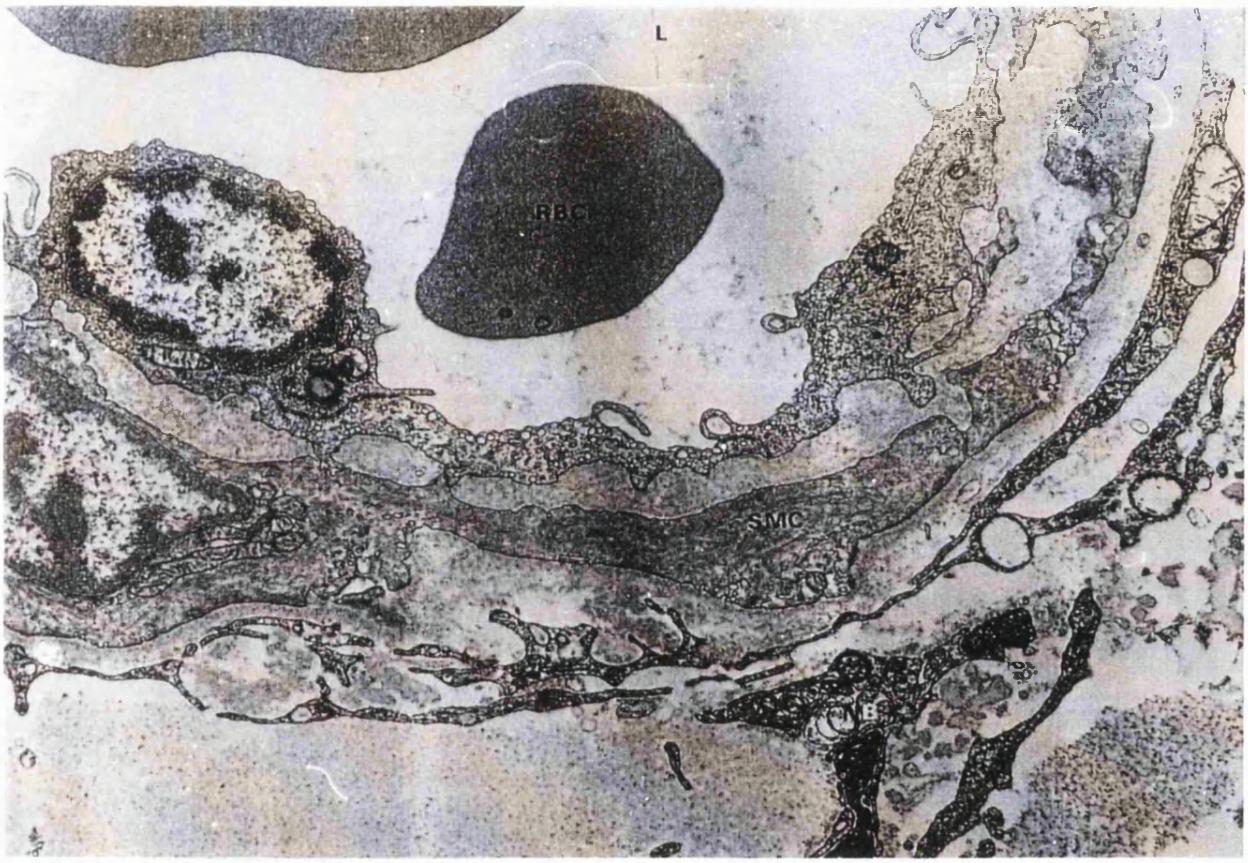


Figure 1: Electron micrograph showing the ultrastructure of an arteriole in the normal liver.

Note the lumen (L) containing a red blood cell (RBC), an endothelial cell (E), a smooth muscle cell of contractile phenotype (SMC) and a nerve bundle (NB). Calibration bar = 1.0 μm.

concerned with the regulation of blood flow are the terminal arterioles , although most of the studies performed have focused on larger vessels.

The perivascular nerves form a plexus of branching terminal fibres. These fibres do not have a schwann cell lining and are covered with varicosities.

It is these varicosities which contain the neurotransmitters which when released control smooth muscle tone.

1.5.2.2 The control of blood vessel tone

It was initially thought that blood vessel tone was predominantly under the control of the sympathetic nervous system via the perivascular nerves with the only neurotransmitters being noradrenaline and acetylcholine. However in the last 15 years a large number of other neurotransmitters have been identified in addition to identifying the pivotal role played by the endothelium in the control of vessel tone.

Sympathetic nerves

In addition to noradrenaline (NA) both adenosine 5-triphosphate (ATP) and neuropeptide Y (NPY) are released at sympathetic terminals causing vasoconstriction (Stjarne et al 1989, Burnstock 1988, Lundberg 1983, Lundberg 1984).

NA and ATP appear to be released as co-transmitters in a variety of vascular beds in a number of different mammals (Burnstock 1988) with the ratios of each released varying depending on the vessel. Both of these agents in addition to acting on the post junctional plate also possess a degree of pre-junctional modulation (Evans et al 1992). This co-transmitter action permits a wider range of control by varying both the ratios and pre and post junctional differences.

NPY is also released at sympathetic nerve endings although alone it has little action (Stjarne et al 1986, Pernow et al 1986). NPY action is that of a neuromodulator enhancing the post-junctional effects of ATP and NA whilst acting pre-synaptically to reduce their release, the predominance of which is dictated by the size of the synaptic cleft(Wahlestedt et al 1986, Saville et al 1990).

5-Hydroxytryptamine (5-HT) is also associated with sympathetic nerves but is not produced here, rather it is taken up from the circulation and released later as a false transmitter (Griffith et al 1983, Jackowski et al 1989). Its action is to constrict large vessels and venules and dilate arterioles, causing movement of fluid out of the microcirculation.

Parasympathetic and sensorimotor nerves

The parasympathetic system secretes vasoactive intestinal peptide (VIP) and acetyl choline (ACh) which have a vasodilatory effect on blood vessels.

The sensorimotor nerves function via an axon reflex which incorporates an afferent sensory and efferent motor limb (Maggi and Meli 1988, Szolcsanyi 1988). Their stimulation leads to the release of a variety of transmitters which include substance P, calcitonin gene related peptide (CGRP) and ATP (Gibbins et al 1985, Lee et al 1985, Krishtal et al 1988) which cause vasodilatation.

The endothelium

The endothelium was first proposed to play an integral role in the control of vasodilation in 1980 (Furchgott and Zawadski) and the agent responsible for this action was identified as nitric oxide (NO) in 1987 (Furchgott et al 1987) with prostaglandins also suggested to play a minor role.

NO is produced by the action of the enzyme nitric oxide synthetase on L-arginine in response to a variety of stimuli. These include ATP, substance-P, vasopressin (Vas), angiotensin-II (AgII), bradykinin, histamine and ACh. It is unlikely that the source of such agents is from the perivascular nerves given the anatomical positions of these two areas. One possible source is the blood and another is the endothelial cells themselves (Ralevic et al 1992). NO is released continuously by vessels, especially arteries, leading to resting tone. If the endothelium is damaged or NO synthesis altered the tone will rise.

The endothelium also produces a variety of vasoconstricting agents which include the family of polypeptides known as the endothelins, with endothelin-1(ET-1) being the most potent. Vasoconstricton is stimulated by a variety of stimuli which include; hypoxia, mechanical stretch, NA and thrombin (Katusic et al 1987, Yanagisawa et al 1988).

1.5.2.3 Endothelin-1

Endothelin-1 structure and function

ET-1 is one of a family of peptides first identified by Yanasigawa et al in 1988. Structurally it is a 21 amino acid peptide produced by vascular endothelial cells. ET-1 is formed from the cleavage of the 212 peptide, preproendothelin-1 to big (pro) ET-1 and then by the cleavage of the Try-Val bond by endothelin-1 converting enzyme to active ET-1.

Its action is pronounced and prolonged vasoconstriction (Levin 1995) which is via actions on two different subtypes of endothelin-1 receptors ETA and ETB, which vary both in their action and their distribution (Lin et al 1991). When administered intravenously, ET-1 causes an initial vasodilation, an effect mediated by the ETB receptors on vascular endothelium (Clozel and Clozel 1989, Baydoun et al 1989). Stimulation of the ETB receptor leads to production of nitric oxide which results in vasodilation (Clozel et al 1992). ET-1 induced vasoconstriction is achieved via stimulation of ETA receptors which cause an intracellular calcium influx and hence, stimulate vascular smooth muscle contraction (Clozel et al 1992). The actions of ET-1 on vasomotor control are mediated by the endothelium as its removal abolishes the effects of ET-1 (Yanasigawa et al 1988, Vallance et al 1989).

ET-1 is also known to be a promoter of mitogenesis and stimulator of the synthesis of a variety of hormones and autocoids (Rossi et al 1994, Belloni et al 1994) which include aldosterone, arginine-vasopressin, nitric oxide and prostacyclins.

Endothelin-1 and benign disease

ET-1 has been implicated in the pathogenesis of a number of conditions whose common denominator appears to be an element of increased vasoconstriction. Such disorders include hypertension, Raynauds phenomenon, pulmonary hypertension, renal failure and ischaemic heart disease (Miyauchi et al 1989, Kamai et al 1990, Cernacek and Stewart 1989). These conditions are all associated with elevated levels of ET-1 in the plasma although in only picomolar concentrations. Such levels would be unlikely to cause vasoconstriction alone but might sensitise blood vessels to the actions of other vasoconstrictors. Undoubtedly these elevated levels of ET-1 represent only an estimate of deranged ET-1 sysnthesis and secretion occuring in the tissues, especially given it s short half life.

Recognition of the altered levels of ET-1 in these conditions has led to trials of ET-1 receptor antagonists in a variety of animal models and ex-vivo and in vitro human studies . Given that ET-1 antagonists are now available orally a number of clinical trials are currently underway.

ET-1 appears to be involved in pathological mitogenesis both in atherosclerosis and in restenosis after coronary angioplasty. In rabbits the tissue levels of ET-1 and ETB receptors were increased after endothelial damage similar to that following angioplasty. Subsequent application of ETA receptor antagonists did not affect neo-intimal formation suggesting a role for ETB receptors in modulating the pathogenesis of restenosis (Azuma et al 1994).

Endothelin-1 and non-colorectal cancer

Prostate cancer

Endothelin-1 is normally found in high concentrations in normal human ejaculate. Nelson et al (1996) demonstrated that all the specimens of both primary and metastatic prostatic cancer had elevated levels of ET-1 present. They also

demonstrated that in tissue culture ET-1 acted as a mitogenic agent directly and enhanced the mitogenic actions of insulin-like growth factor I, insulin-like growth factor II, platelet derived growth factor, basic fibroblast growth factor and epidermal growth factor in cell lines of prostate cancer. These effects were not reduced by application of ET B receptor antagonists but were by ET A antagonists, implying that receptor A type is responsible for modulating ET-1 actions in prostate cancer. In addition none of the cell lines possessed ETB receptors, reinforcing its apparent lack of importance in regard to these tumours.

Plasma levels of ET-1 in patients with bony metastases from prostatic cancer are elevated, which is of some interest given its stimulatory effect on osteoblasts (Nelson et al 1995). ET-1 inhibition in this instance may provide another treatment for symptomatic bony involvement.

Breast cancer

ET-1 is elevated in primary breast cancers and might be useful as a prognostic indicator. Kojima et al (1995) showed that tumours positive for ET-1 had both a higher local recurrence rate and lower 5 year survival than ET-1 negative patients. It has also been demonstrated using immunohistochemistry that the level of ET-1 found within cancer cells is inversely proportional to the degree of tumour differentiation (Lu et al 1995) and that over production of ET-1 may result in higher rates of breast growth and hence contribute to malignant transformation.

One postulated mechanism for the regulation of ET-1 production in breast cancers is via protein kinase A and C signalling pathways. Patel et al (1997) demonstrated that stimulation of either system increases ET-1 secretion. Also human breast fibroblasts release prostaglandin E2 after treatment with ET-1 and PGE2 stimulates ET-1 production in breast cancer cells. This paracrine loop maybe important given the elevated levels of both ET-1 and PGE2 found in breast cancers.

Non-colorectal gastrointestinal tumours

ET-1 and ETA receptors are upregulated in gastric carcinoma cell lines demonstrated using the reverse transcriptase polymerase chain reaction (Mathieu and Chevillard 1995). Pancreatic cell lines also produce ET-1 as shown by Oikawa et al (1994). Plasma levels of ET-1 are elevated in patients with hepatocellular carcinoma when compared to patients with cirrhosis and controls (Nakamuta et al 1993). There was, however, no correlation with other biochemical prognostic indicators in the hepatocellular carcinoma group. ET-1 production in these patients may have some bearing on systemic metastatic development.

Gynaecological tumours

Ovarian cancer cell lines produce ET-1, possess both ETB and ETA receptors and exhibit increased mitogenesis in a dose dependent manner with the addition of exogenous ET-1. In addition it has been shown that ETA receptors are the main sites of action for ET-1 in tumour mitogenesis utilising an as yet unidentified tyrosine kinase(Bagnato et al 1997).

Endometrial carcinoma cell lines also produce ET-1 which is speculated to be involved in the angiogenic process occuring during tumour growth (Economos et al 1992). ET-1 is known to be both directly and indirectly angiogenic. ET-1 is a direct endothelial cell mitogen and indirectly stimulates angiogenesis by causing monocytes to produce interleukin-8, a potent angiogenic agent (Giaid et al 1995, Huribal et al 1994).

Endothelin-1 and colorectal cancer

Since earlier studies demonstrated that cell lines, including those from colorectal cancers, produce and respond to ET-1, attempts to identify the sites of production and action of ET-1 have been made (Shichiri et al 1991a, Shichiri et al 1991b). Inagaki et al (1992) demonstrated a high density of ET-1 binding sites over tumour

vessels and stromal tissues surrounding primary colorectal cancers, suggesting ET-1 might be acting in a paracrine fashion.

Some groups have suggested that a primary tumour may influence the growth of its metastases by a positive or negative feedback circuit (Prehn 1991). If one were to propose such a mechanism then a variety of signalling agents would be required to be secreted into the systemic circulation. ET-1 could be one such messenger. As already mentioned the HPI is elevated in patients with colorectal liver metastases. Since ET-1 is a potent vasoconstrictor with marked effects on the splanchnic bed and maybe produced by colorectal cancers, it might play a role in this phenomenon.

What needs to be assessed is whether plasma levels of ET-1 are elevated in patients with colorectal cancer with and without liver metastases and also whether ET-1 is present in colorectal cancers and their metastases. The metastases represent the more interesting group since these tumours are those with the highest metastatic potential. What also needs to be determined is which cell types, if any, produce ET-1within the tumour mass.

The importance of ET-1 within liver metastases was assessed by Loesch et al (1997) using the MC28 animal model. This showed elevated expression of ET-1 in the endothelium of the tumour vessels and tumour cells.

If ET-1 is a metastatic promoter then inhibition of its action would be expected to reduce the incidence of liver metastases. The most appropriate time for such intervention would be at the micrometastatic phase of development, during the perioperative period using the portal vein.

1.5.3 The control of liver blood flow in health

1.5.3.1 Introduction

The liver receives 25% of cardiac output, with 25% of the liver weight being blood and as such is the most vascularised organ in the body. The blood supply of the liver

is from 2 sources; the hepatic artery and portal vein, with the venous component accounting for over 2/3 of the total.

The portal circulation is normally a low pressure system, usually maintained at about 7mmHg, with the pressure gradient across the liver to the inferior vena cava being only a few mm of Hg. This contrasts to the high pressure arterial system of approximately 100mmHg which mixes with the portal venous blood in the sinusoids before exiting via the hepatic veins. The control of blood flow to the liver is via the hepatic artery rather than portal vein, which in turn is regulated by the hepatic arterial buffer response (HABR).

1.5.3.2 The hepatic microcirculation

The functional unit of the liver is the hepatic acinus of which there are approximately 100, 000 in the human liver. The acinus is composed of parenchymal cells clustered around the terminal branches of the hepatic arterioles and portal venules. These branches enter at the center of the acinus where free mixture of portal and arterial blood occurs. Blood then flows past the hepatic sinusoids to the periphery where it drains into branches of the hepatic veins. The one way flow of blood in the acinus prevents any backflow of metabolites from the hepatic venous side (Lerner et al 1974) which might influence the HABR (see later).

The distribution of blood flow throughout the liver is uniform (Greenaway and Oshiro 1972) regardless of whether the source is the hepatic artery or portal vein. Equal distribution of blood flow persists despite sympathetic nerve stimulation, noradrenaline infusion and alterations in portal vein perfusion pressure (Greenaway and Oshiro 1972, Cousineau et al 1985). Such localised changes in portal blood flow are compensated for by changes in acinar hepatic arteriolar blood flow.

1.5.3.3 The control of hepatic blood flow

The majority of hepatic blood flow comes from the portal vein which is not under the control of the liver and is simply the result of output from the extra-hepatic splanchnic

organs. Rather, all the control of hepatic blood flow occurs via the hepatic artery (Lautt 1996).

The intrinsic control of hepatic blood flow

The intrinsic system operates via two mechanisms, both of which are linked by the concentration of adenosine. In the phenomenon of 'arterial autoregulation' changes in arterial perfusion pressure and hence flow, directly lead to reciprocal alterations in arterial tone to maintain the status quo ie hepatic arterial constriction if arterial pressure rises. The second arm of the intrinsic mechanism again utilises compensatory alterations in hepatic arterial flow but this time in response to changes in portal venous flow. If portal flow reduces then this leads to hepatic arterial dilation with subsequent increased arterial flow and visa versa. Both of the intrinsic systems of autoregulation rely on the adenosine washout principle (Lautt 1985, Lautt 1996): adenosine is released at a constant rate into the space of mall which lies adjacent to the hepatic arterial resistance vessels and portal venules. The concentration of adenosine is therefore related to the rate of removal which in turn is proportional to blood flow from the hepatic artery and portal vein. If adenosine levels rise due to a fall in blood flow from either the arterial or portal system there is a compensatory increase in hepatic arterial flow due to hepatic arterial dilatation. As levels fall the hepatic artery constricts.

The extrinsic control of hepatic blood flow

This system is less well understood and is often superseded by the effects of the HABR. The hepatic artery responds much like other vessels to intraportal and intrahepatic arterial infusions of vassopressors (Richardson and Withrington, 1981, Israel and Orrego, 1981). These responses are only found when pharmacological doses are administered and not with normal physiological concentrations. The exception to this is found in the post-prandial state when the hepatic artery is relatively resistant to constriction, presumably due to a released vasodilator. A

number of agents have been suggested including gastrin, secretin and glucagon (Richardson and Withrington, 1981, Israel and Orrego, 1981).

The hepatic artery receives a rich supply of sympathetic fibres which act on alpha adrenoceptors to cause varying degrees of vasoconstriction, although its function in the normal state is incompletely understood (Lautt 1983).

The hepatic venous sphincters

The hepatic venous sphincters encompass those found within the portal veins, sinusoids and terminal hepatic venules, with vascular resistance being negligible proximal to this point. These sphincters can undergo active constriction leading to raised intrahepatic pressure. Agents that have been shown to produce marked constriction of these sphincters in the cat and dog include angiotensin-II and noradrenaline.

These sphincters regulate intrahepatic and portal pressures, protecting these pressures from passive changes in the central venous pressure(Lautt et al 1987, Lautt and Legare 1987).

1.5.4 The Structure and control of tumour neovasculature

Several early studies suggest that the morphological pattern of blood vessels is altered within tumours (Krylova 1969, Krylova and Presnov 1964, Huesby et al 1975).

Most of these studies utilised tumours grown in rodents. Krylova and Presnov (1964) demonstrated that blood vessels in a sarcoma-45 when implanted intramuscularly in a rat consisted of a single layer of endothelial cells upon a basement membrane with no surrounding contractile tissue. Such findings were also found in the vessels of adenocarcinomas implanted in the thyroid gland of the rat (Krylova and Dmitrieva 1967).

Huesby et al (1967) reported that blood vessels in mammary carcinomas explanted in mice were immature. Again these vessels lacked contractile elements and had a sinusoidal architecture. Konerding et al (1991) looking at xenotransplanted tumours in mice (human melanomas and head and neck tumours) found similar vessels to those described above, with the only exceptions being those at the tumour periphery which represented host vessels being incorporated into the tumour.

In addition to work on animal tumours, studies have also reported results from investigations utilising human tumours. Long (1973) using electron microscopy demonstrated that the blood vessels within meningiomas had a primitive sinusoidal appearance. Chaudhry et al (1978) reported similar results with cerebellar haemangiomas. Suzuki et al (1987) reported that blood vessels within hepatocellular carcinomas consisted of endothelial cells resting on a basement membrane associated with occasional hypoplastic smooth muscle cells in their walls. Similar features were observed in the branches of the hepatic artery supplying the tumour.

The innervation of tumour vessels has been looked at in a number of studies since 1955. Coutelle (1955) reported that nerves found in Erlich's ascites cancer, when grown intracutaneously in mice, moved away from their original site , had disrupted connections and finally degenerated. Krylova (1967 and 1977) also reported the absence of perivascular nerves in neoplastic vessels in a variety of tumours.

Mattson et al (1977 and 1979), using hepatoma and 20-methylcholantherene sarcoma implanted intramuscularly in rats, demonstrated that tumour blood vessels had no evidence of perivascular innervation apart from at the periphery.

Hafstrom et al (1980) reported that adrenergic nerves were absent in the rat hepatic tumours hepatoma (Hep-H) and adenocarcinoma (NG-W).

Mitchell et al (1994a and 1994b) demonstrated that perivascular nerves were absent in the main parts of human primary breast and colon carcinomas and in colorectal and breast cell line tumours in immunodefficient mice.

Ashraf et al (1996 and 1997) performed immunofluorescent light microscopy on both human colorectal liver metastases and MC28 tumours from the livers of Hooded Lister rats looking at tumour vessel innervation. This study looked at markers for sympathetic, parasympathetic, sensorimotor and general neuronal markers in both the tumour and adjacent normal liver. The tumour vessels did not stain positively for any of the neuronal markers, while the normal liver vessels stained positive for all in differing degrees. The only exception was at the periphery of the tumour where some vessels stained weakly positive for some markers. Such vessels may represent normal adjacent vessels becoming incorporated into the tumour. These findings applied to both the human and animal tumours. Such results support the hypothesis that tumour vessels lack innervation and that this animal model could be used to assess pharmacological manipulation of tumour blood flow.

The same group also performed electron-microscopy on human colorectal liver metastases and animal hepatic tumours. They demonstrated that tumour vessels had a primitive endothelium and lacked a smooth muscle coat and neuronal innervation (Ashraf et al 1996 and 1997).

These more recent findings in conjunction with the earlier results suggest tumour vessels may exist in a state of perpetual dilation and would be unable to vasoconstrict regardless of stimulus. This dilated state would aid blood flow and hence promote tumour growth.

If this were to be the case then co-administration of a vasoconstrictor agent with HAI should lead to selective constriction of the hepatic vessels whilst leaving the tumour vessels unaffected. This in turn should lead to blood being ' shunted' into the tumour from the hepatic bed and hence improve drug delivery. This is appropriately called the shunting theory (figure 2).

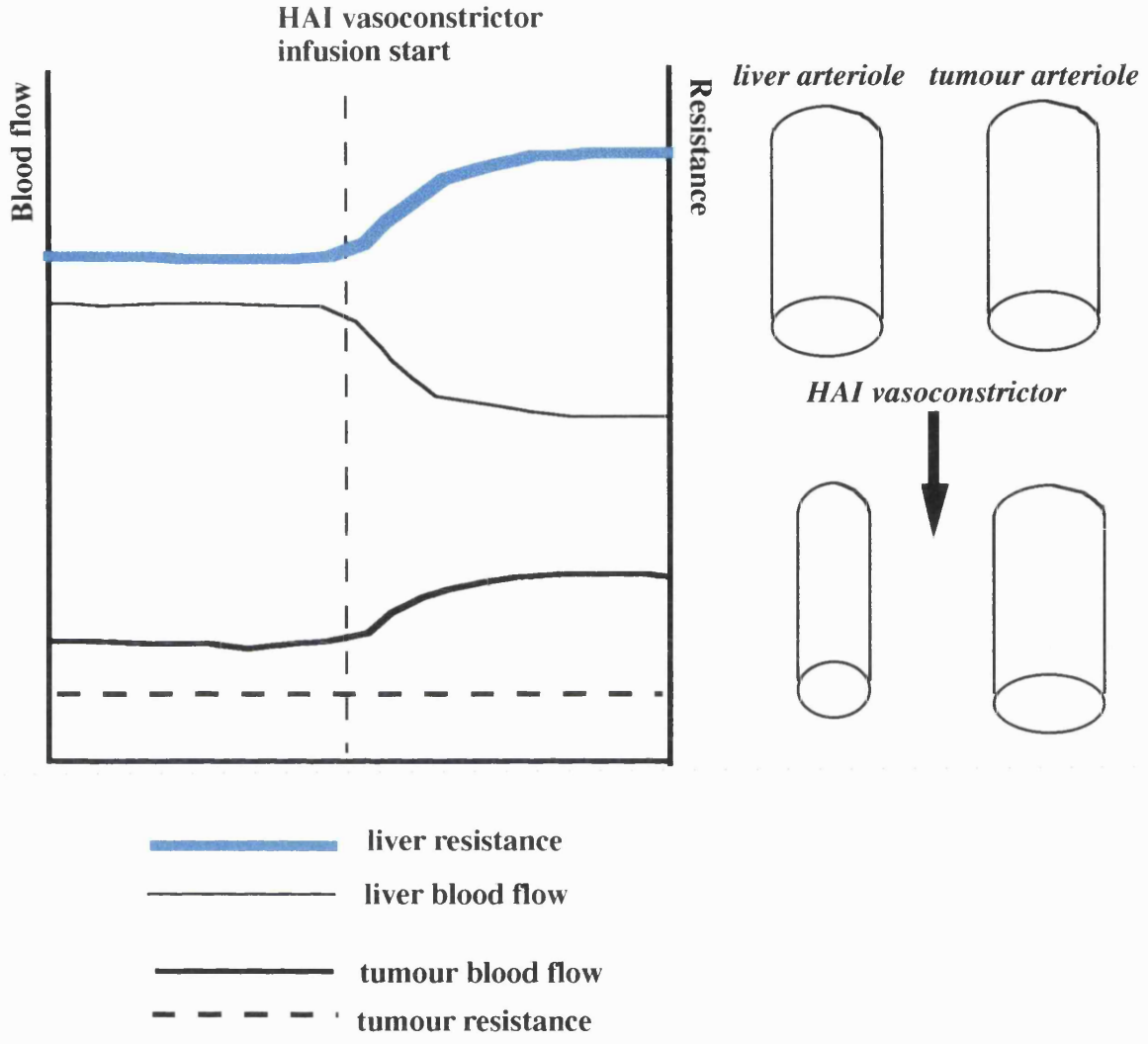


Figure 2: Diagram illustrating the principle of the shunting theory.

1.5.5 The pharmacological manipulation of blood flow in liver tumours

Numerous vasopressors have been applied in a variety of models both within the liver and elsewhere in an attempt to alter tumour blood flow and so increase drug delivery. Most of these studies are based on the presumption that tumour vessels are unresponsive and exist in a state of maximal dilatation and therefore constriction of the surrounding normal vasculature should result in shunting of blood into the tumour. Whether or not the improved tumour blood flow is related simply to shunting or whether there is an actual change in vessel tone is unknown.

In their normal state colorectal liver metastases have a characteristic rim of vascular tissue and an avascular core on angiography. The central theory of shunting is that the vasoconstriction of hepatic vasculature reverses the gradient and forces open the normally closed central tumour vessels, thereby providing access to an area which is hard to treat with conventional chemotherapy. It is also postulated that these vasopressors may act by simply raising the systemic blood pressure, and therefore improving the perfusion gradient as part of a generalised rather than a local effect. This is unlikely however since changes observed in the tumour circulation do not mirror alterations in blood pressure.

Abrams et al (1964) demonstrated altered responsiveness of tumour blood vessels to vasopressors and since then many other groups have presented similar results. Ackerman et al (1980 and 1984) demonstrated improved perfusion of liver metastases with infusion of adrenaline in an animal model. These experiments utilised microfil to demonstrate improved tumour vessel filling when adrenaline is administered. The actions of adrenaline are mediated by alpha-1 receptors present on normal smooth muscle and are abolished by alpha -1 receptor blockers. It has long been accepted that the tumour blush of colorectal liver metastases on angiography is improved by the administration of adrenaline (Ackerman and Makohon 1984, Ackerman and Hechmer

1980). This effect does not appear to be due to decreased normal vessel filling alone, but also to increased tumour vessel flow.

Iwaki et al (1978) demonstrated improved hepatic angiography and uptake of mitomycin C in VX2 carcinomas in the livers of rabbits by the infusion of adrenaline . Improvements on the effect produced by adrenaline have been attempted by applying other alpha receptor agonists with a variety of success.

Hafstrom et al (1980) applied both adrenaline and noradrenaline in a rat model of liver metastases with blood distribution measured with radiolabelled microspheres. In this study both produced improvements in the tumour/normal liver blood flow with noradrenaline having the greatest effect.

Similar results have been reported in head and neck tumours, with noradrenaline improving blood flow to localised tumours with relatively isolated arterial supplies (Ziesssman et al 1985).

Ackerman et al (1988) using laser doppler flowmetry to measure hepatic blood flow assessed the effects of bolus infusions of intraportal adrenaline and noradrenaline on liver tumour blood flow. Both agents (adrenaline>noradrenaline)produced short lived improvements in the T/N ratio with marked increases in tumour blood flow.

Zlotecki et al (1985) reported similar results using VX2 carcinomas in rabbits. Burton and Gray (1987) again using VX2 carcinomas implanted into the liver of rabbits measured the uptake of systemically administered radiolabelled microspheres with and without systemically administered noradrenaline. Noradrenaline alone produced a reduction of uptake by the tumour but when co-administered with propanolol-a beta receptor antagonist-produced an improvement in uptake. The supposition being that when noradrenalines beta effects are removed the alpha constricting actions predominate. This conflicting evidence with regard to noradrenaline is found in other studies.

Grady et al (1981) produced similar results in patients with liver tumours, in this instance using the uptake of radioactive microspheres as a measure of blood flow.

Young et al (1979) showed a reduction in the T/N ratio in rats and rabbits with hepatic tumours with noradrenaline.

Vasopressin is a vasopressor agent used in the management of portal hypertension and variceal bleeding. When infused at a low rate it leads to a reduction in portal pressure (and hence its clinical usefulness) but when infused more quickly it leads to portal hypertension secondary to vasoconstriction (Jenkins et al 1984 and 1985). This effect on hepatic haemodynamics has led to a number of groups assessing its use in manipulating hepatic tumour blood flow.

Hemmingway et al (1991) systemically infused vasopressin for 10 minutes into rats with HSN hepatic tumours and demonstrated an increase in tumour blood flow using radiolabelled microspheres.

Hennigan et al (1994) compared the effects of 40 minute hepatic arterial infusions of vasopressin (Vas), endothelin-1 and angiotensin-II (Ag-II) on liver blood flow in non-tumour bearing rats. Blood flow was measured continuously using laser doppler probes. ET-1 was found to have the greatest duration of action, although none of the agents effects lasted throughout their infusions.

The effects on tumour bearing rats using similar techniques was assessed by Dworkin et al (1995). Vasopressin was infused via the hepatic artery and changes in blood flow in both HSN tumour and liver were measured using a laser doppler. Again the effect of vasopressin was limited although it did produce improvements in the T/N ratio. Of more interest is that the addition of a nitric oxide synthase inhibitor, l-*N* - nitro-L-arginine methyl ester (l-NAME) prolonged the action of vasopressin, suggesting nitric oxide release may play a part in reducing the effects of vasopressors. Also of note is that although an improvement in the T/N ratio was achieved, the tumour blood flow was reduced during the infusion suggesting perhaps an element of tumour vessel vasoconstriction or perhaps constriction of 'normal' feeding vessels. This same group using the same model demonstrated that both angiotensin-II and vasopressin produced rapid falls in hepatic and tumour blood flow as measured using laser doppler flowmetry, whilst endothelin-1 produced the greatest duration of action, although the

onset of its effect was delayed. The actions of angiotensin-II and vasopressin were limited in duration despite continuous infusion, with only ET-1 having a prolonged effect. All three vasoconstricting agents improved the T/N ratio (although only ET-1 produced significant effects), with none actually increasing the tumour blood flow overall (Dworkin et al 1997). Dworkin et al (1996) demonstrated, using the same model, that although HAI angiotensin-II improved the T/N ratio this did not lead to significant improvements in tumour drug uptake.

Nitric oxide has been implicated by a number of studies in the control of tumour vascular tone. Wood et al (1993) and Andrade et al (1992) in different tumour models demonstrated that inhibition of nitric oxide synthase led to increased tumour vessel resistance. Tozer et al (1995) using ex-vivo rat carcinomas also showed increased tumour vessel resistance with nitric oxide synthase inhibitors. Given that nitric oxide is predominantly produced by vascular endothelial cells its importance in colorectal liver metastases is debatable given the primitive and incomplete nature of these cells found by Ashraf et al (1997).

Angiotensin-II has been widely used in both animal models (see Hennigan et al 1994 Dworkin et al 1992 above) and patients with hepatic tumours. Burton et al (1985) demonstrated an improved uptake of radioactive microspheres by liver tumours in rats and rabbits using intravenous angiotensin-II with similar results produced by Suzuki et al (1981).

Sasaki et al (1985) infused angiotensin-II into the hepatic artery of patients with both primary and secondary hepatic tumours. Using radioactive isotopes they demonstrated a doubling of tumour uptake and greater than trebling of the T/N ratio. This was compared to intravenous infusions of Ag-II which produced greater systemic hypertension with reduced effects on tumour blood flow.

Hemmingway et al (1992) using intraoperative laser doppler have confirmed this action in patients with colorectal liver metastases. Patients undergoing placement of hepatic arterial catheters underwent a 90 second infusion of Ag-II. The tumour blood

flow increased by up to ten times the baseline during the infusion, with the effects being most prominent in the smaller lesions.

Leen et al (1993) studied patients with colorectal liver metastases who already had hepatic artery catheters in place for regional chemotherapy. They were infused with Ag-II over 90 seconds with changes in hepatic arterial flow measured using an external duplex ultrasound probe before and during the infusion. As hepatic arterial flow is a direct measure of intrahepatic resistance, which itself is secondary to arteriolar tone, the efficacy of the infusion can be assessed. In this case the flow was reduced by 75-80 percent.

An important question is whether increased blood flow leads to greater drug delivery in man. A number of studies have shown improved delivery of a tracer substance when combined with vasopressors. Goldberg et al (1990) injected radiolabelled colloid into the hepatic artery of patients with colorectal liver metastases. When combined with a bolus infusion of angiotensin the uptake was doubled, as determined by external radiocounting. The same group subsequently showed that if angiotensin is given with microspheres into the hepatic artery, a 3 fold improvement in uptake occurs as determined by tumour analysis after excision.

The question that remains unanswered from these experiments is whether an increased uptake of agents by the tumour corresponds to an improved response? Bloom et al (1987) showed an improved level of tumour cell death when intraportal adrenaline was combined with doxorubicin in a Walker carcinoma innoculated rat model.

Goldberg et al (1990) in a phase 2 clinical trial gave patients bolus treatments of intrahepatic arterial angiotensin-II, microspheres and 5FU and showed some improvement over chemotherapy alone. Anderson et al (1992) administered glass yttrium-90 microspheres [a beta radiation emitter] with Ag-II via tha hepatic artery in patients with colorectal liver metastases. Of the 7 patients studied, 6 developed extrahepatic disease with a median survival of 11 months.

Before entering into a clinical control trial of vasoactive agents to augment HAC, the pharmacokinetic profile of these agents needs to be assessed. The comparibility of the animal models used so far to the human situation are open to speculation. It is already known that vessels within the same tumour are markedly heterogenous and it is likely that the physiological and anatomical profile of individual tumour vessels pocess similar differences.

Conclusion

Each of the studies mentioned have used varying doses of vasopressor agents infused over different periods of time. Many of the studies have utilised systemic administration which is known to produce greater systemic hypertension and less improvement in tumour blood flow. The degree of systemic hypertension reported in most publications would be unacceptable in a clinical setting.

The MC28 tumour when developed by intraportal inoculation in Hooded Lister rats, as already discussed, possesses blood vessels with an almost identical profile to those found in colorectal liver metastases and therefore offers a representative model wih which to study the effects of HAI vasoconstrictors.

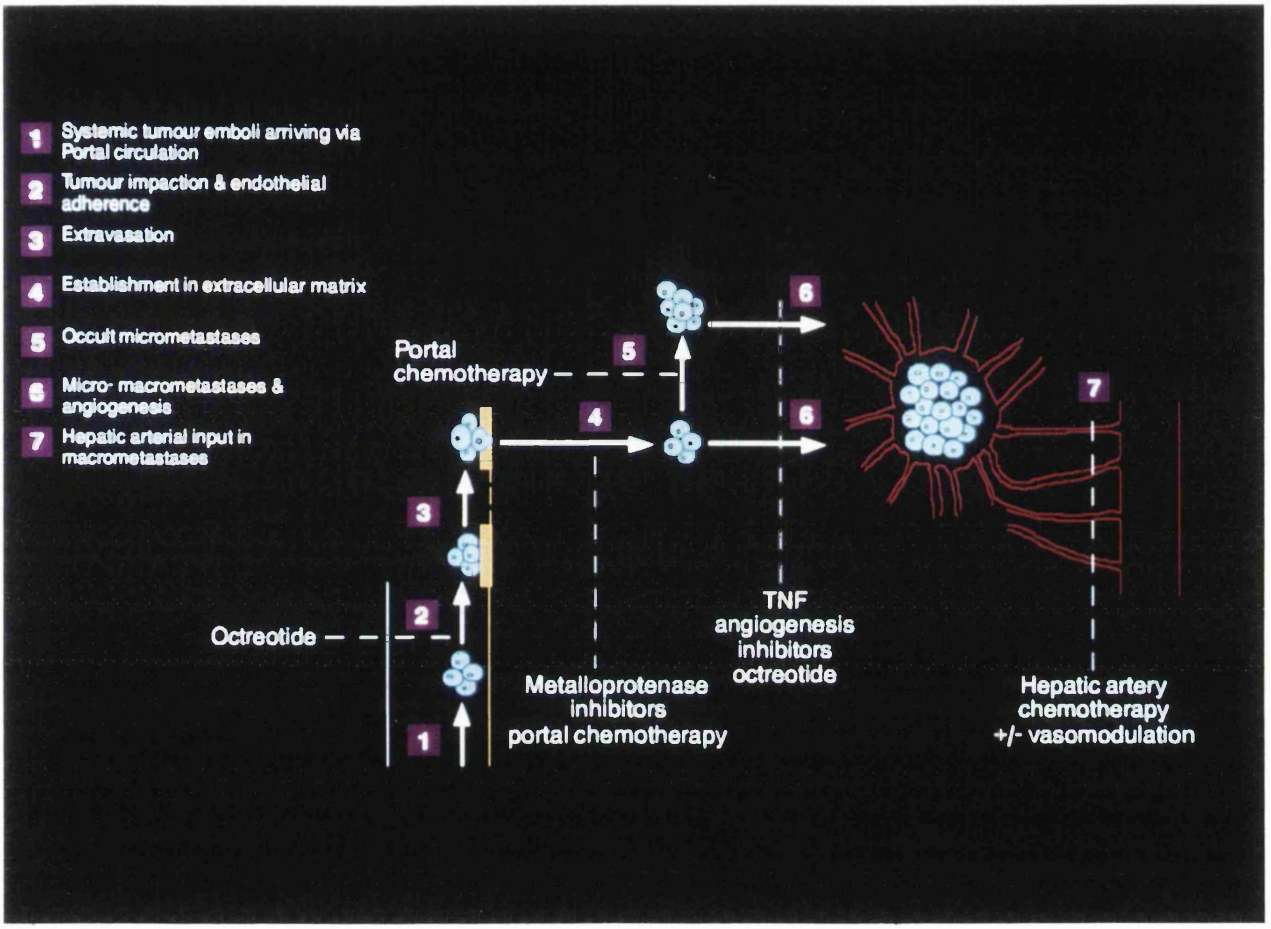


Figure 3: Diagram illlustrating the sites of possible therapeutic intervention in both prophylaxis and treatment of colorectal liver metastases.

Chapter II

The manipulation of blood flow through liver metastases in a animal model

Introduction

Hepatic arterial chemotherapy produces a higher locoregional response rate than conventional systemic chemotherapy in patients with colorectal liver metastases. Initial clinical studies suggest this may translate into improved survival (Chang et al, 1987; Martin et al, 1990) and enhanced quality of life (Allen-mersh et al, 1990). The rationale behind this route of therapy is that liver metastases receive up to 95% of their blood supply via the hepatic artery, hence a greater proportion of a chemotherapeutic agent will be delivered to the tumour.

Further improvements in drug delivery might be achieved by a greater understanding of the differences that exist between tumour and normal hepatic vessels.

It has been previously demonstrated -using electron microscopy- that blood vessels within colorectal liver metastases lack both innervation and a developed smooth muscle coat (Ashraf et al, 1996). As such these 'tumour' blood vessels are unlikely to possess any significant neurogenic vasoconstrictor capability.

Appreciation of this observation may allow selective vasoconstriction of normal hepatic vessels whilst leaving tumour vessels unaffected. This in turn should lead to shunting of blood into the tumour with a concomitant improvement in drug delivery. Regionally infused vasopressors improve the uptake of chemotherapeutic agents by liver metastases presumably by enhancing tumour blood flow (Hemmingway et al, 1993) . However their short duration of action is a limiting factor. In this chapter an animal model of colorectal liver metastases is used to assess the mechanism and efficacy of regionally infused vasopressors . The blood vessels within the tumour have a similar anatomical and physiological profile to those in human colorectal liver metastases (Loesch et al, 1997; Ashraf et al, 1997).

In **section I** the blood flow in the liver of healthy Hooded Lister rats is manipulated using hepatic arterially infused vasopressors. In addition to investigating the effects on blood flow and blood pressure this section also addresses the standardisation of the

model which is essential to obtaining reproducible results in the subsequent experiments.

Section II deals with measuring changes in blood flow through both liver metastases and adjacent normal liver to assess whether such changes in blood flow translate into changes in uptake of radiolabelled 5-fluorouracil by both the tumour and liver.

Aims of study

1. To determine the effect of hepatic arterial infusion of vasoconstrictors on liver blood flow in non-tumour bearing animals.

2. To determine the effect of a standard dose of the same hepatic arterially infused vasoconstrictors on hepatic tumour and adjacent liver blood flow .

3. To determine whether hepatic arterially infused vasoconstrictors affects the uptake of radiolabelled 5-FU into both normal liver and hepatic tumours in an animal model.

Section I- The manipulation of hepatic blood flow through the liver of Hooded Lister rats by hepatic arterially infused vasopressors.

2.1 Methods

2.1.1 Hepatic arterial cannulation

Male Hooded Lister rats weighing between 350-400g were used for all the experiments and were fed a diet of rat pellets and water ad libitum.
All animals underwent induction of anaesthesia with 3% halothane and were maintained on 1.5% halothane and 5L/min oxygen administered via a face mask.
The animals were laid upon a heated mat to ensure a consistent core temperature, which was confirmed initially using a rectal thermometer.
Once adequately anaesthetised, the carotid artery was cannulated as described below and continuous readings were recorded on a computer logging system.
A upper transverse abdominal incision was then performed and cannulation of the gastroduodenal artery was performed using a similar technique to that described by Leivested and Malt (1973) as illustrated in figure 4.
Cotton wool was used to pack the liver cranially, providing access to the lesser omentum. Artery forceps were then applied to the first part of the duodenum and used to retract the stomach caudally. This allowed the common hepatic artery and its branches to be placed under tension, which in turn provided greater access to the gastroduodenal artery. Cotton wool buds were then used to sweep the fascia from the vessel and identify its origin. The distal portion of the gastroduodenal artery was then lifted off the underlying tissues and tied with a 6/0 silk ligature. An artery forcep was then applied to the tie, enabling the vessel to be retracted and a further ligature placed under the vessel but not tied. A cotton wool ball soaked in 5% procaine was then placed on the vessel and left for 5 minutes to induce vasodilatation.

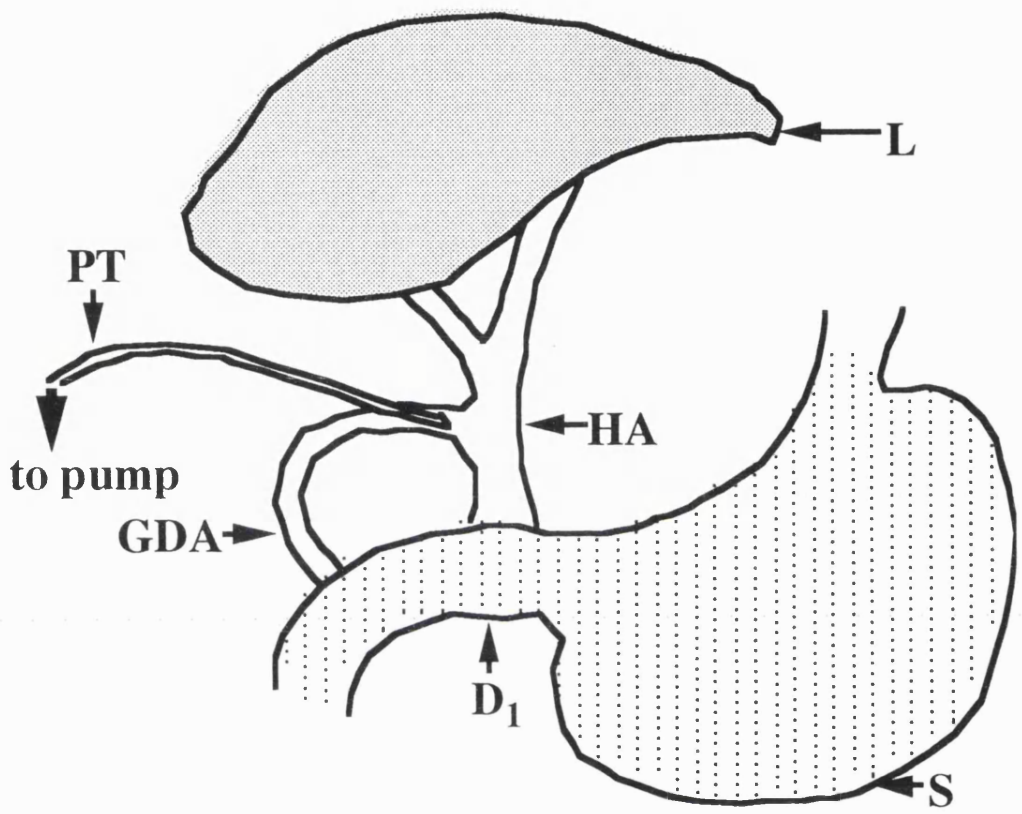


Figure 4: Diagram illustrating the principle of gastroduodenal artery cannulation in the animal model.

Gastroduodenal artery (GDA), hepatic artery (HA), first part of duodenum (D_1), portex tubing (PT), stomach (S) and liver (L).

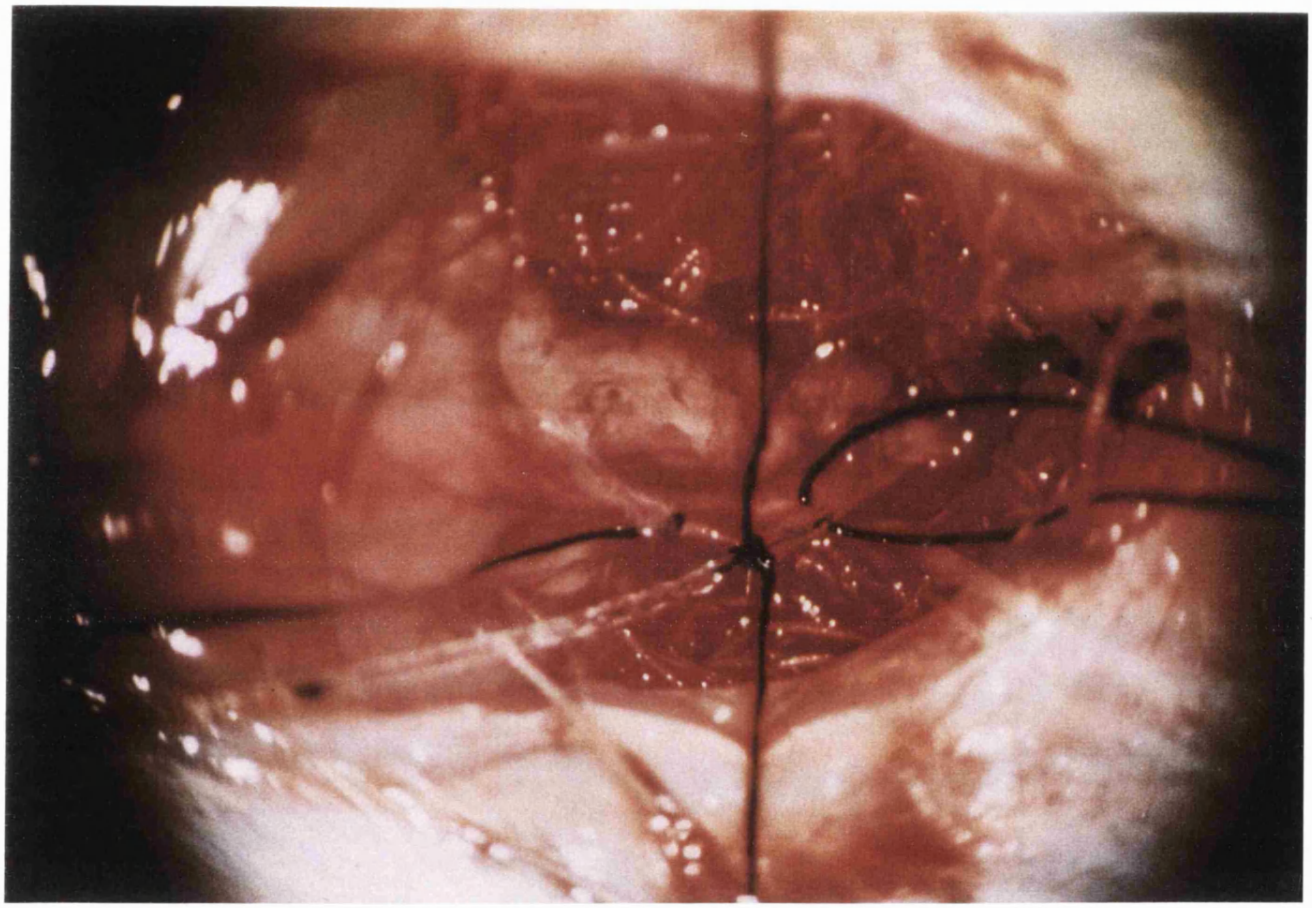


Figure 5: Photograph demonstrating the cannulation of the hepatic artery in the animal model through the operating microscope.

Using an operating microscope a fine curved forcep was placed under the vessel allowing flow to be controlled and a small hole produced using a 27G needle as in figure 5. A bevelled 2/0 gauge portex (Portex, UK) tube (which had previously been stretched to approximately half its original diameter) was then introduced and tied in such a position that it did not affect flow through the hepatic artery.

One ml of normal saline (warmed to core temperature) was then flushed into the vessel to ensure patency and distribution throughout the hepatic artery. In addition the presence of adequate pulsatile backflow was confirmed.

2.1.2 Carotid artery cannulation

A transverse incision was made in the anterior triangle of the neck on the right side at the base of the sternocleidomastoid muscle. The carotid artery was then dissected free and untied ligatures placed distally and proximally to allow control of blood flow prior to vessel puncture.

A blood pressure monitor (Harvard instruments, UK) was used to measure the mean systemic arterial blood pressure and was coupled to the Mac lab computer (Maclab, UK) to provide a continuous readout. Prior to each experiment the machine was recalibrated and set to zero.

The carotid artery was then punctured using a 27G needle, while blood loss was prevented using the untied slings placed previously. A heparinised saline filled 2/0 gauge portex tube, which was connected to the pressure transducer via a luerlocked membrane, was then inserted into the puncture site and tied in place. A series of readings were then taken to ensure a constant baseline.

2.1.3 Laser doppler flowmetry

Laser doppler flowmetry allows measurement of blood flow within the microcirculation without interfering with it. This technique has been used in a number of studies and found to provide an accurate and sensitive measure of both tumour and hepatic blood flow (Ackerman et al 1988, Dworkin et al 1995, Dworkin et al 1997).

The principle of its action relies upon the fact that laser light when reflected from perfused tissue undergoes a doppler shift in its wave form. The degree of shift is determined by both the velocity and density of red blood cells within the sample volume which is expressed as flux units (an arbitary measure of flow) on the monitor. The instrument used in this study was an MBF3D (Moor Instruments Ltd, UK) laser doppler flowmeter (wavelength 780-820nm and 15KHz bandwidth) with a dual channel source which permits the simultaneous operation of two probes (30 x 1mm). The probes emit laser light over a 1mm radius (Acker et al 1990) which allow measurement of blood flow to a depth of 1-2mm. In order to minimise movement artefact (especially that due to respiratory movement) a time constant of 3.0 seconds was used with a frequency of 0.25Hz.

The probes were placed in specially designed holders so that the tip of the probe was allowed to rest on the liver surface, whilst still being able to visualise the area of contact via a window cut in its base (figure 6). Each probe was calibrated before every experiment using a standard solution of polystyrene spheres in solution. Adhesion was maintained by surface tension between the liver surface and the probe holder, utilising the film of fluid between the two surfaces.

In order to reduce and standardise the pressure of the probe holder and hence the probe tip-which lay flush with the holder base-on the surface, it was suspended from a clamp above the animal, with the probe wire kept at the same length.

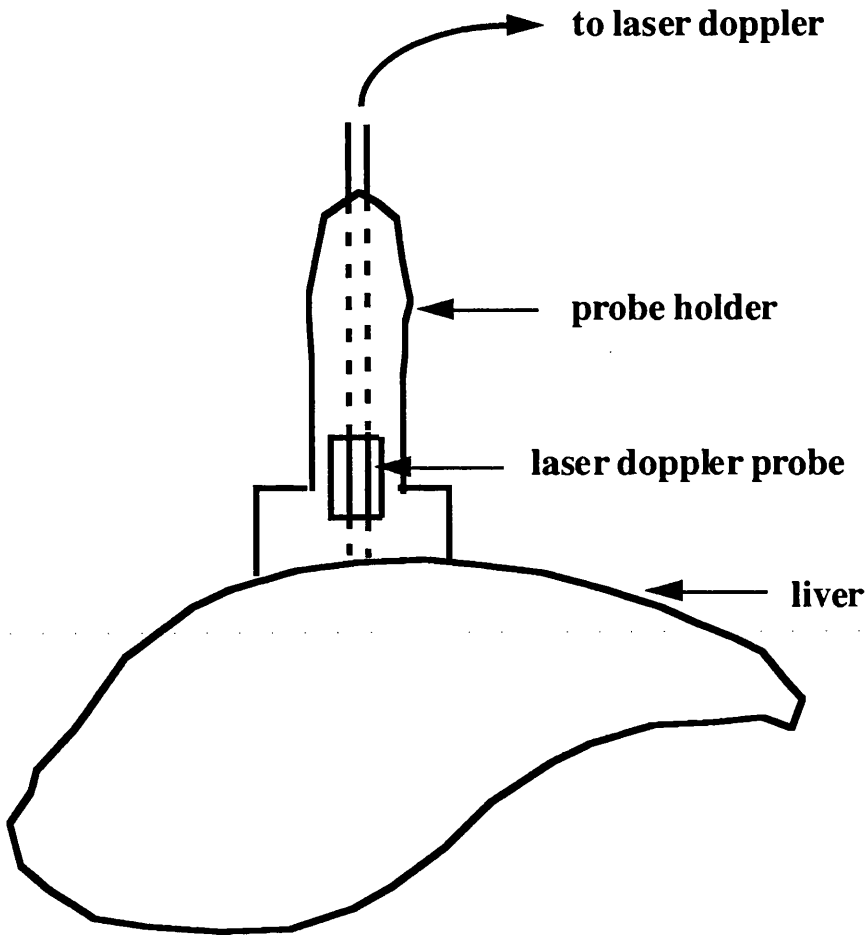


Figure 6: Diagram illustrating placement and design of the laser doppler probe holder on the liver surface.

2.1.4 The hepatic arterial infusion of vasoactive agents

Each animal underwent gastroduodenal and carotid artery cannulation (as described previously) to allow infusion of a pharmacological agent into the hepatic arterial circulation, whilst measuring systemic blood pressure. Each animal received one agent only.
Animals were divided into six groups as follows;

Group 1: Saline only (n=6)
Group 2: Angiotensin II (n=24) dose range= 0.2 - 0.45micrograms/kg/min
Group 3: Endothelin -1 (n=24) dose range= 2 - 8micrograms/kg/min
Group 4: Noradrenaline (n=24) dose range= 2.5 -10micrograms/kg/min
Group 5: L-Nitro-n-Arginine (n=24) dose range= 0.5 -2milligrams/kg/min
Group 6: Vasopressin (n=24) dose range= 0.6 - 1.2micrograms/kg/min

All chemicals were obtained from Sigma, Poole, UK, unless otherwise stated.

Each group (apart from the saline group) was then subdivided into four further groups(n=6 in each), and received a different dose of the same drug.
All agents chosen have previously been used in similar studies in different tumour models to assess effects on hepatic tumour and liver blood flow (Ackerman et al 1988, Bloom et al 1987, Burton 1987, Burton et al 1985, Carter et al 1992, Dworkin et al 1995, Dworkin et al 1996, Hemmingway et al 1991, Hennigan et al 1993, Hennigan et al 1994). Each agent is known to produce vasoconstriction in the hepatic vascular bed, although with differing potency and duration of action. The doses chosen were based on those used in previous studies, with special consideration given to the fact that an ideal dose should not produce more than a 20% rise in systemic blood pressure.

Reasons for the use of agents chosen in this study

Angiotensin-II has been given via the hepatic artery in a number of studies on both hepatic tumours in animal models and in human colorectal liver metastases. This has led to variable changes in both tumour blood flow and drug delivery depending on the study (Burton et al 1985, Carter et al 1992, Dworkin et al 1996, Hennigan et al 1994). Given its wide use in other studies it was applied here, since some have shown an improvement in tumour blood flow and drug delivery.

Noradrenaline is one of the most potent physiological vasoconstrictors known and has been shown to produce improvements in hepatic tumour blood flow and drug delivery (Ackerman et al 1988, Burton 1987, Hafstrom et al 1980). In addition Ashraf et al (1996 and 1997) demonstrated an absence of sympathetic innervation in colorectal liver metastases. Given that noradrenaline is a sympathetic transmitter, its absence in the tumour might lead to a profound effect on liver blood flow whilst leaving the hepatic tumour unaffected(see shunting theory described earlier) and was therefore included in the study.

Endothelin-1 is a potent, long acting vasoconstrictor agent, produced by vascular endothelial cells and produces changes in both liver and hepatic tumour blood flow (Hennigan et al 1994, Dworkin et al 1997). In addition since Ashraf et al (1996 and 1997) demonstrated an absence of a developed endothelium within colorectal liver metastases, its receptors might also be down - regulated in such tumours. As with noradrenaline ET-1 might therefore also produce improvements in tumour blood flow by the same mechanism and was therefore included in the study.

Nitric oxide is produced by vascular endothelial cells and mediates vasodilation via a second messenger. Inhibition of its production leads to vasoconstriction and possible improvements in tumour blood flow as above. As the tumour lacks an endothelial cell layer, inhibition of nitric oxide production should not affect tumour blood flow and hence might improve tumour blood flow via the shunting phenomenon and was therefore used in the study. Dworkin et al (1995) have already demonstrated such an

effect using a nitric oxide synthase inhibitor in an animal model of colorectal liver metastases.

Vasopressin is a recognised vasoconstrictor agent which is routinely used in the management of variceal bleeding. It has also been shown to produce changes in liver and hepatic tumour blood flow over a prolonged period relative to other vasoconstricting agents (Hennigan et al 1994, Dworkin et al (1995). Given its potent longlasting effects on hepatic tumour blood flow vasopressin was also used in the study.

Technique

The changes in liver blood flow were measured using a laser doppler probe placed on the left lobe of the liver (having previously demonstrated that flux measurements change uniformly throughout all lobes). All agents were dissolved in 0.9% saline and each infusate was prepared immediately prior to infusion. All samples were kept in a water bath in order to maintain at rat core temperature. Failure to do this results in vasoconstriction secondary to cooling, independent of any intrinsic vasoconstrictor action the agent may have.

The laser doppler probes were placed on the surface of the left lobe of the liver and connected to the data logging system as previously described.

Once in place the abdomen was closed in two layers with 3/0 silk to prevent drying and cooling of the liver surface. Failure to do this results in a steady spontaneous reduction of flux values regardless of infusion, secondary to vasoconstriction, drying of the surface and cooling, with subsequent falls in hepatic microcirculatory blood flow.

The flux measurements were recorded on a data logging system as above. A 5 minute consistent baseline was obtained prior to commencement of the infusion. Each infusion then lasted 30 minutes (all agents being dissolved in normal saline) in a volume of 1ml, with a further 5 minute recording after this point. On completion of the study the animals were killed by cervical dislocation.

2.2 Results

Five vasoactive agents, at different doses, were infused over 30 minutes in normal rat livers via the hepatic artery and their effect on blood flow was investigated and compared to normal saline. The typical flux patterns seen in the liver during the hepatic arterial infusion are illustrated in figures 7 and 8.

For each animal a number of different parameters were calculated (as demonstrated in figure 7). These were;

a) the maximum flux drop from the baseline (expressed as a percentage of the baseline)

b) the time taken to return to the original baseline once the infusion was commenced(which in each case was less than the 30 minute infusion period)

c) the area between the flux line and the original baseline over the period the vasopressor has its effect (area 1) expressed as a percentage of the entire area (ie area 1/area 1+area 2 x 100) over the 30 minute period

d) the maximum blood pressure rise obtained (expressed as a percentage of the baseline) was also determined.

For each agent a different pattern of flux drop was obtained as illustrated in figure 8. All the agents apart from l-NAME produced a flux drop followed by a recovery over a variable time back to the baseline. l-NAME on commencement of the infusion did not produce a flux drop initially but later produced a flux drop which lasted throughout the duration of the infusion. If the l-NAME infusion was stopped at any stage the flux line would return to the baseline.

These values are all demonstrated on the dose response curves illustrated in figures 9-28.

To perform further experiments on the tumour vasculature it was necessary to choose a dose for each of the vasopressors studied which would produce no more than a 20%

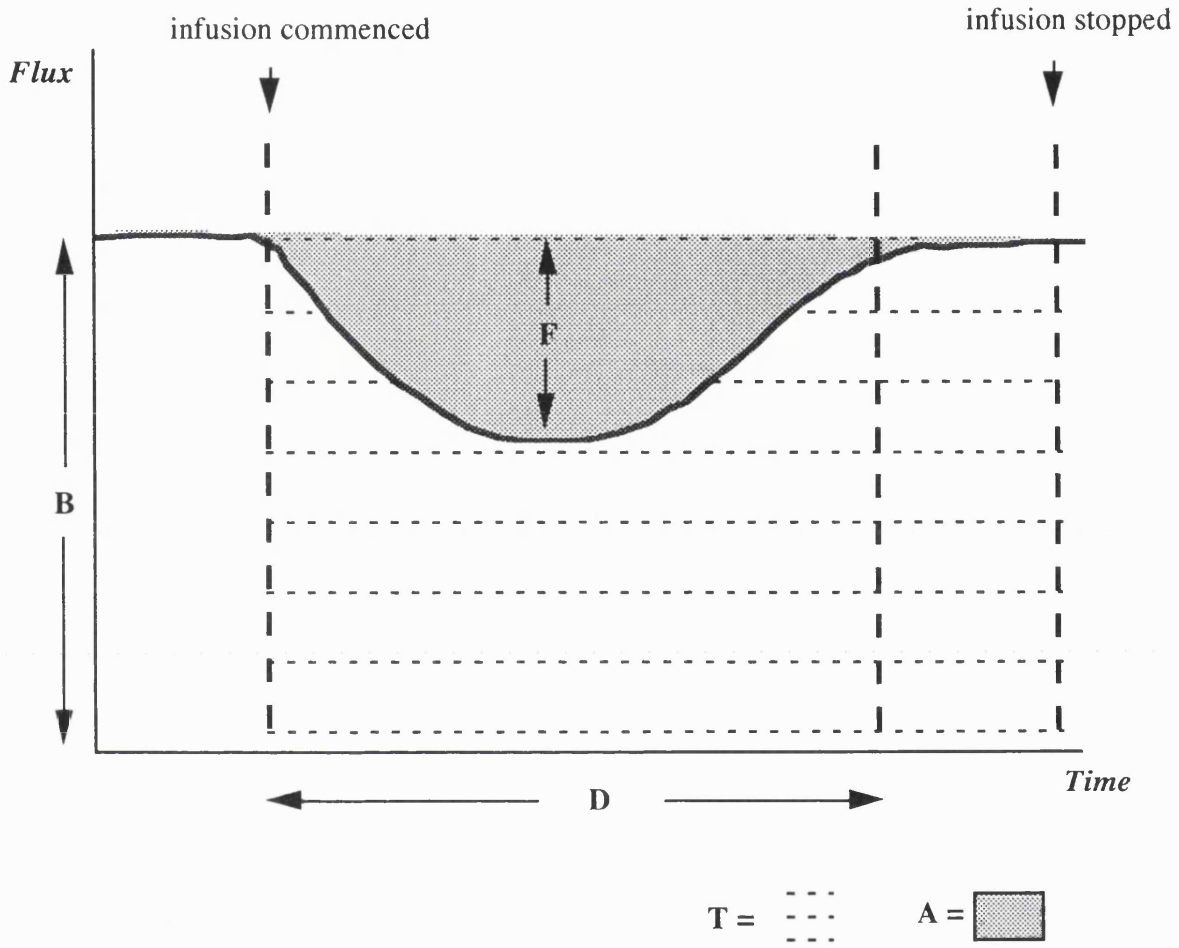


Figure 7: Schematic representation of typical flux pattern during infusion of hepatic arterial vasoconstricting agents in non-tumour bearing animals.

The maximum flux drop is labelled F, baseline flux labelled B (with the % flux drop calculated by F/B x 100), duration of action labelled D and area change produced by vasoconstrictors is the shaded area labelled A and the total area, if there had been no infusion, is the area under the flux line (T) plus A, therefore % area change = [A/(T+A)] x 100.

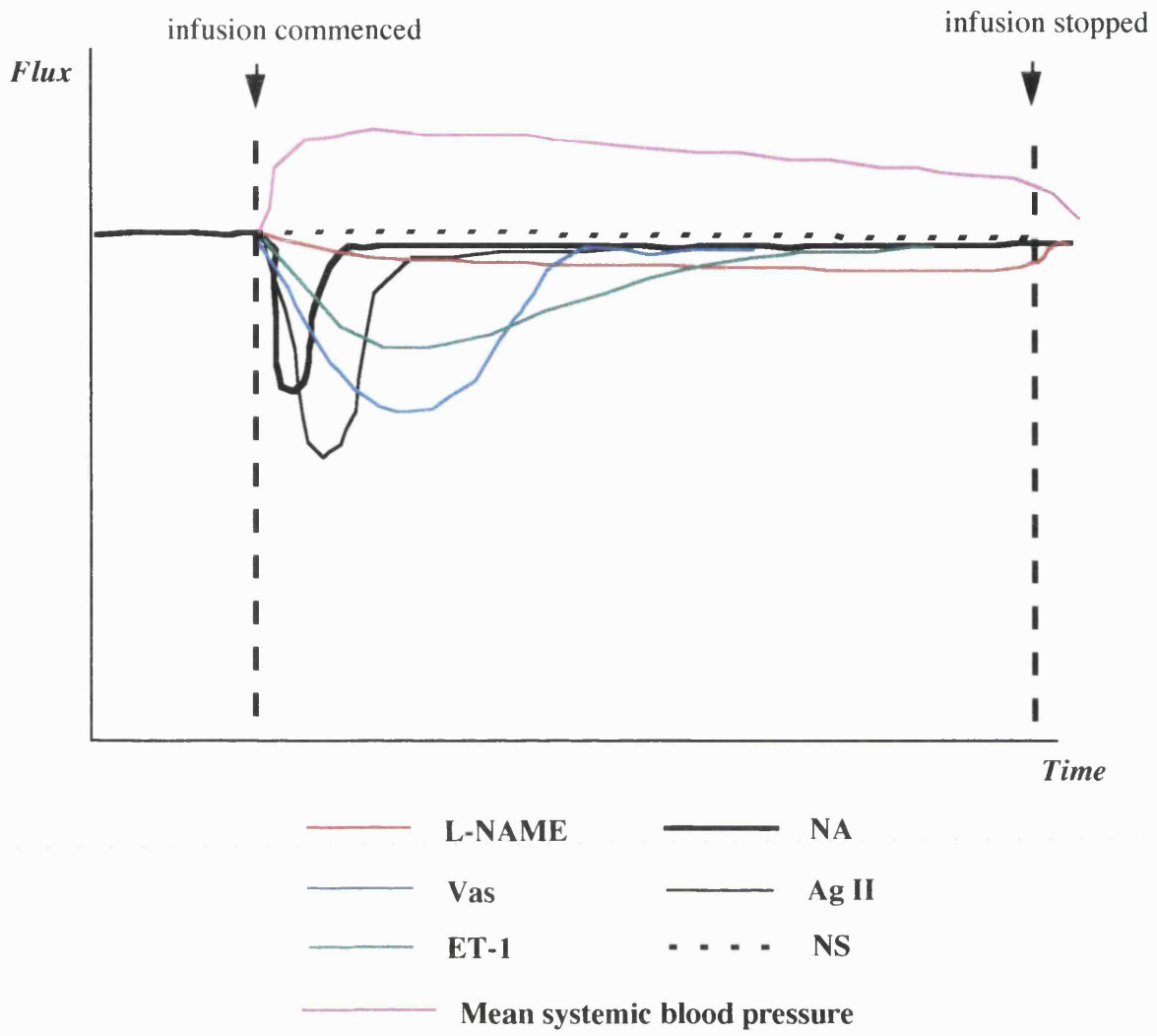


Figure 8: Schematic representation of typical flux patterns seen during the hepatic arterial infusion in non-tumour bearing animals for the various agents studied.

rise in systemic blood pressure. This threshold was felt to be the maximum level of sustained hypertension which would be acceptable. These doses are;

vasopressin 1mcg/kg/min
noradrenaline 5mcg/kg/min
angiotensin-II 0.35mcg/kg/min
l-NAME 1.5 mg/kg/min
endothelin-1 0.5mcg/kg/min

Graphs comparing the results obtained using these doses are also shown for duration, maximum flux drop and % area change (figures 29-31).

For the optimum doses the agent with the greatest duration (mean ± SDmins) was l-NAME 24.8 ±1.7, ET-1 21.2±1.9, Vas 12.6±1.4, AgII 4.8±0.7 and NA 2.4±0.6.
The maximal flux drop recorded(mean±SD% of baseline) was for AgII 43.7± 4.86, Vas 37.2±5.6, NA 29.5±2.6, ET-1 22.1± 2.8 and l-NAME 5.2±0.6.
The agent producing the greatest % area change over the 30 minute period (mean±SD%)was Vas 13±2.4, joint second were ET-1 and l-NAME with 7.2±1.3 and 7.2±1.8 respectively, AgII 4.4±1.36 and NA 2.7±0.8.
Saline overall produced no significant change in flux over the period studied (as represented on the idealised flux diagram).
For these doses there was no significant difference between the blood pressure changes produced by these agents giving values approximately 20% above the baseline ($p>0.05$). The blood pressure elevation produced by the vasopressors in all the infusions lasted until the end of the infusions and did not follow the pattern of flux changes seen.

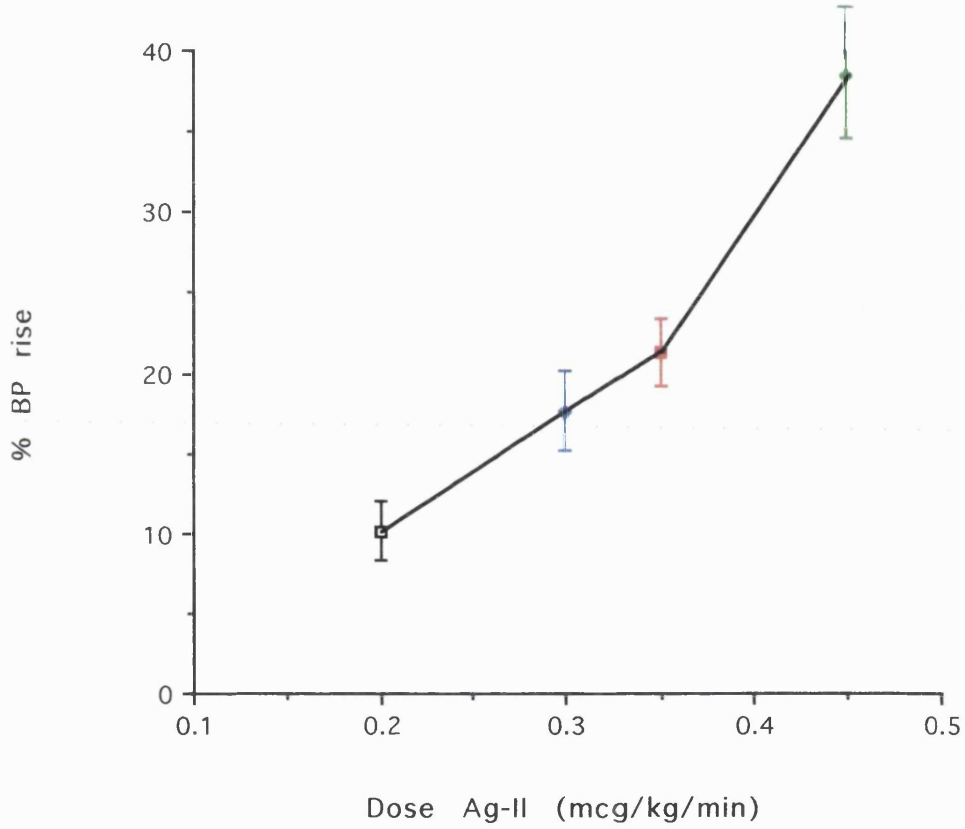


Figure 9: Dose curve for % BP rise due to HAI Ag-II in non-tumour bearing animals.

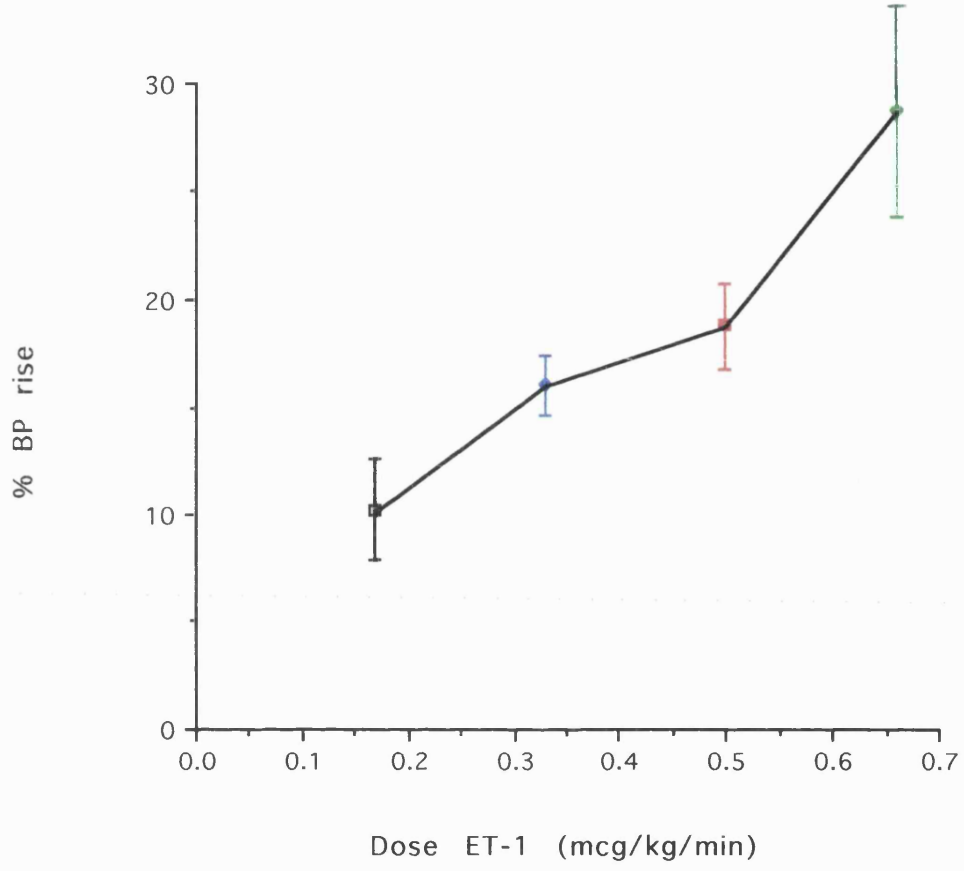


Figure 10: Dose curve for % BP rise due to HAI ET-1 in non-tumour bearing animals.

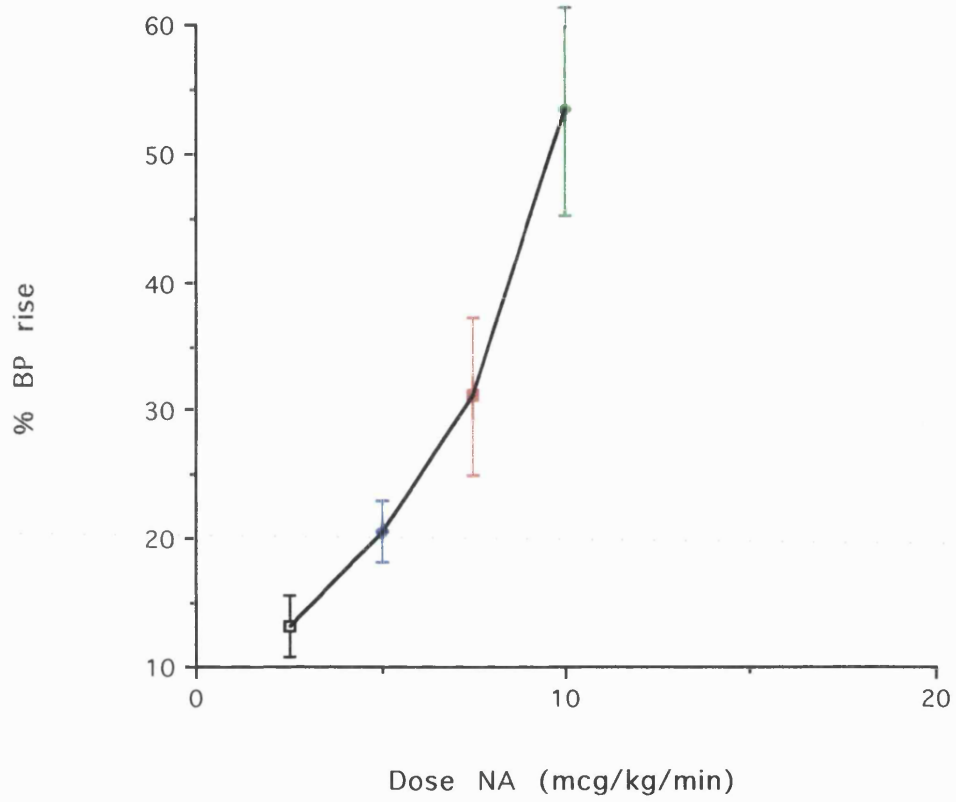


Figure 11: Dose curve for % BP rise due to HAI NA in non-tumour bearing animals.

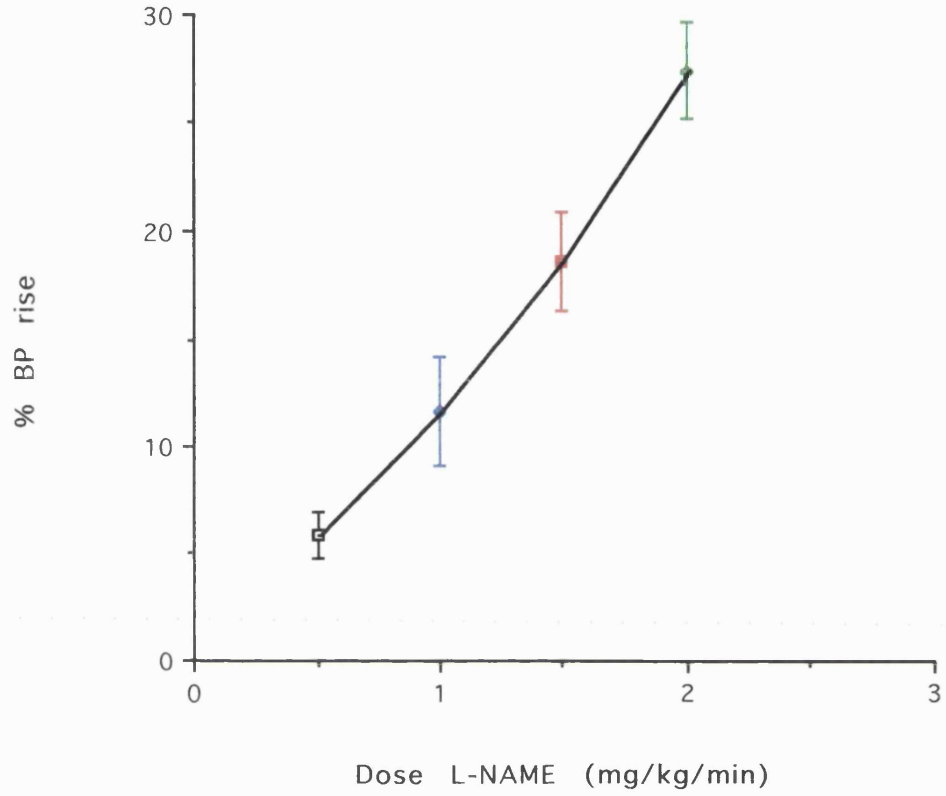


Figure 12: Dose curve for %BP rise due to HAI L-NAME in non-tumour bearing animals.

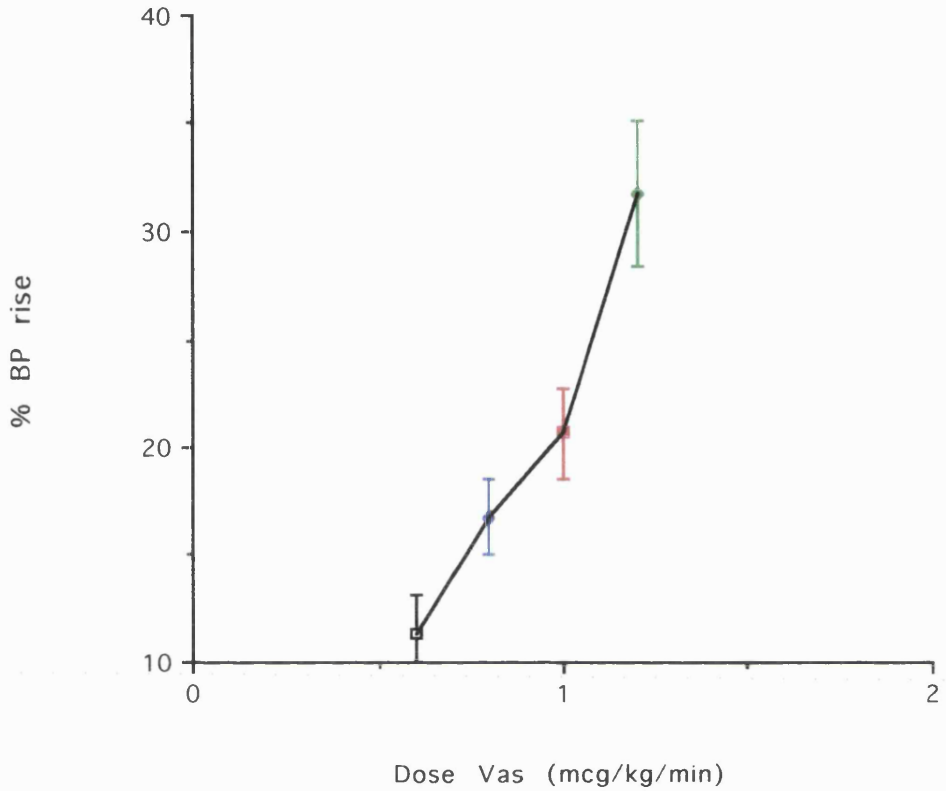


Figure 13: Dose curve for %BP rise due to HAI Vas in non-tumour bearing animals.

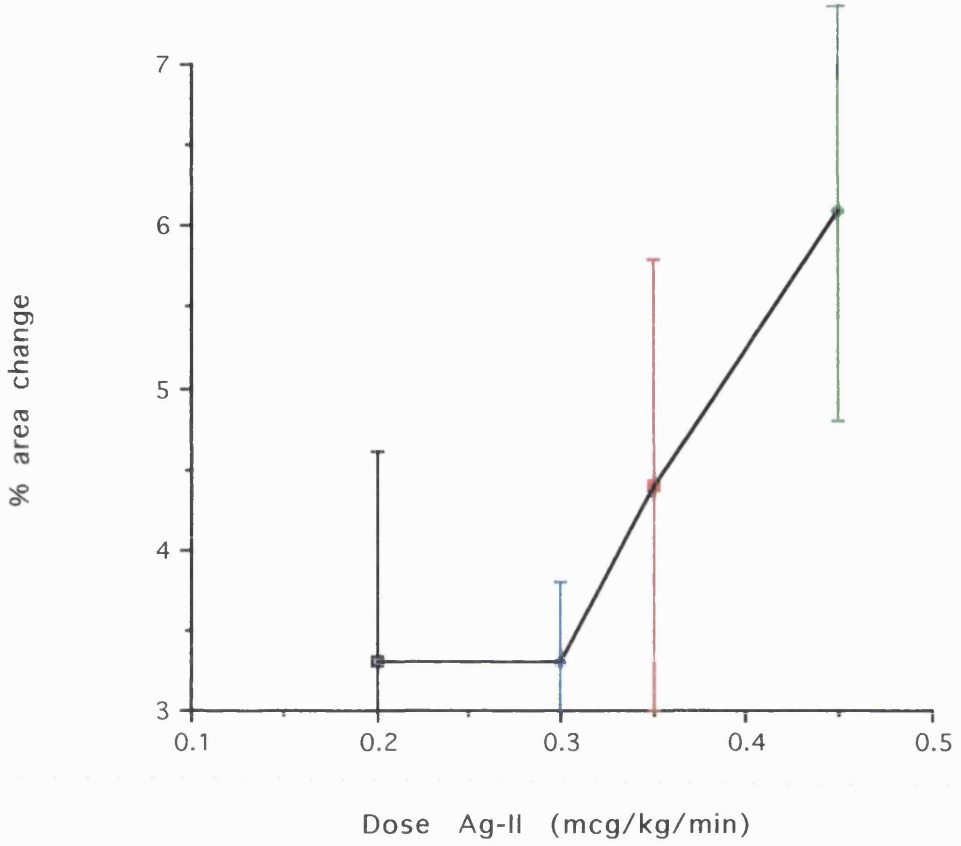


Figure 14: Dose curve for % area change due to HAI Ag II in non--tumour bearing animals.

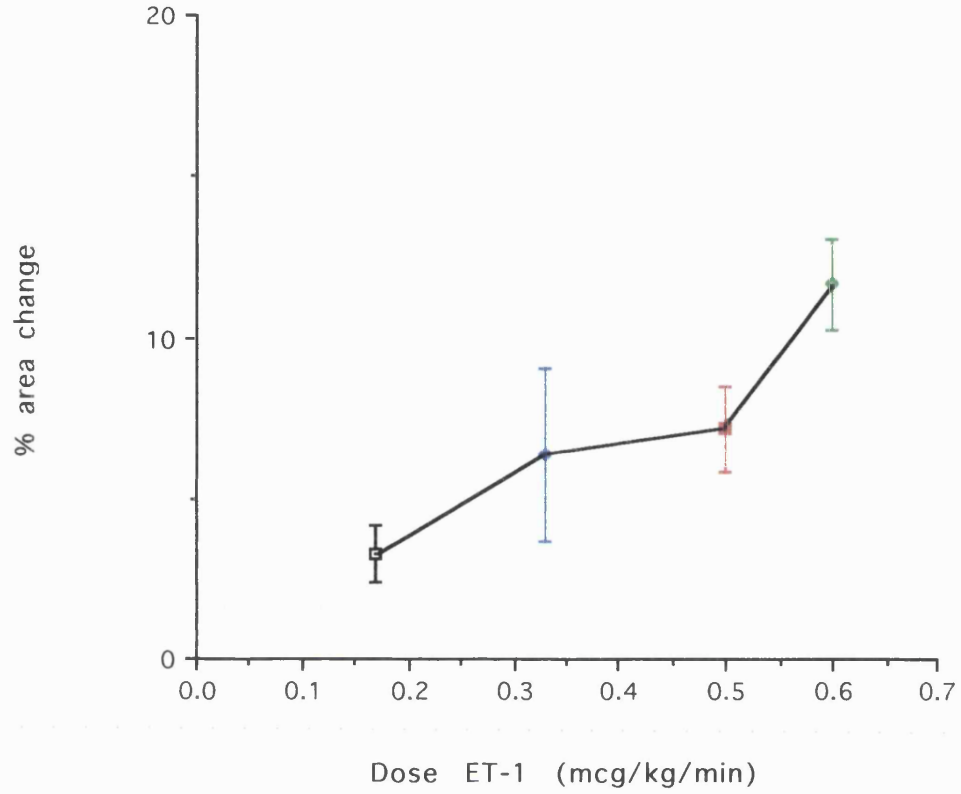


Figure 15: Dose curve for % area change due to HAI ET-1 in non-tumour bearing animals.

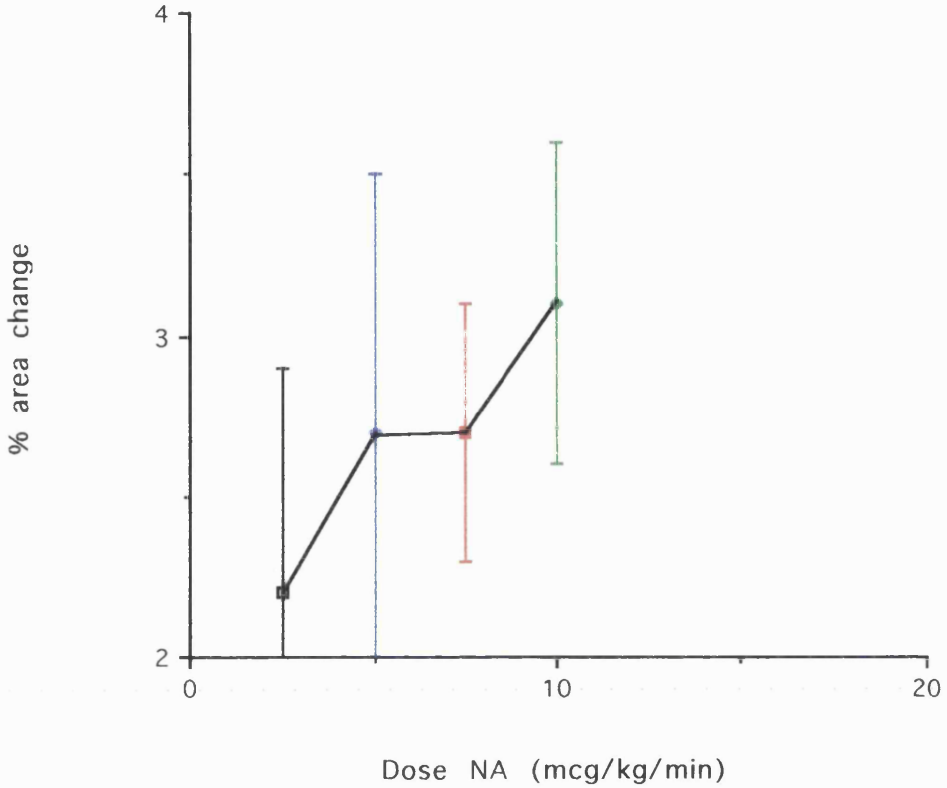


Figure 16: Dose curve for % area change due to HAI NA in non-tumour bearing animals.

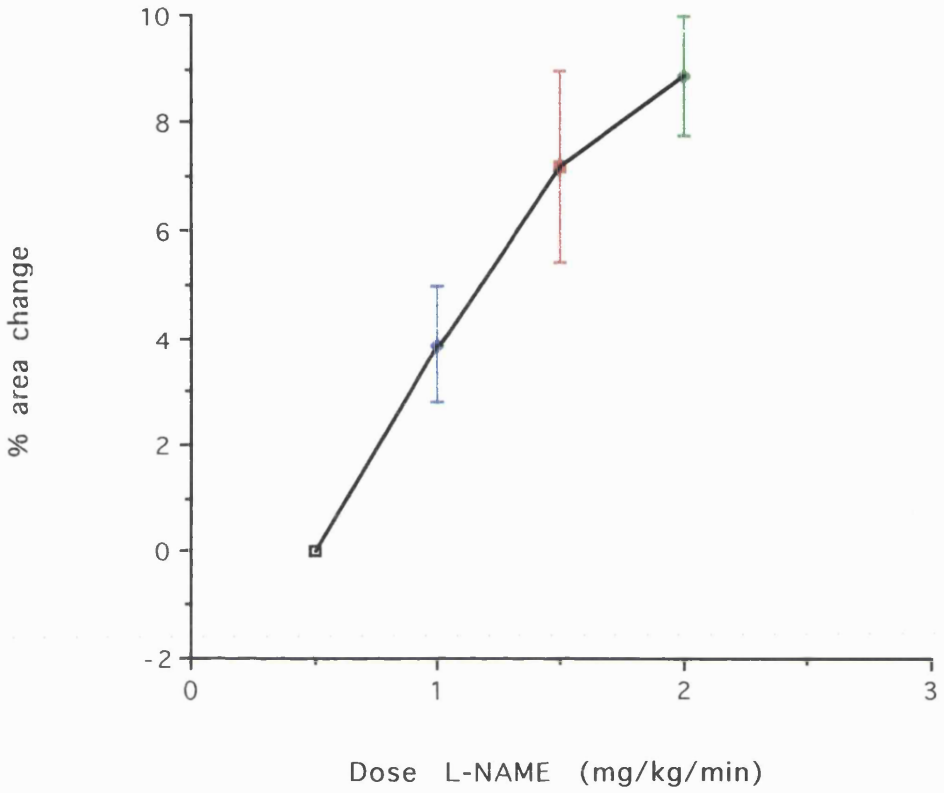


Figure 17: Dose curve for % area change due to HAI L-NAME in non-tumour bearing animals.

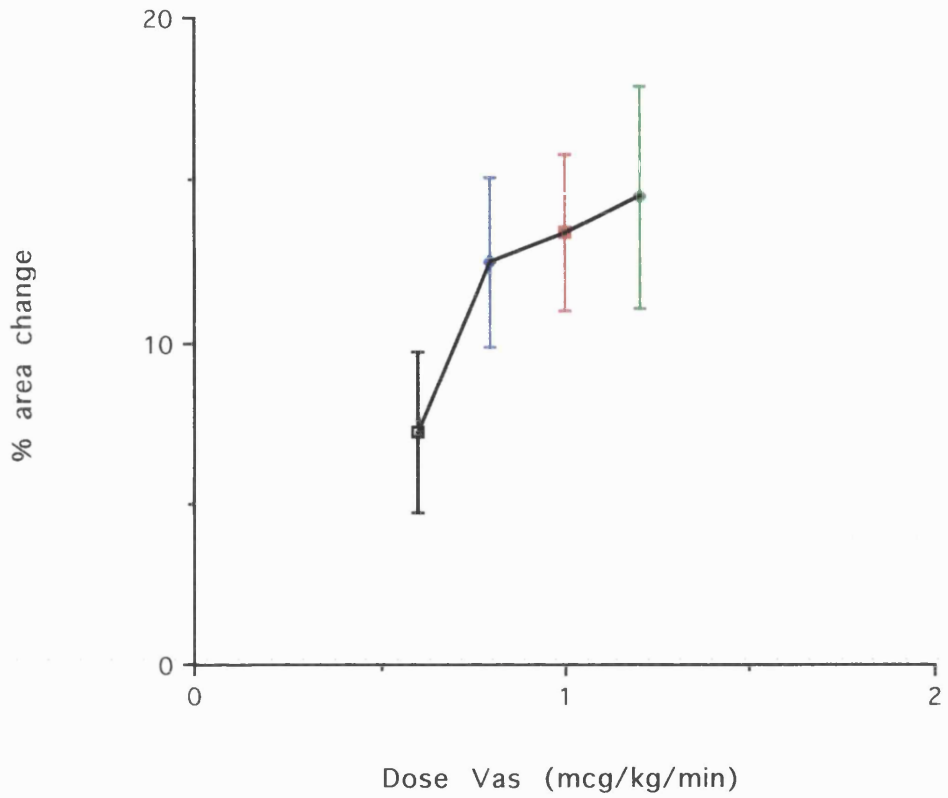


Figure 18: Dose curve for % area change due to HAI Vas in non-tumour bearing animals.

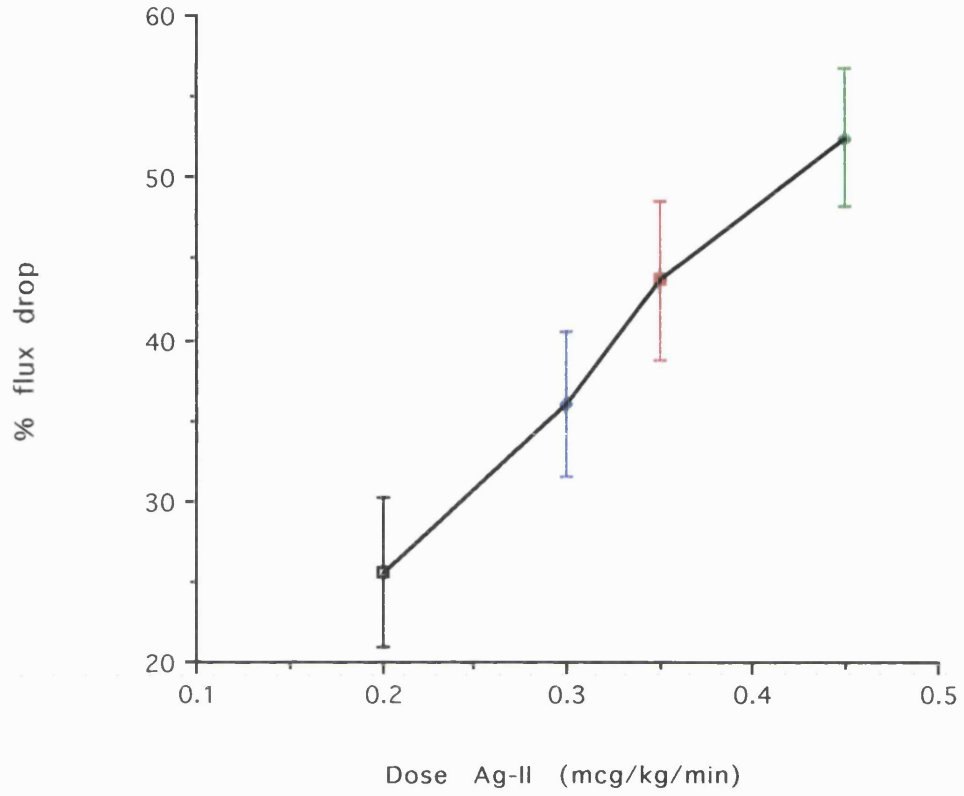


Figure 19: Dose curve for maximal % flux drop due to HAI Ag-II in non-tumour bearing animals.

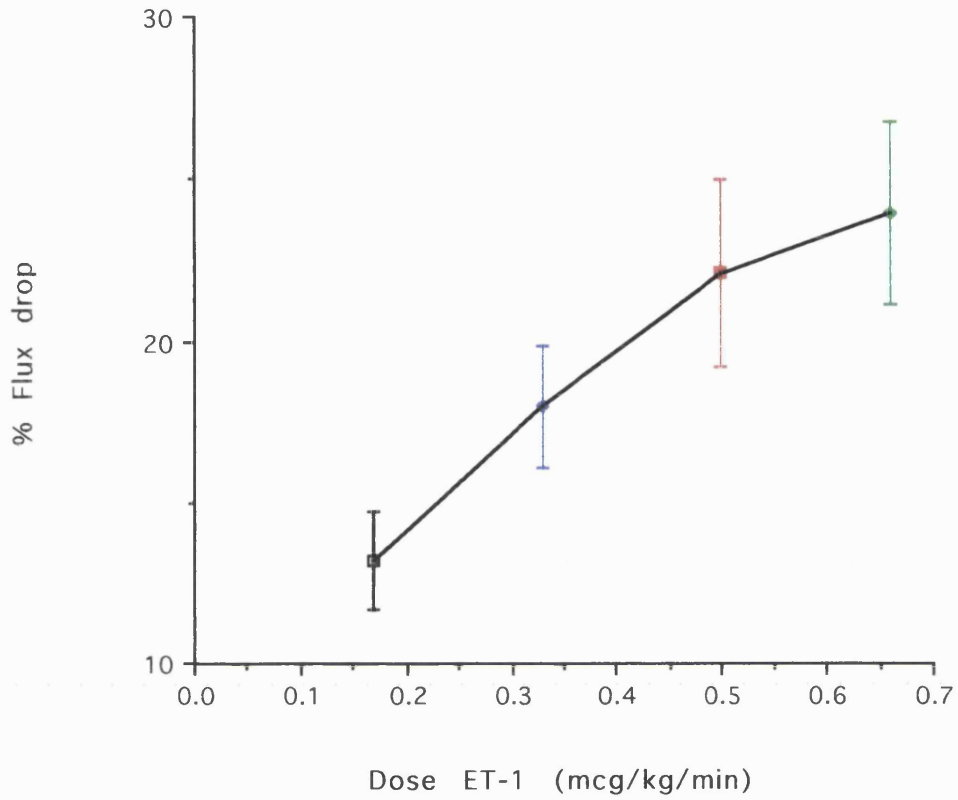


Figure 20: Dose curve for maximal % flux drop due to HAI ET-1 in non-tumour bearing animals.

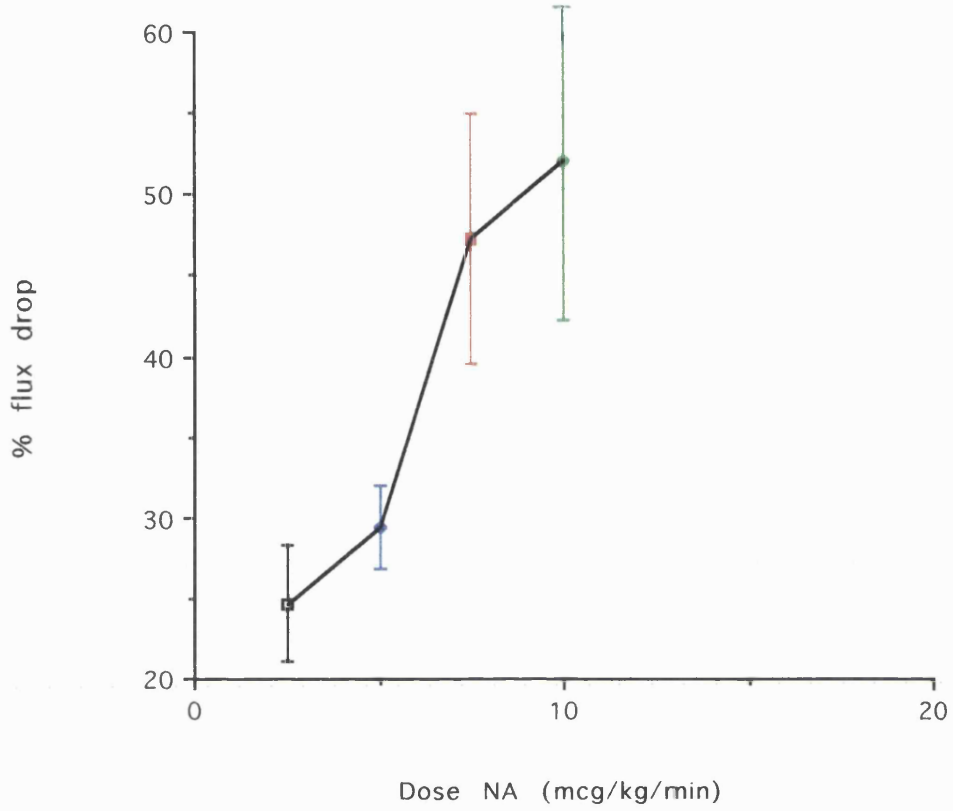


Figure 21: Dose curve for maximal % flux drop due to HAI NA in non-tumour bearing animals.

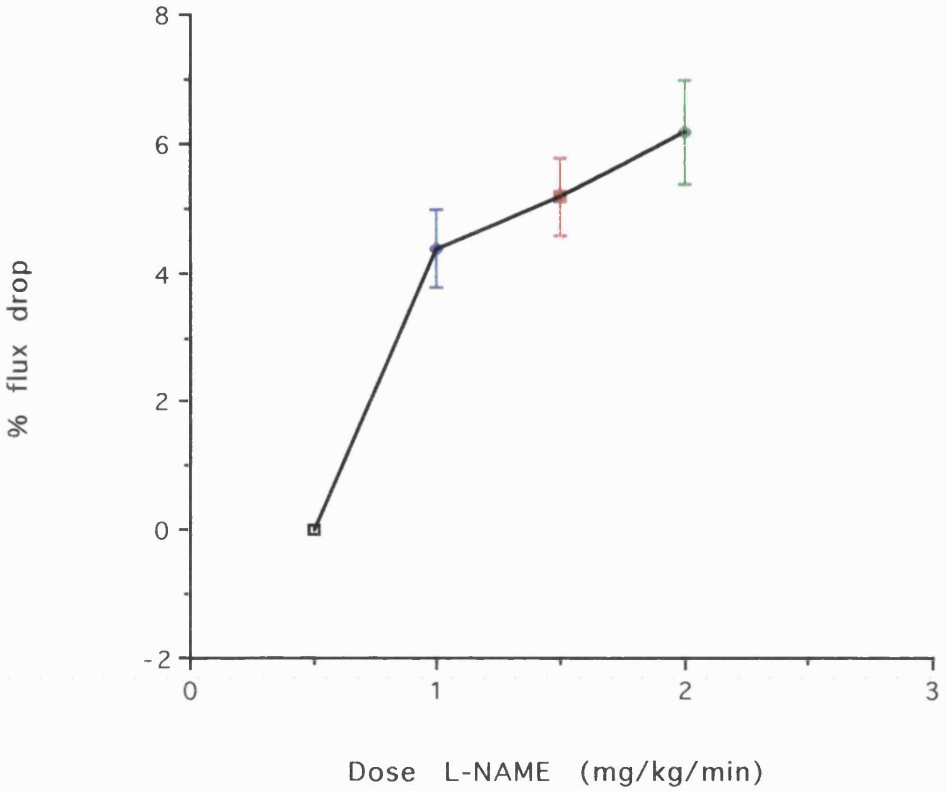


Figure 22: Dose curve for maximal % flux drop with HAI l-NAME in non-tumour bearing animals.

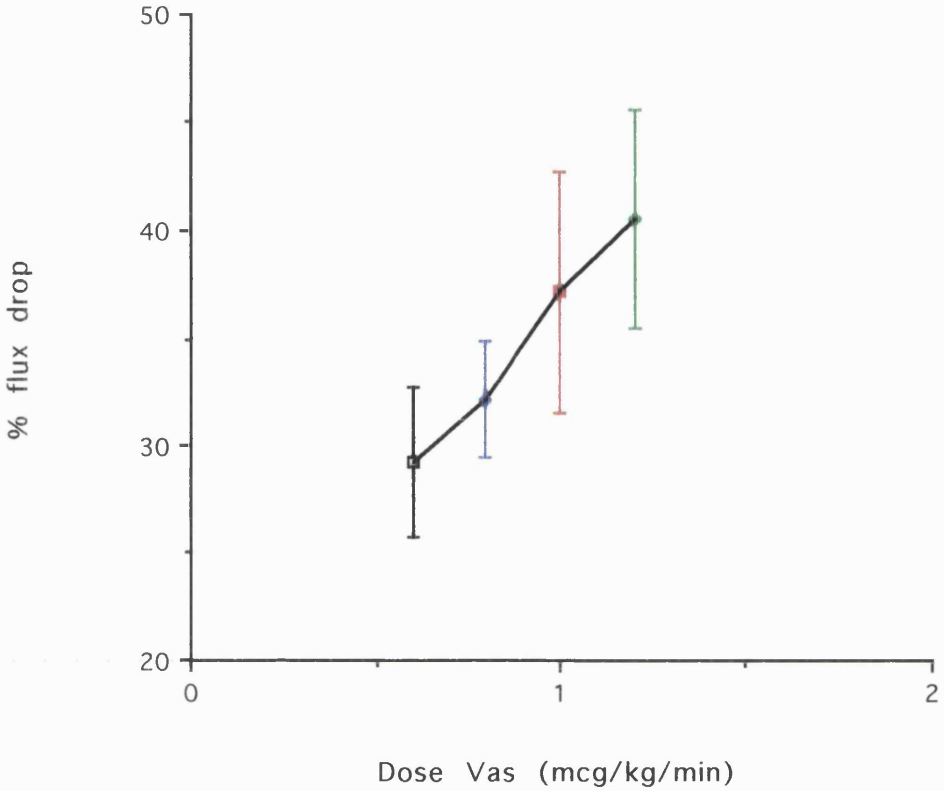


Figure 23: Dose curve for maximal % flux drop due to HAI Vas in non-tumour bearing animals.

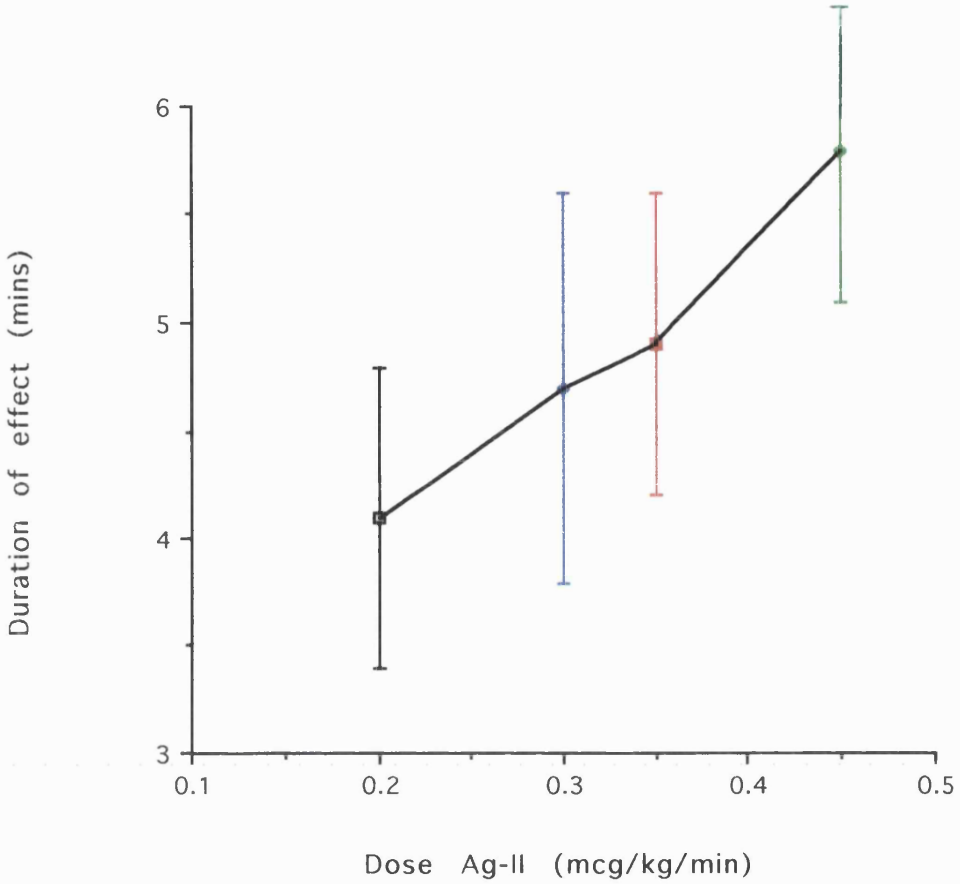


Figure 24: Dose curve for duration of effect of HAI Ag-II on liver blood flow in non-tumour bearing animals.

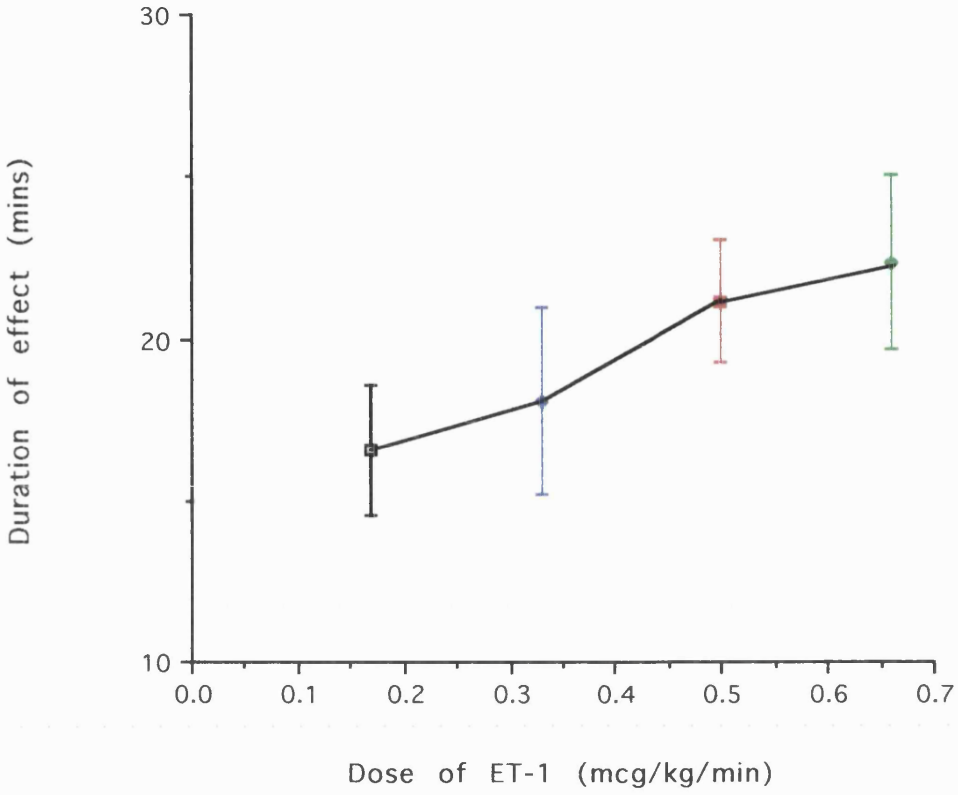


Figure 25: Dose curve for duration of action of HAI ET-1 on liver blood in non-tumour bearing animals.

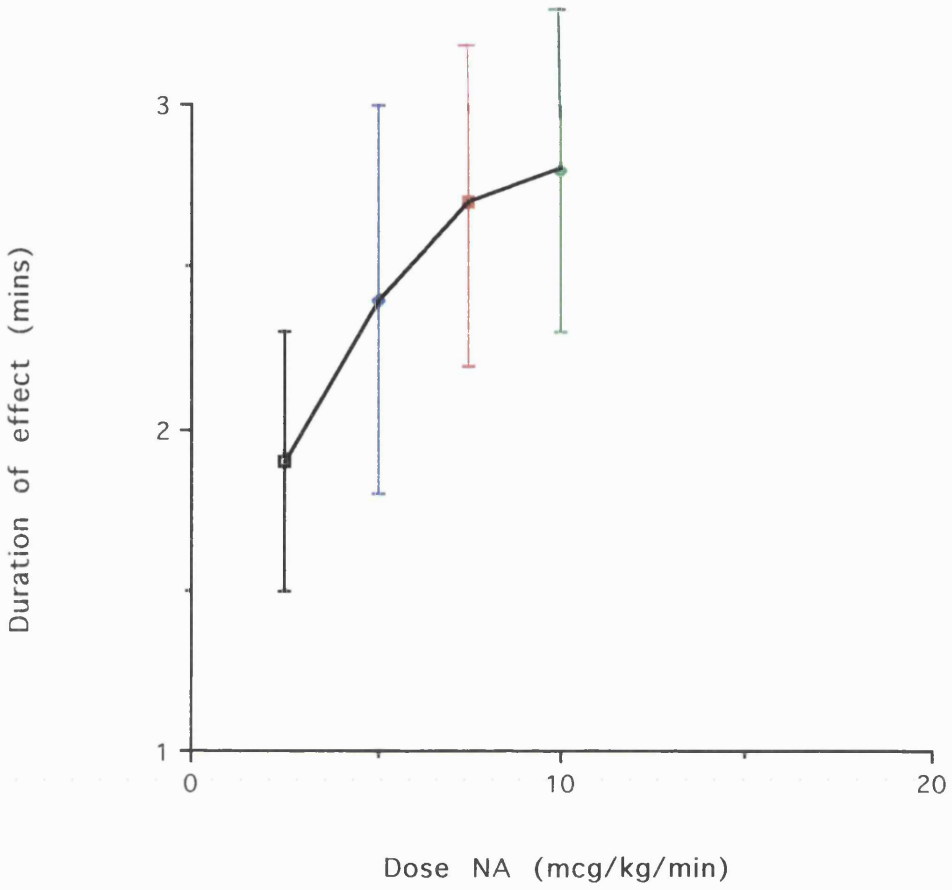


Figure 26: Dose curve for the duration of action of HAI NA on liver blood flow in non-tumour bearing animals.

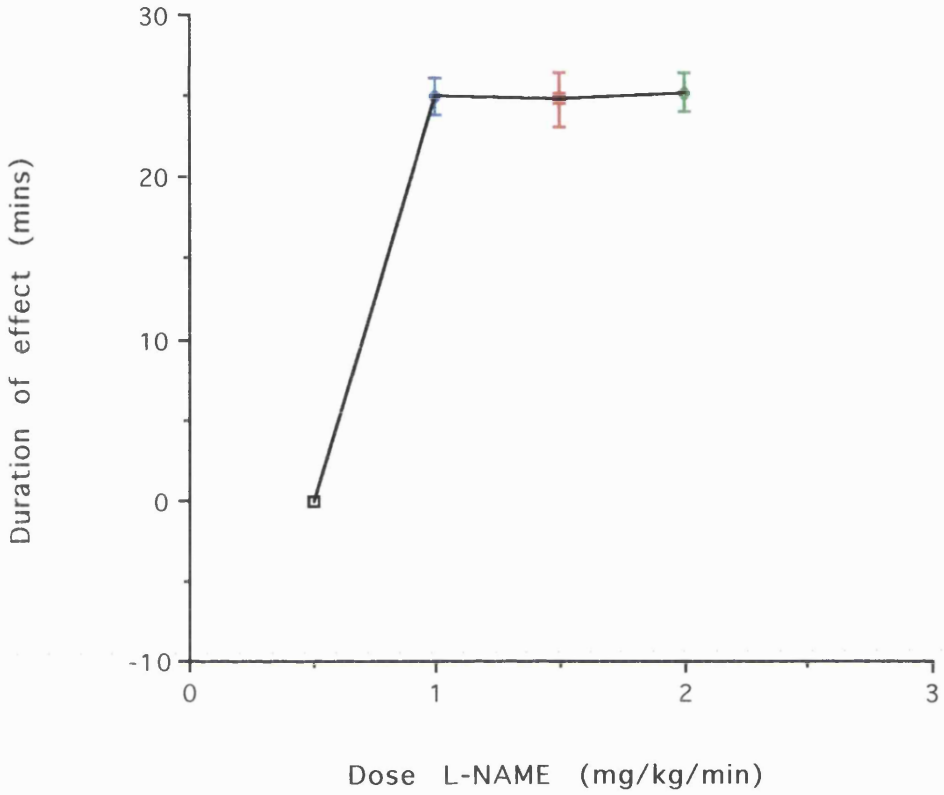


Figure 27: Dose curve for the duration of action of HAI L-NAME on liver blood flow in non-tumour bearing animals.

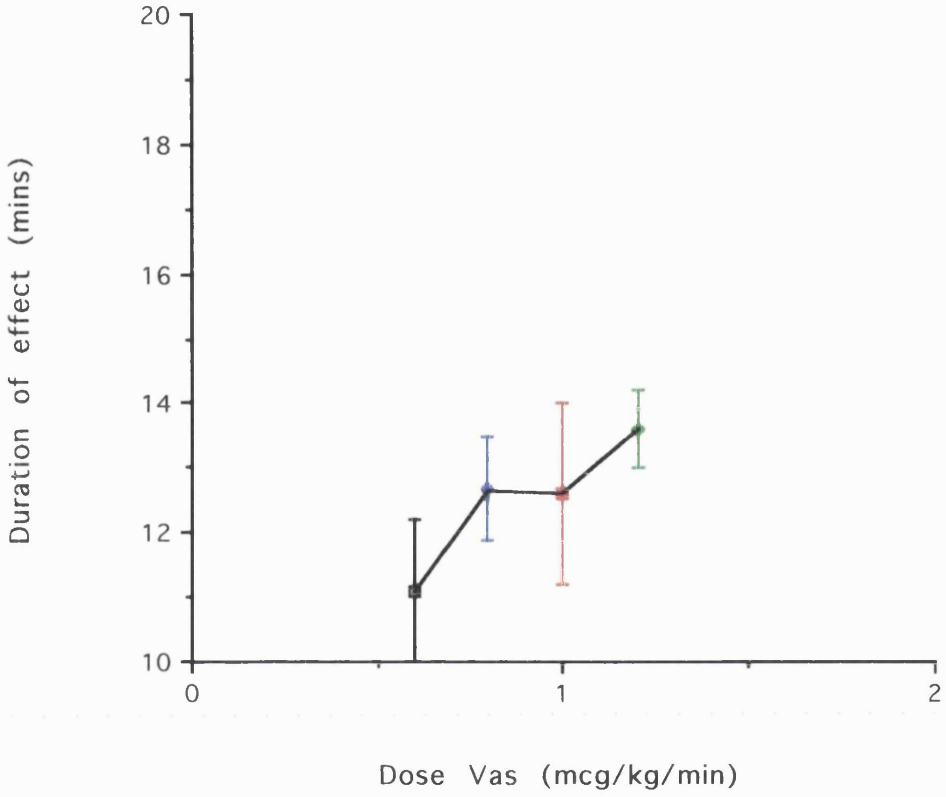


Figure 28: Dose curve for the duration of action of HAI Vas on liver blood flow in non-tumour bearing animals.

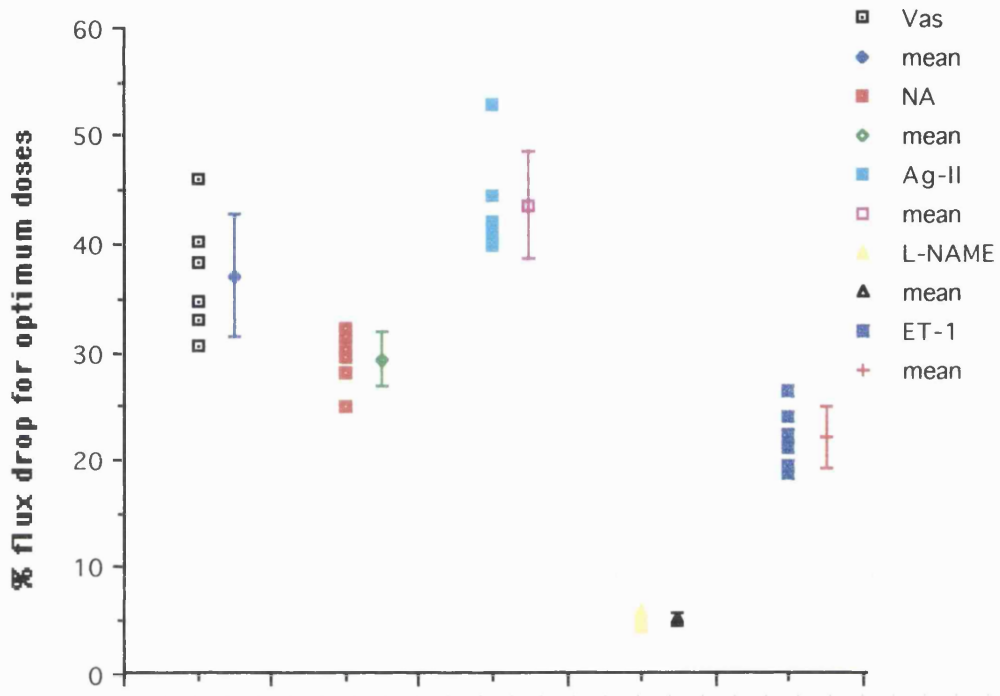


Figure 29: Graph demonstrating the maximum % flux drop for the optimum (20% BP rise) doses during HAI of the vasopressors

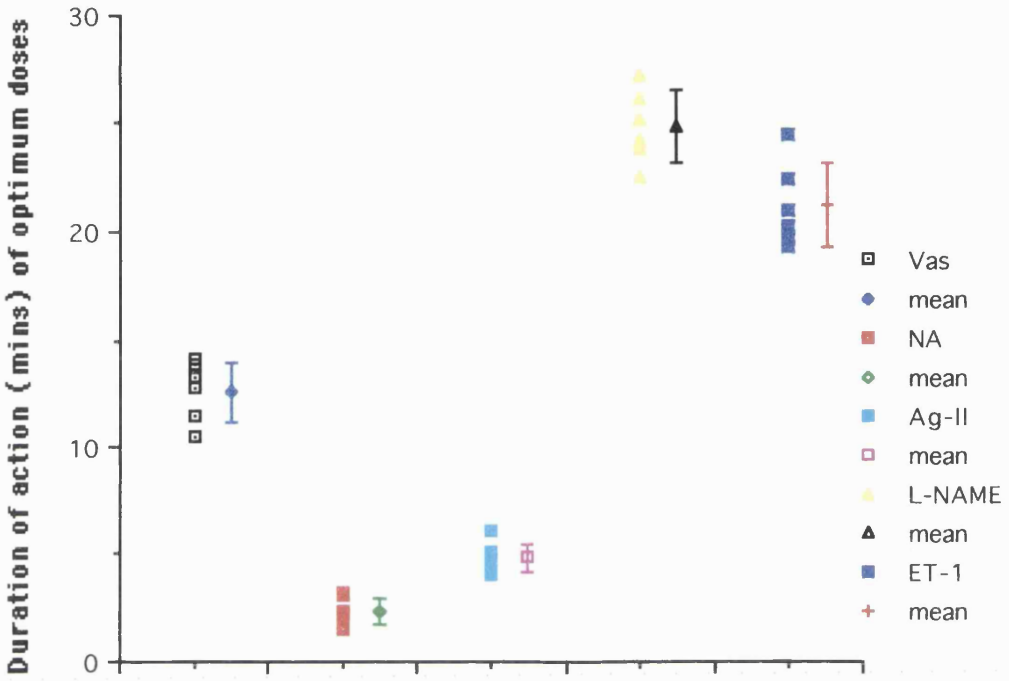


Figure 30: Graph demonstrating duration of action for the optimum (20% BP rise) doses of HAI vasopressors.

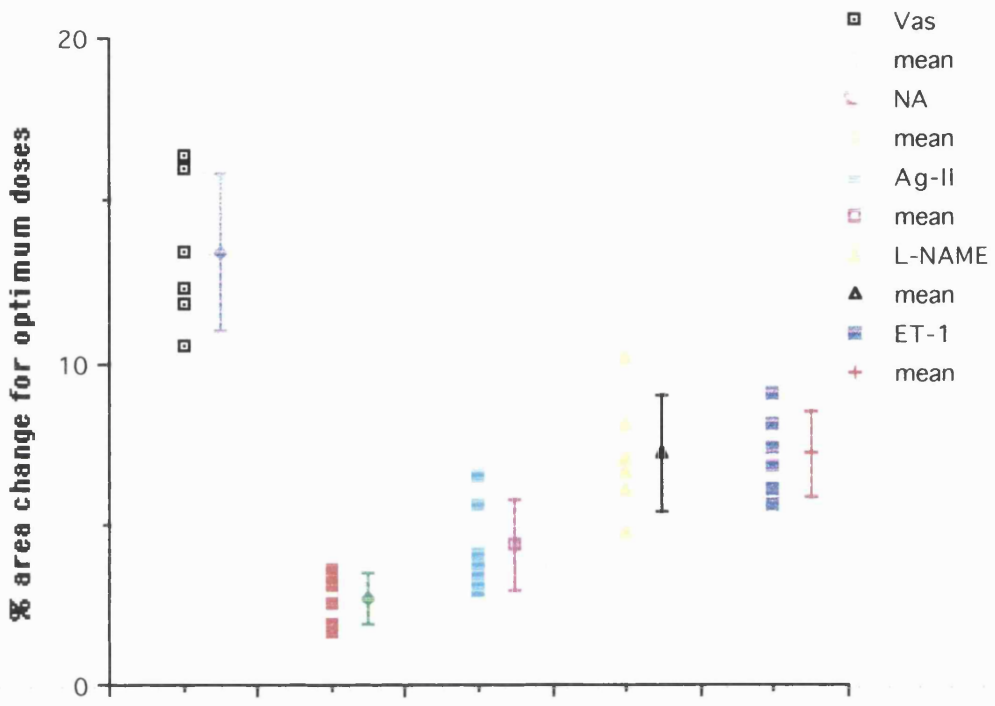


Figure 31: Graph demonstrating the % area change for the optimum doses (20% BP rise) caused by HAI of vasopressor agents.

2.3 Discussion

Given that blood vessels within colorectal liver metastases possess neither a smooth muscle coat nor evidence of innervation, such vessels should not be able to undergo vasoconstriction. Hence administration of hepatic arterial vasopressor agents should lead to selective constriction of hepatic vessels whilst leaving tumour vessels unaffected.

This in turn should lead to shunting of blood from the liver into the tumour and therefore improve drug delivery in hepatic arterial chemotherapy (as illustrated in figure 2).

The agent which would be expected to have the greatest effect on blood flow in the tumour -if indeed the shunting phenomenon were to exist with no intrinsic tumour vessel constriction- would be vasopressin since this produced the greatest % area change. Vasopressin produced almost double the % area change over the second most potent agents ET-1 and l-NAME.

Vasopressin is known to be a potent vasoconstrictor in the portal system if infused at a sufficient rate (Hennigan et al 1994 and Dworkin et al 1995). The period of action and maximal flux drop for vasopressin are higher in this study although this is probably due to different dosage regimens. Angiotensin-II, endothelin-1 and noradrenaline all produced marked hepatic vasoconstriction in relation to the optimal dose, although less than reported in some previous publications . All these agents are known to be potent hypertensive agents via their action on peripheral vascular resistance and to a lesser extent on cardiac output. One reason for the differences between these results and previous work is that nearly all the reported studies used higher doses (many not stating the dose in terms of g/kg/min) than described here with subsequent systemic hypertensive effects. Also the animals studied varied in strain and the routes of administration were different (eg portal vein).

The interesting feature found with all the agents apart from l-NAME is the short duration of action despite continued infusion of the agent. This phenomenon is reported in other studies and has been attributed to either portal vein or hepatic artery vasodilation or to a combination of the two. This ' escape' phenomenon probably forms part of an intrinsic protective feed back mechanism to prevent prolonged periods of hepatic ischaemia. The mechanism underlying this process may be partly explained by the findings of Dworkin et al (1995) who demonstrated that the addition of a nitric oxide synthase inhibitor at the time the flux began to return to the baseline reversed the "escape" phenomenon.

Nitric oxide is a potent vasodilator secreted by vascular endothelial cells and is recognised to be the endothelially derived relaxing factor. In this study the findings were in keeping with this hypothesis. Here l-NAME had a delayed but continuous effect on hepatic blood flow suggesting a basal secretion of nitric oxide by the hepatic arterial system. Removal of this effect lead to a small but consistent vasoconstriction; perhaps the relatively small effect it had allowed it to evade the threshold of the "escape" phenomenon.

Combination of a nitric oxide synthase inhibitor with a vasoconstrictor may prolong and increase the effects in the presence of tumour.

Section II-The pharmacological manipulation of blood flow in liver metastases in an animal model and changes in the uptake of radiolabelled 5-FU

2.4 Methods

2.4.1 In vitro culture of MC28 tumour cells

All culture grade plastics were purchased from Becton-Dickinson (Oxford, UK) and all solutions and drugs were purchased from Gibco BRL (Paisley, UK), unless otherwise stated.

Syngeneic MC28 cells (Institute of Rancer Research, Sutton, UK) of passage number 15-18 were cultured in $75cm^2$ plastic flasks (Gibco BRL, Paisley, UK) in Dulbecco's modified Eagles medium(DMEM) with Glutamax, fortified with 10% fetal calf serum(Gibco BRL) and penicillin and streptomycin(100 mcg/ml each). The cells were then maintained in a humidified atmosphere at $37C^0$.

Once the cell cultures became confluent they were washed twice in phosphate buffered saline (PBS) to remove the serum containing medium. The cells were then disaggregated by incubating in 1ml of trypsin-EDTA, containing 500μg/ml of trypsin and 0.02% EDTA in PBS at $37C^0$ for 3 minutes. This enzyme reaction was then partially stopped by the addition of 10ml of DMEM containing 10% fetal calf serum. The cell suspension was centrifuged for 5mins at 1000rpm (300g), the supernatant discarded and the resultant cell pellet washed twice in PBS to remove any remaining trypsin (each time being centrifuged as before). After the final wash the cells were resuspended in PBS. Cell numbers were calculated by mixing equal volumes of cells with a tryphan blue solution and counted on a haemocytometer. Cell viability was determined using the tryphan blue dye exclusion method and was >95%. Usually 1/5 of cells were used for in vitro passage. The concentration of cells used for experiments in vitro was 5×10^6 cells/ml.

2.4.2 The intraportal injection of MC28 tumour cells

The animals were anaesthetised with a mixture of 3% halothane and oxygen and maintained on 1.5% halothane and oxygen. A lower midline incision was made and the caecum and terminal ileum delivered.

A large venous tributary was identified in the mesentery and the tumour cells injected using a fine gauge needle(27G). Each animal received 0.2mls of suspension described above (ie $1x10^6$ cells) and once injected a cotton wool swab was applied to prevent backbleeding. The two control groups received 0.2mls of dead cells and normal saline respectively.

The wounds were then closed in 2 layers with continuous 4/0 silk sutures and the animals allowed to recover. Rats inoculated with live tumour cells developed superficial tumours (between 1-5) after 14 days.

2.4.3 The measurement of blood flow in tumour and liver

Hepatic metastases were established in Hooded Lister rats weighing between 350-400gs as described above. Animals were divided into three groups; Group 1: Tumour bearing, Group 2: Injected with dead cells, Group 3: Injected with saline.

Fourteen days after inoculation the animals underwent carotid and gastroduodenal cannulation as previously described. A Laser doppler probe was placed on the surface of an accessible tumour over its central portion- having first been calibrated- and another probe placed 2cms away on an adjacent area of macroscopically healthy liver in the same lobe.

The abdomen was then closed as before and the optimum doses (determined from the previous experiment as being that dose which did not cause greater than a 20% rise in blood pressure) of the same agents administered in 1ml of normal saline. Once again

the infusions lasted a total of 30minutes and results were collected on a computer data logging system.

2.4.4 The measurement of the uptake of radiolabelled 5-fluorouracil by MC28 hepatic metastases when combined with vasoconstricting agents.

This experiment involved only animals that had liver tumours. 14 days after the injection of MC28 tumour cells, animals that had developed tumours underwent gastroduodenal artery cannulation (described above) and a combination of ^{3}H radiolabelled 5-fluorouracil with and without the optimum doses of the vasoconstricting agents were infused over 30 minutes in 1ml.
The groups of animals were as follows;

Group 1=5FU and normal saline[n=6].
Group 2=5FU and Angiotensin II[n=6].
Group 3=5FU and Noradrenaline[n=6].
Group 4=5FU and Vasopressin[n=6].
Group 5=5FU and Endothelin -1[n=6].
Group 6=5FU and L-NAME[n=6].

Once the infusions were completed the animals were sacrificed and the liver removed. Tumour and liver from the same lobe (taken 2 cm away from the tumour) were removed and weighed. The tissue was solubilised and diluted in scintillant (Toluene Scintillator, Packard, Reading, UK). Samples were then radiocounted and values expressed as counts per gram of tissue per minute (cpm/g) .

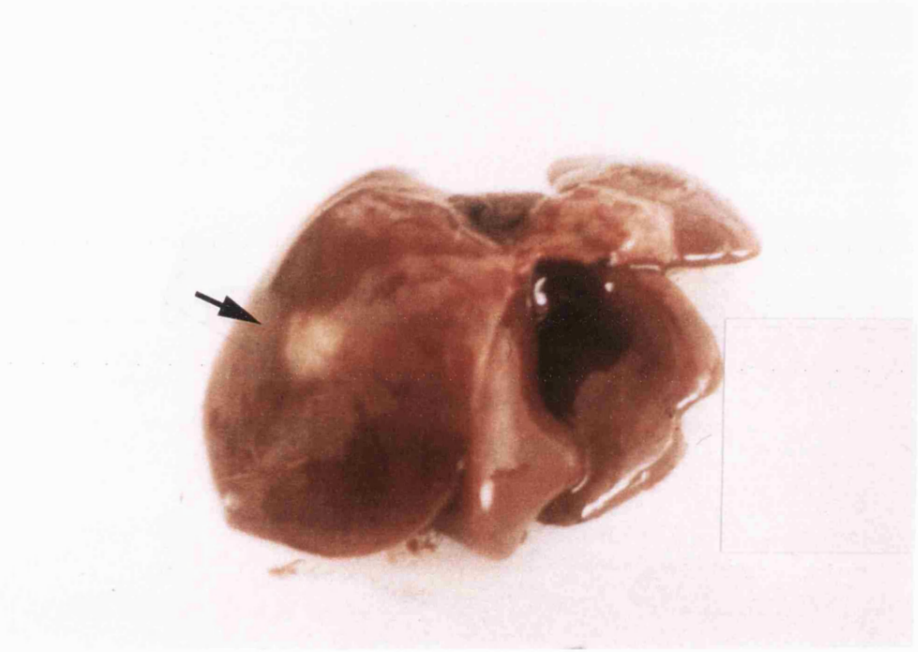


Figure 32: Photograph of liver tumour in the MC28 animal model on day 10 post-inoculation (arrow).

2.4.5 Calculations and statistical analysis

The results underwent oneway ANOVA analysis followed by unpaired t-test corrected for unequal variances. A probability of $p<0.05$ was considered significant. The tumour/normal liver blood flow ratio (T/N) was calculated in two time periods. Firstly the total T/N over the whole 30 minute infusion period and secondly the effective period of the vasopressor agent. The effective period is defined as the time over which the vasopressor has its effect ie the time taken for the flux line to return to the baseline value. Blood flow is represented by the area between the flux line and zero in each of these periods for both tumour and normal liver.

2.5 Results

Animals were infused with vasoactive agents to assess their effect on blood flow in both liver tumours and normal liver tissue.

All vasopressors studied produced a variable drop in flux measurement in both tumour and normal liver (all agents apart from l-NAME producing almost immediate effects and the time course in both tumour and liver being equal). None of the agents produced an absolute rise in tumour blood flow.

Prior to commencing the infusions the tumour/normal blood flow ratio gave a mean of 0.3, SD 0.04, which is similar to that found in human colorectal liver metastases.

The percentage changes in the tumour to normal ratio (T/N) during the infusion periods can be considered in two areas. Firstly when taken over the whole 30 min infusion period none of the agents produced a statistically significant change (figure 34) giving values of (mean±SD%) Ag-II 1.5±0.3, NA 3.5± 0.6, Vas 2.7± 0.8, ET-1

5.26±2.28 and l-NAME 1.95±0.1. However if the effective period for each agent is considered seperately (figure 35) the mean % changes in T/N ratios and durations of action(which were not significantly different from the non-tumour bearing animals) are as follows; angiotensin-II 10 ± 2.4 % $p<0.05$ and 4.2 ± 0.2 mins, noradrenaline 34 ± 5.1 % $p<0.05$ and 2.9 ± 0.4 mins, vasopressin 6.6 ± 1.9 % $p<0.05$ and 11.1 ± 0.9 mins, endothelin-1 13.8 ± 5.3 % $p<0.05$ and 21.5 ± 2.3 mins and L-NAME 2.4 ± 0.6 % p=NS and 22.6±3.3 mins. Therefore the only agent that did not produce a significant elevation in the T/N ratio was l-NAME.

Animals with liver metastases were given radiolabelled 5FU with and without the vasoactive agents tested in the previous study, to assess whether changes in blood flow resulted in changes in 5FU delivery.

None of the agents produced a significant difference in the uptake of [^{3}H]-5FU in the normal liver compared to the saline group (figure 36).

The uptake in the tumour gave means (cpm/g ± SD x10^5) as follows; saline 5.1 ± 3.2, angiotensin II 5.1± 1.4, endothelin-1 15.8 ± 14.2, L-NAME 3.5 ± 1.3, noradrenaline 19.1 ± 9.8 and vasopressin 6.8 ± 3.5.

Noradrenaline was the only agent to produce a significant increase in radiolabelled 5-fluorouracil uptake in the tumour ($p<0.05$) as illustrated in Figure 37.

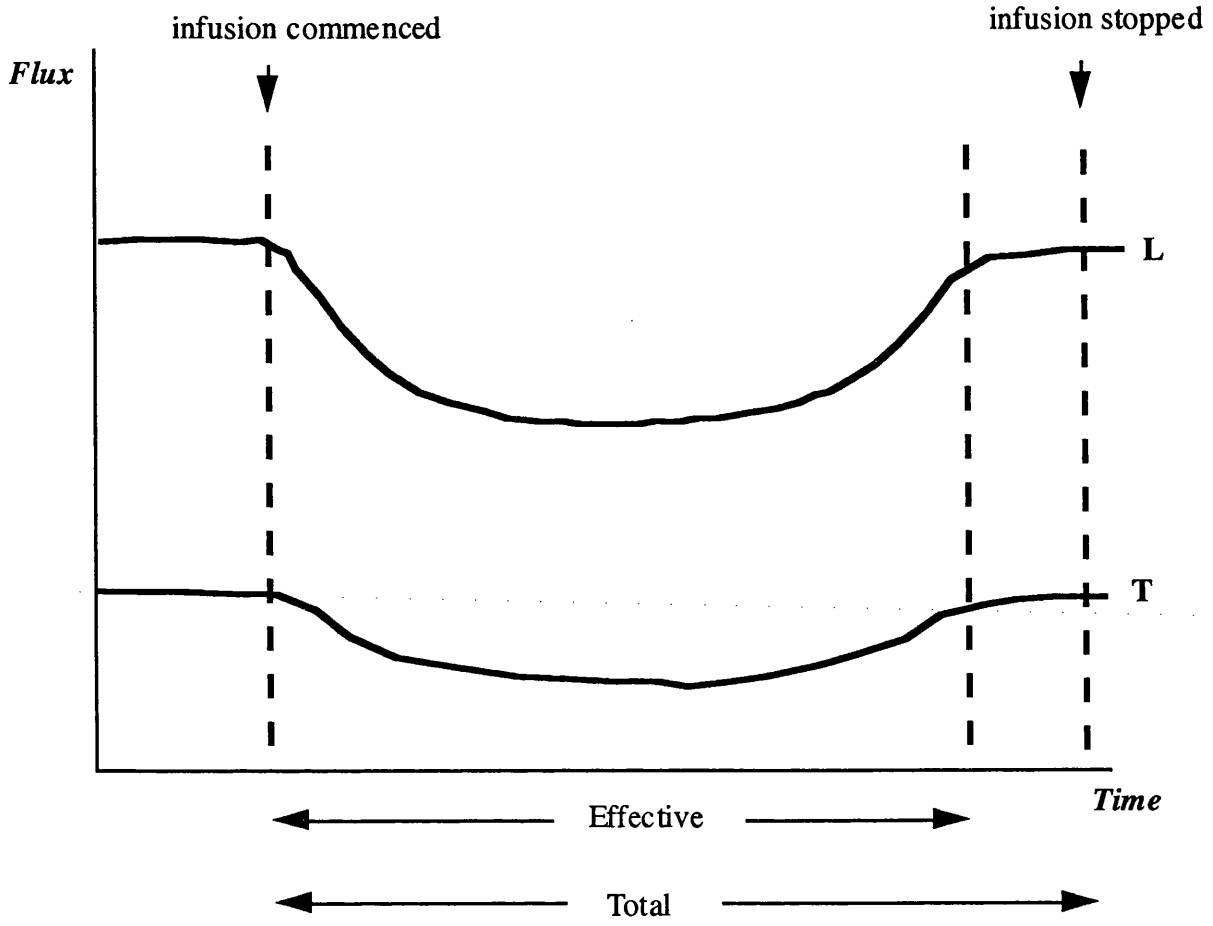


Figure 33: Schematic representation of the typical flux pattern seen during hepatic arterial infusion of a vasoconstricting agent in tumour bearing animals.

The flux pattern in the liver is labelled L and the tumour labelled T. The area under the curves represents blood flow in both the liver and tumour. The T/N blood flow ratio is calculated in 2 time periods; T=total 30 min infusion period and E=effective period of the vasoconstrictor (taken as the time the flux line takes to return to the original baseline preinfusional point).

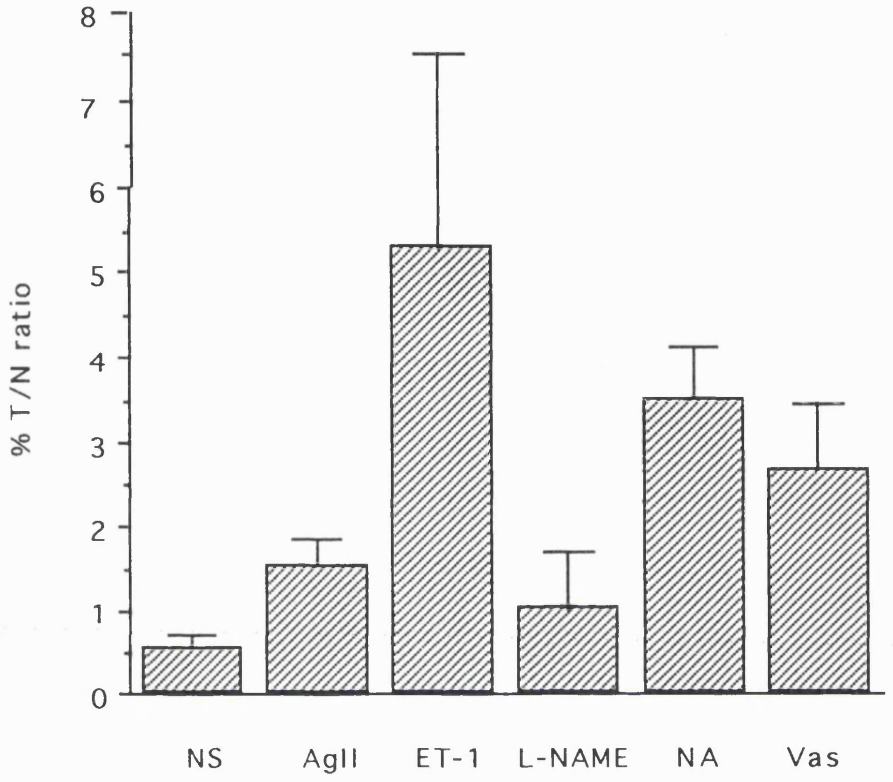


Figure 34:Tumour/normal blood flow ratio during the entire 30min period of hepatic arterial infusion.

The % change in blood flow ratio (%T/N) during the 30 minute infusion period is shown on the Y-axis. All vasoconstricting agents are shown on the X axis.

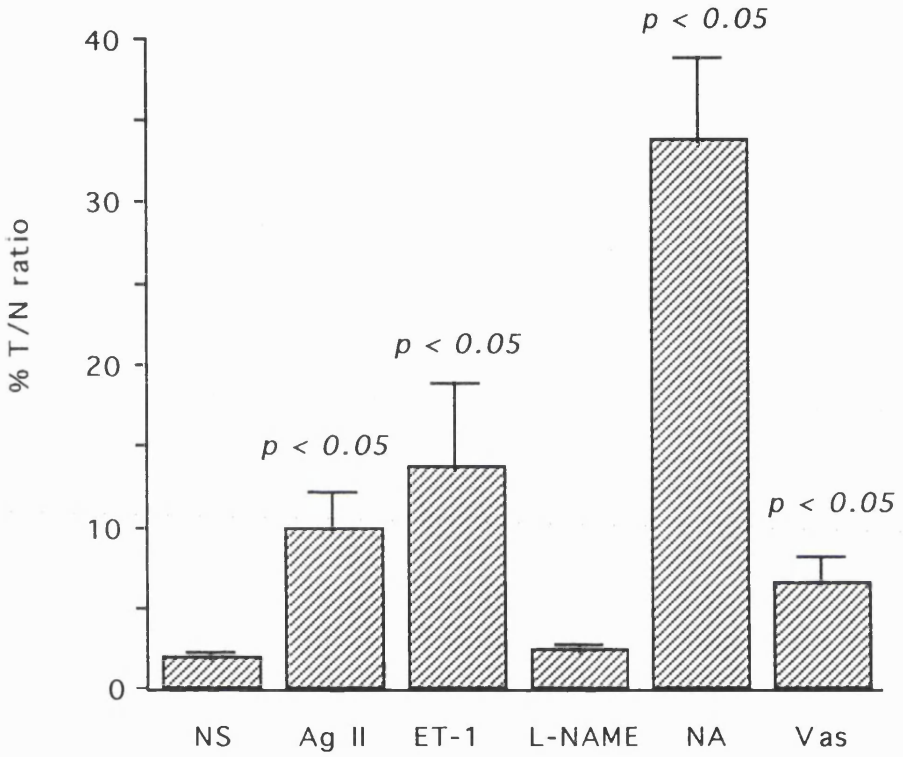


Figure 35: Tumour / normal blood flow ratio during the effective period of vasoconstriction.

The % change in blood flow ratio between tumour and normal liver (% T/N ratio) is shown on the Y-axis. All vasoconstrictor agents are shown on the X-axis.

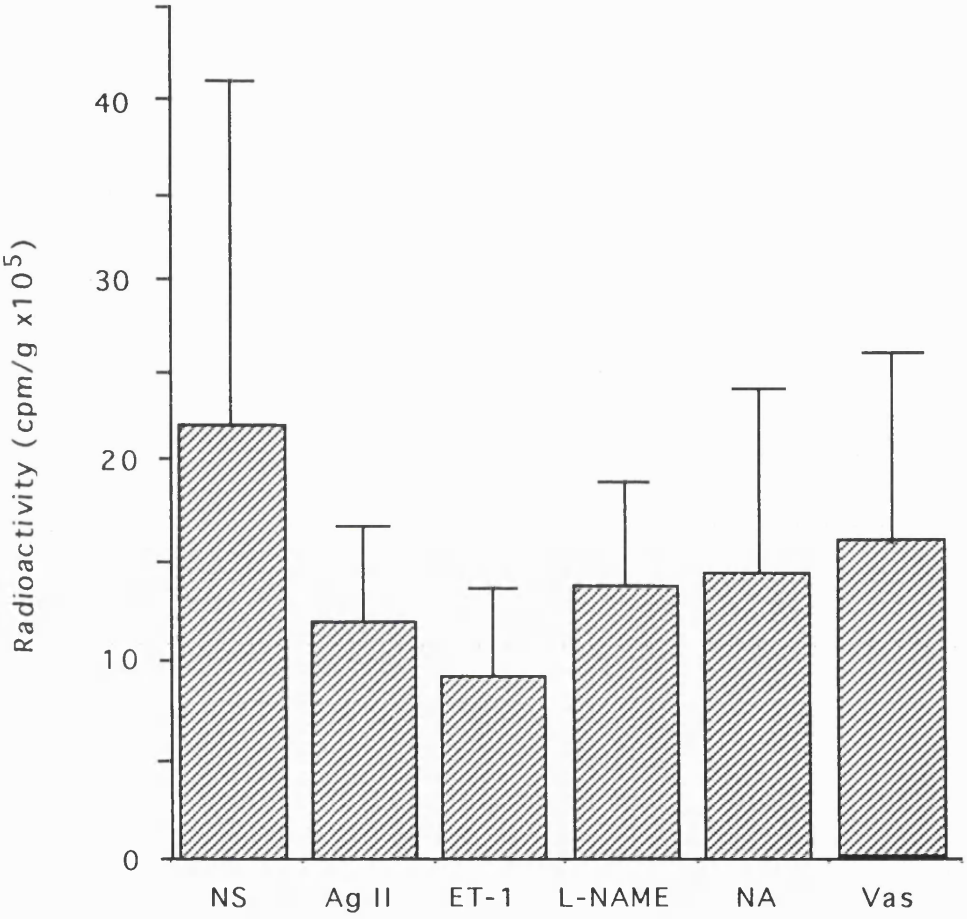


Figure 36: Uptake of [^{3}H]-5-fluorouracil in normal liver, under the influence of vasoconstrictors.

The radioactivity present per unit of weight (g), per unit time (min), depicted as radioactivity (cpm/gx10^5) is shown on the Y-axis. Vasoconstrictor agents are shown on the X-axis.

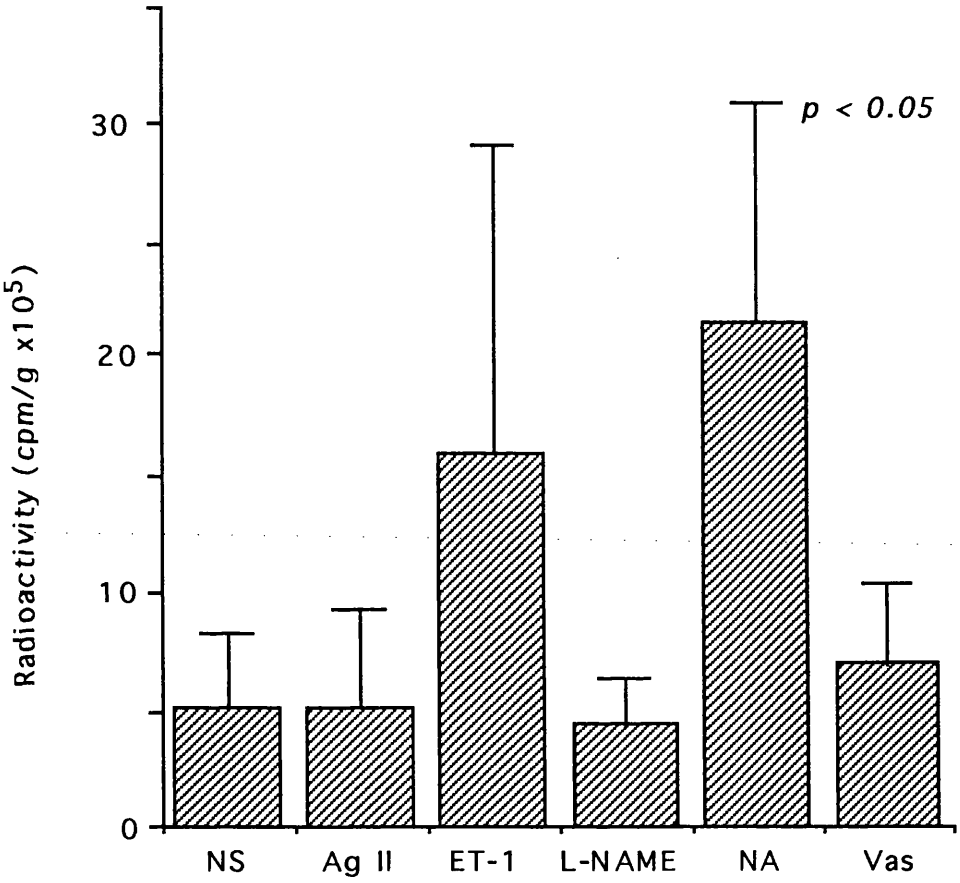


Figure 37: Uptake of [^{3}H]-5-fluorouracil in tumour, under the influence of vasoconstrictors.

The radioactivity present per unit of weight (g), per unit time (min), depicted as radioactivity (cpm/gx10^5) is shown on the Y-axis. Vasoconstrictor agents are shown on the X-axis.

2.6 Discussion

Differences between blood vessels in tumour and normal hepatic tissue have been suggested since 1969 (Krylova, 1969). More recently our group demonstrated, using electron microscopy and immunohistochemistry, that blood vessels in colorectal liver metastases lack both a complete smooth muscle wall and neuronal innervation (Ashraf et al, 1996). A similar profile has been demonstrated in the blood vessels supplying MC28 tumours, suggesting a comparable situation to that in man (Loesch et al, 1997; Ashraf et al, 1997).

These findings lend support to the hypothesis that selective vasoconstriction of normal, but not tumour, hepatic vessels achieved by the administration of regionally infused vasopressors, would result in blood being shunted from the hepatic vascular bed into the tumour and hence improve drug delivery.

Adrenaline has long been known to improve the tumour blush in hepatic angiography of colorectal liver metastases, beyond that which might be expected from vasoconstriction of normal surrounding vessels alone.

Noradrenaline has been shown to produce increased uptake of radiolabelled microspheres when co-administered in conjuction with propanolol via the hepatic artery in an animal model (Burton et al, 1987), although its duration of action was limited. Ackerman et al (1988) demonstrated increased capillary blood flow, using laser doppler flowmetry, within intra-hepatic tumours due to infused catecholamines in an animal model. Goldberg et al (1990) demonstrated improved uptake of radioactive tracers with HAI angiotensin-II in patients with colorectal liver metastases receiving hepatic arterial chemotherapy. Use of intraoperative laser dopplers in patients with colorectal liver metastases has demonstrated improved tumour blood flow when angiotensin-II was given into the hepatic artery (Hemmingway et al, 1993).

It has been suggested that part of the effect is due to the elevated systemic blood pressure and hence increased tumour perfusion pressure, producing a subsequent opening up of tumour vessels. This is unlikely, however, as the changes in pressure do not mirror the changes in tumour blood flow demonstrated (as seen in this study). Dworkin et al (1995), using an animal model, showed improvements in the T/N blood flow ratio of intrahepatic tumours using vasopressin with and without a nitric oxide synthase inhibitor. This study suggested that the addition of NOS inhibitors prolongs the effect of vasopressin and implicates nitric oxide production within hepatic vessels as a possible cause of the limited duration of action seen with HAI vasoconstrictors. Dworkin et al (1996) using the same model demonstrated that although HAI angiotensin-II led to improvements in the T/N ratio it did not lead to a significant improvement in drug uptake. This same group (Dworkin et al 1997), again using HAI vasoconstrictors in the same animal model, demonstrated that only ET-1 produced a significant improvement in the T/N ratio when infused over 30 minutes. One of the limitations of most studies to date is that vasopressors have been given as either a bolus or short infusion. Given that hepatic arterial chemotherapy is administered over a prolonged period, assessment of the action of regionally infused vasopressors over this time course would be required prior to contemplating a clinical trial.

This study confirms the limited duration of action of the vasopressors studied and illustrates their ability to elevate the tumour to normal blood flow ratio. The reduction in blood flow seen (although less than that in the liver) in the tumour circulation might be due to constriction of feeding vessels coming from the normal adjacent liver or might represent an element of tumour vessel constriction.

Down regulation of sympathetic innervation in tumour vessels, as demostrated previously (Ashraf et al, 1996; Ashraf et al, 1997), might explain why noradrenaline -a sympathetic neurotransmitter- produced the greatest rise in the T/N ratio. If noradrenaline receptors were down regulated to a greater extent than for other neurotransmitters then its administration would lead to less intrinsic tumour vessel

constriction and hence produce a greater T/N elevation. This might also explain why noradrenaline alone produced significant improvements in drug delivery, despite the other agents also producing significant changes in the T/N ratio. These other agents might produce heterogeneous constriction of the tumour as well as hepatic vessels, but is not detected by the laser doppler, hence reducing drug delivery.

Other groups have demonstrated improved drug uptake and blood flow in liver tumours using endothelin-1, angiotensin-II and vasopressin. The animal studies involving these agents used different tumours to the one employed in this study, which may explain some of the differences reported here. It should be noted that the blood vessels found within MC28 tumours possess a similar profile with regard to both structure and innervation to those found in human colorectal liver metastases (Ashraf et al 1996, Ashraf et al 1997). The issue of doses used is relevant when comparing the results of this study to those from similar investigations in animal and humans. Many of these studies used higher doses of vasopressors than described here, producing marked hypertension (greater than the 20% level in this study). Such effects would be unacceptable in a clinical setting. Such variations in doses may account for the greater effectiveness noted for agents such as angiotensin-II in previous studies.

As none of the agents produced improvements in tumour blood flow in real terms it is surprising that noradrenaline caused an increased tumour uptake of radiolabelled 5-flurouracil.

Jain (1991 and1994) demonstrated that these tumours exist in a state of interstitial hypertension, which retards the movement of molecules such as chemotherapeutic agents through the interstitium. This interstitial hypertension leads to radially outward convection within the tumour periphery which opposes inward diffusion. Part of the effect produced by vasopressors might be a reversal of the pressure gradient around the tumour leading to improved inward diffusion and concomitant increased drug

delivery. Since noradrenaline produced the greatest change in the T/N ratio it maybe the only agent to have passed the threshold required to reverse the gradient.

Vasopressors increase the T/N ratio and noradrenaline especially appears to improve drug delivery when co-administered with hepatic arterial chemotherapy. However as hepatic arterial chemotherapy is usually given over a prolonged infusion the limited duration of action of the vasopressors studied may preclude their use in a clinical setting. A greater understanding of their pharmacokinetic profile, especially with regard to their refractory period, may allow their incorporation into a clinical regional chemotherapeutic trial.

ET-1 as demonstrated in this section has a considerable vasoconstricting effect within the liver and perhaps within the tumour. It has been suggested that ET-1 in addition to acting as vasoconstrictor, may also play a role in regulating tumour growth in other systems.

The intention of chapter III is to determine whether ET-1 is produced by colorectal cancers, both primary and secondary. In the first section, using immuno-electronmicroscopy, colorectal liver metastases and adjacent liver were stained for ET-1. The second section determines whether or not such production leads to elevated systemic levels of ET-1, which may have implications on distant growth of tumours once shed from the primary site. Therefore the plasma levels of ET-1 in patients with both primary and secondary tumours were measured.

Chapter III

Endothelin-1 and human colorectal cancer

Introduction

Endothelin-1 (ET-1), a peptide with recognised vasoconstrictor properties, is known to be elevated in the plasma of patients with a variety of conditions associated with vasospasm including; pregnancy induced hypertension, myocardial infarction and cardiogenic shock (Miyauchi et al 1989, Kamai et al 1990, Cernacek and Stewart 1989). Apart from its involvement in non-neoplastic conditions, ET-1 plasma levels are elevated in patients with hepatocellular carcinoma (Nakamuta et al 1993).

ET-1 also stimulates cellular proliferation *in vitro*, in a variety of normal cell types including smooth muscle, fibroblasts, renal mesangial cells (Hirak et al 1989, Simonson et al 1989, Kusuhara et al 1989) and several tumour cell lines, including some derived from colorectal cancers (Masayoshi et al 1991).

Histochemical analysis of different types of cancer, eg hepatocellular carcinoma (Kar et al 1995), show elevated levels of ET-1 in the tumour compared with normal tissue. In addition immunohistochemical analysis on primary colorectal cancer specimens have demonstrated a heterogenous distribution of ET-1 binding sites around tumour cell nests and associated vessels (Inagaki et al 1992). These findings suggest that ET-1 may perform a paracrine function in such tumours, in relation to both mitogenesis and blood supply.

The first part of this chapter assesses the importance of ET-1 in colorectal cancer- with particular emphasis on liver metastases. Immuno electronmicroscopy for ET-1 was performed on colorectal liver metastases and adjacent normal liver obtained at hepatectomy. Immunoelectron microscopy was chosen, as opposed to immunohistochemistry, so that not only the presence of ET-1 could be detected, but also the cell types associated with it could be identified.

The second part of this chapter measures the plasma levels of ET-1 in patients with colorectal cancer, with and without liver metastases, and compare these results with an age and sex matched control group.

Aims of study

1. Using immuno-electronmicroscopy to determine whether ET-1 is produced by cells within human colorectal liver metastases and cells in adjacent normal liver.

2. To measure the plasma levels of ET-1 and thrombomodulin in patients with colorectal cancer with and without hepatic metastases and to compare with a control group.

Section I- Immunoelectron microscopy of colorectal liver metastases

3.1 Methods

3.1.1 Tissue samples

Six patients with colorectal liver metastases had specimens taken from both macroscopically normal liver and from metastases within the same lobe during surgery for colorectal liver metastases or resection of the primary tumour. The patient details are in table 3.

3.1.2 Immunohistochemistry for electron microscopy.

Specimens were taken from metastatic tissue and from normal liver (at least 5cm away from the tumour) at the time of hepatic resection, once the specimen had been removed. These were fixed, within 30 minutes of excision, in 4% paraformaldehyde (Sigma, UK) and 0.25% glutaraldehyde (Sigma) in 0.1M cacodylate buffer at pH 7.3 for no less than 4-6 hours at 4°C[11]. Tissue was then transferred to 0.1M cacodylate buffer and stored overnight at 4°C.

3.1.3 Pre-embedding immunohistochemistry

For localization of ET-1, tissue was processed according to the peroxidase-antiperoxidase (PAP) method of Sternberger (1979), modified as previously reported (Loesch et al 1991a, Loesch et al 1991b). Samples were exposed to 0.3% hydrogen peroxide in 50% methanol for 30 minutes (for blocking endogenous peroxidase activity), washed in 0.1M Tris buffer at pH 7.4, and then exposed to normal goat serum (NGS, Nordic Immunology Tilberg, The Netherlands), diluted 1:40 in Tris containing 0.1% sodium azide and 0.5% bovine albumin (Sigma, Poole,

Patients initials	Age/sex	Operation	Duration from 1^0 resection	1^0 histology
DN	75	Liver and met Bx at resection of 1^0 12/7/95	Sync	Dukes B
CT	55	Liver and met Bx during HA cannulation 19/7/95	Sync Ant Res June 97'	Dukes B
CS	50	Liver and met Bx at resection of 1^0 21/8/95	Sync	Dukes C
JM	49	VI and VII resection 13/9/95	Ant res Jan 92'	Dukes C
AW	56	Rt Hep 25/10/95	Rt Hemi Mar 94'	Dukes B
TP	58	Rt Hep 15/11/95	Lt Hemi Apr 94'	Dukes C

Table 3. Details of patients with colorectal liver metastases undergoing immuno-electronmicroscopy for ET-1.

U.K.) for 1.5h, and then rinsed in Tris. Samples were incubated for 48h at 4°C with a rabbit antibody for ET-1 at a dilution of 1:1000, in Tris containing 0.1% sodium azide and 0.5% bovine albumin. The specimens were washed in Tris and exposed for 1.5h to goat-anti-rabbit immunoglobulin G serum (Cappel Labs, West Chester, USA) diluted 1:40 in Tris containing 0.1% sodium azide and 0.5% bovine albumin, washed in Tris, and incubated for 3h with rabbit PAP complex (Dakopatts, Glostup, Denmark) diluted 1:60 in Tris. After exposure to 3',3'-diaminobenzidine (Sigma) and hydrogen peroxide, the specimens were washed in Tris and then post fixed with 2.5% glutaraldehyde in cacodylated buffer (pH 7.4) overnight. The specimens were washed with phosphate buffer and postfixed in 1% OsO_4, dehydrated through a graded series of ethanol and propylene oxide and then embedded in epoxy resin (Durcupan A/M, UK, Ltd), for transmission electron microscopy (Aliev et al 1993). Semi-thin sections for preliminary electron microscopy analysis were stained with 1% toluidene blue and examined under a Ziess light microscope. Ultra-thin sections were stained with uranyl acetate and lead citrate and investigated using a Jeol TEM-1010 microscope (Japan).

3.2 Results

Normal liver

The blood vessels within the portal tracts corresponded to the typical phenotype found elsewhere. There was a regular layer of endothelial cells surrounded by contractile smooth muscle cells, with an outer connective tissue adventitia. Associated with these vessels were both peri and paravascular nerves which were unmyelinated and found within the medial adventitial border and adventitia respectively. Innervation was denser around the branches of the hepatic artery than portal vein.

Only few endothelial cells lining these vessels showed immunopositivity for ET-1(figure not shown). When present the level of labelling was low when compared with similar areas in the tumour.

Liver metastases

The colorectal liver metastases and their contained blood vessels had a markedly different morphological structure. A heterogenous distribution of blood vessels was observed throughout the tumours, with the central portions showing areas lacking any differentiation or cellular boundaries, as described previously (Ashraf et al 1996). The tumour blood vessels appeared uniformly primitive lacking both a continuous smooth muscle coat and any evidence of innervation . The only exception to this pattern was at the junction with the adjacent liver, where presumably normal blood vessels were being incorporated into the tumour and as such still retained some normal structure. It was also noted that the tumour appeared markedly less vascular than the surrounding tissue with large numbers of myofibroblasts and connective tissue surrounding the tumour cell nests.

The distribution of ET-1 was very different from the normal liver with ET-1 staining strongly positive within the tumour cells (figure 38) and the surrounding fibroblasts (figure 39). In addition there was an increase in the percentage of the population of ET-1 positive endothelial cells in these tumours (figure 40).

Liver ET-1 staining	Tumour ET-1 staining
Hepatic vessel endothelial cells +	Tumour vessels ++
	Tumour cells +++
	Fibroblasts +++

Table 4. The degree of staining for ET-1 using immuno-electronmicroscopy in colorectal liver metastases and adjacent normal liver.

Liver	Tumour
Hepatic artery and vein lined by single layer of endothelial cells	Heterogeneous vessels Primitive, discontinuous endothelial cell layer
Peri and para vascular nerves	No evidence of neuronal structures
	Hypovascular compared to liver
	Large amount of surrounding fibroblasts and connective tissue

Table 5. Summary of morphological differences at immuno-electronmicroscopy between liver and colorectal liver metastases.

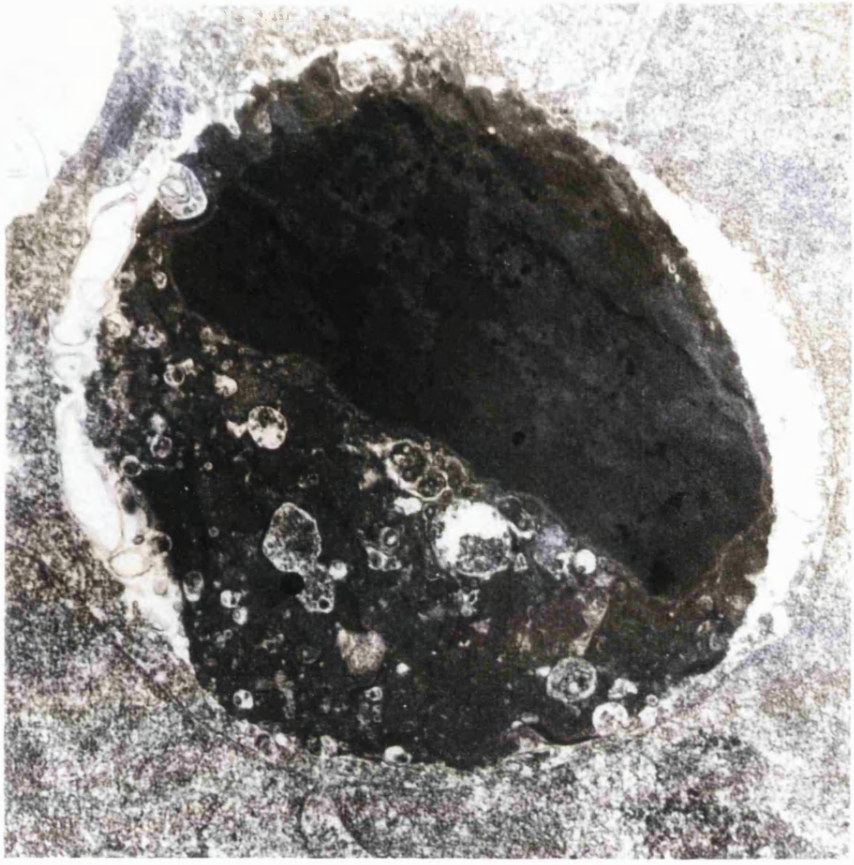


Figure 38: Electron micrograph showing a tumour cell labelled for ET-1 in metastatic tissue.

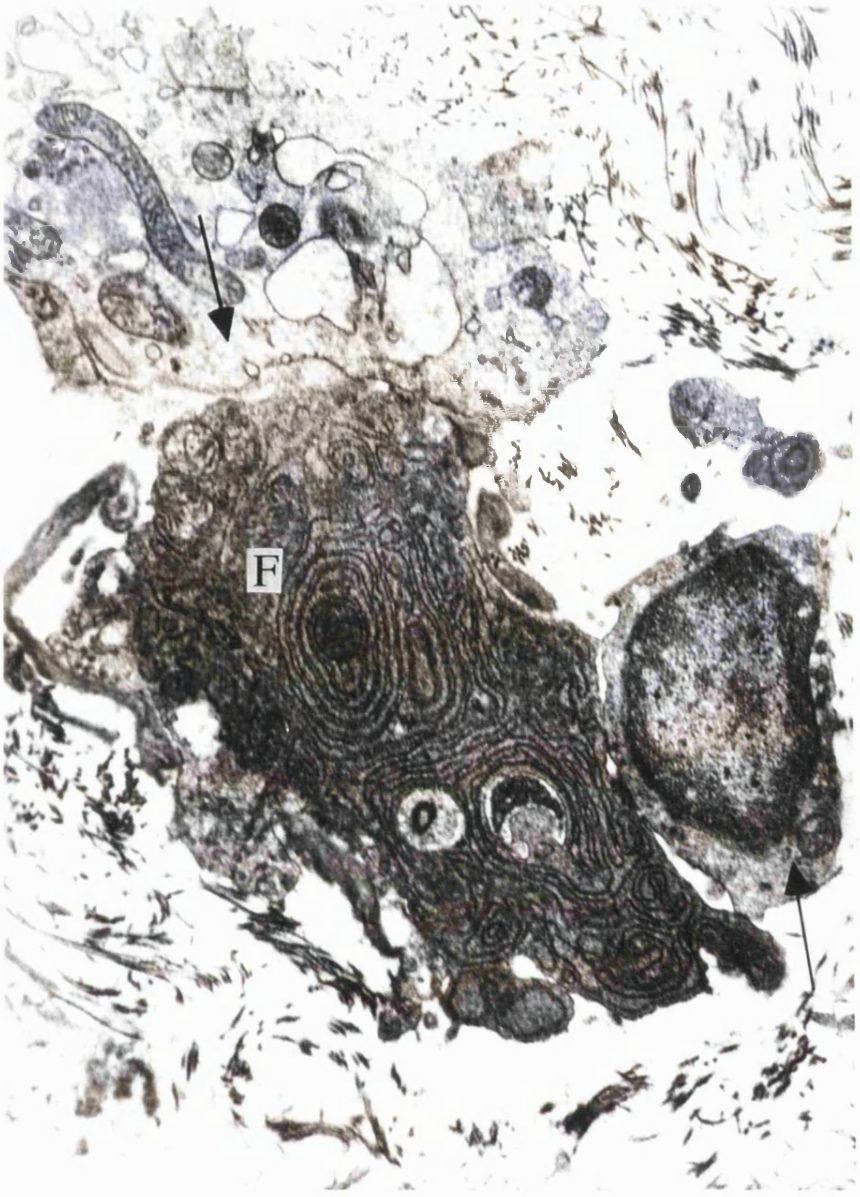


Figure 39: Electron micrograph showing an ET-1 positive labelled fibroblast (F) within the liver metastasis.

Note the adjacent unlabelled cells (arrows).

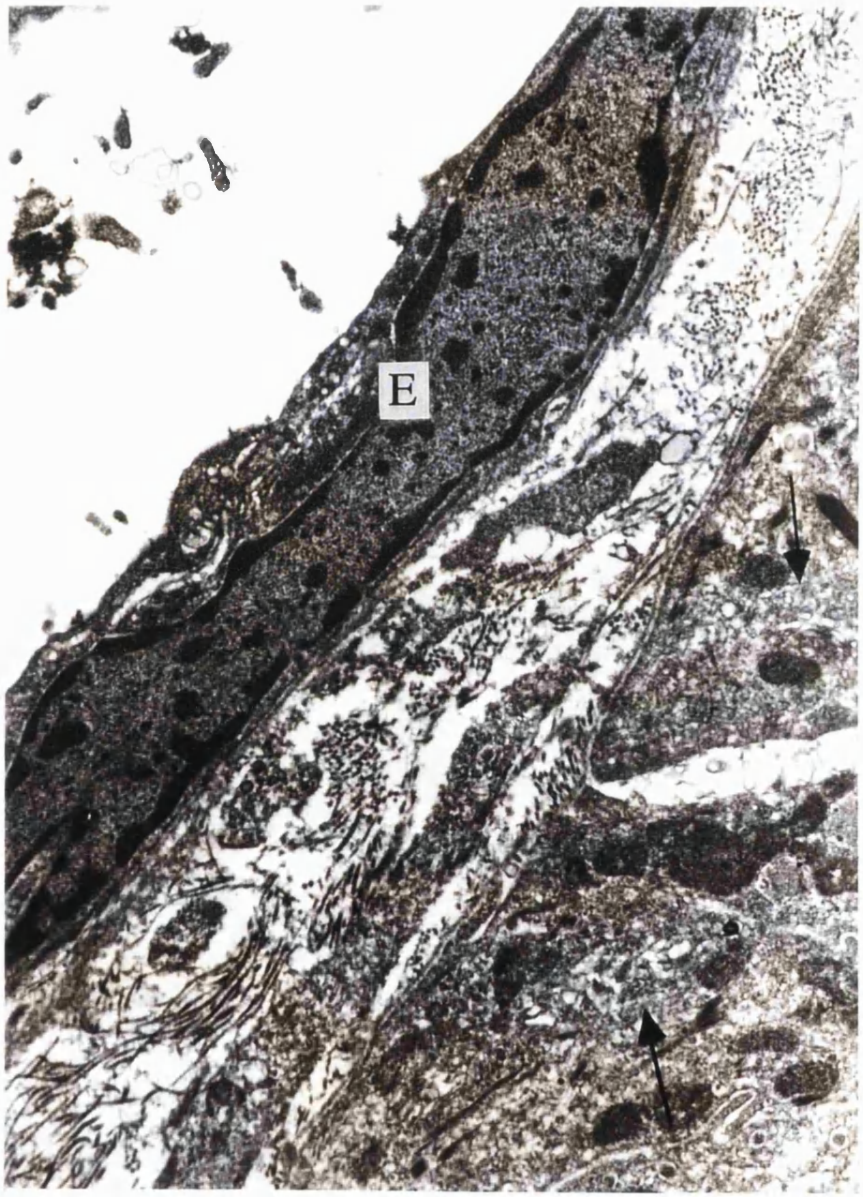


Figure 40: Electron micrograph of a blood vessel within a liver metastasis, showing typical ET-1 immunostaining of an endothelial cell (E).

Note the adjacent unlabelled cells (arrows).

Section II - The measurement of plasma levels of endothelin-1 in patients with colorectal cancer.

3.3 Methods

3.3.1 Blood collection

Three groups of patients were studied:
Group 1-controls (n=22) which were patients admitted for elective non colorectal surgery
Group 2-patients with primary colorectal cancer without liver metastases (n=12)
Group 3-patients with liver metastases who had already had their primary resected and had been referred for liver resection (n=18)
All patient details are provided in appendices 10-12.

Hypertension, renal failure, heart disease, pre-existing liver disease and Raynauds phenomenon are known to be independently associated with elevated levels of ET-1 and hence patients with these conditions were excluded from the study.

Clinical information including carcinoembryonic antigen levels (CEA) and tumour load were recorded for each patient.

All patients had a fasting venous specimen taken prior to surgery which was collected into precooled blood bottles; blood for Thrombomodulin (TM) assay was collected into bottles containing 0.109M trisodium citrate and ET-1 in ethylene diamine tetra-acetic acid. Thrombomodulin was used as a marker of endothelial damage, as ET-1 levels are elevated in the presence of endothelial trauma. If the levels of ET-1 were elevated in the presence of normal thrombomodulin then the source could not be

endothelial cell injury. The specimens were then centrifuged in a chillspin (4° for 15 minutes at 1000 G) and the plasma frozen at -70° within 1 hour of collection.

3.3.2 Endothelin-1 radioimmunoassay

Plasma ET-1 was measured by radioimmunoassay (RIA,Nichols Institute Diagnostics Ltd, U.K.) according to the manufacturer's instructions.
Before performing the assay large proteins were separated from the peptides in each sample (sample extraction). The assay was then performed on the semipurified samples. The whole RIA process is summarised in figure 42.

Sample extraction procedure
Plasma samples were thawed on ice. Once thawed 2ml of sample were pipetted into an 8ml polypropylene round bottom tube. 3ml of 4% acetic acid was then added to each tube and mixed thoroughly. Samples were kept on ice until ready to use.
Sep-pak cartridges are plastic cylinders packed with hydrophobic C-18 matrix. These were shipped in dried form and were activated by the addition of methanol. Each column was flushed through with 5ml 100% methanol followed by 5ml deionised water and finally by 5ml 4% acetic acid. The acidified sample was applied to the cartridge and allowed to elute through under gravity and the empty tube was flushed out by adding 3ml of water to the cartridge. The cartridge was eluted with 3ml of 25% ethanol and then with 86% ethanol and the sample collected in a polypropylene tube. This ethanolic sample was converted to complete dryness in an evacuated centrifuge. Dried samples were reconstituted with borate buffer and stored at -20C.

Radioimmunoassay procedure
Conical polypropylene tubes (4.5ml) were labelled in duplicate, for each sample, standard and control. Additional tubes(T) were used for total counts and for non-specific binding(NSB). Borate buffer (0.3ml) was pipetted into each NSB tube.

0.2mls of standards, sample and control extracts were pipetted into the appropriate tubes. 0.1mls of ^{125}I-ET was added to each assay tube and then 0.1ml of rabbit anti-ET antibody was added to each tube except T and NSB. The tubes were mixed thoroughly and incubated overnight at 2-8C^0. Precipitation of the ET-1-anti-ET-1 complex was performed by the addition of anti-rabbit IgG (donkey). This precipitant was added to all tubes except T. The reagent was mixed continually during addition to ensure a uniform suspension. Each tube was vortex mixed and incubated for 30minutes at room temperature. Deionised water was added to each assay tube except T and vortexed. Assay tubes were centrifuged at room temperature at 2-8C^0 for 15mins at 1500-2000g and the supernatant from each tube except T was decanted. The radioactivity was determined in each assay tube on the Cobra Autogamma 5005 counter. Radioactivity in the bound fraction was quantified in counts per second (cpm).

A calibration curve was constructed measuring cpm against known concentrations of ET-1(ie standards) and samples were referenced against this calibration curve(appendix 13). This procedure was performed on a computer using a four parameter logistical fitting program, RIA smart (Canberra-Packard).

3.3.3 Thrombomodulin ELISA assay

Plasma TM was measured by an enzyme linked immunoabsorbant assay kit (ELISA, Diagnostica Stago, Asnieres, France) according to the manufacturer's instructions (summarised in figure 41)

Eack kit contains a microtitre plate coated with anti-TM antibody. 1ml samples or standards were added in duplicate to the wells of the plate and then incubated at room temperature for 2 hours. This was then washed and a second (anti-TM) antibody conjugated to horseradish peroxidase was added, followed by a further 2 hour

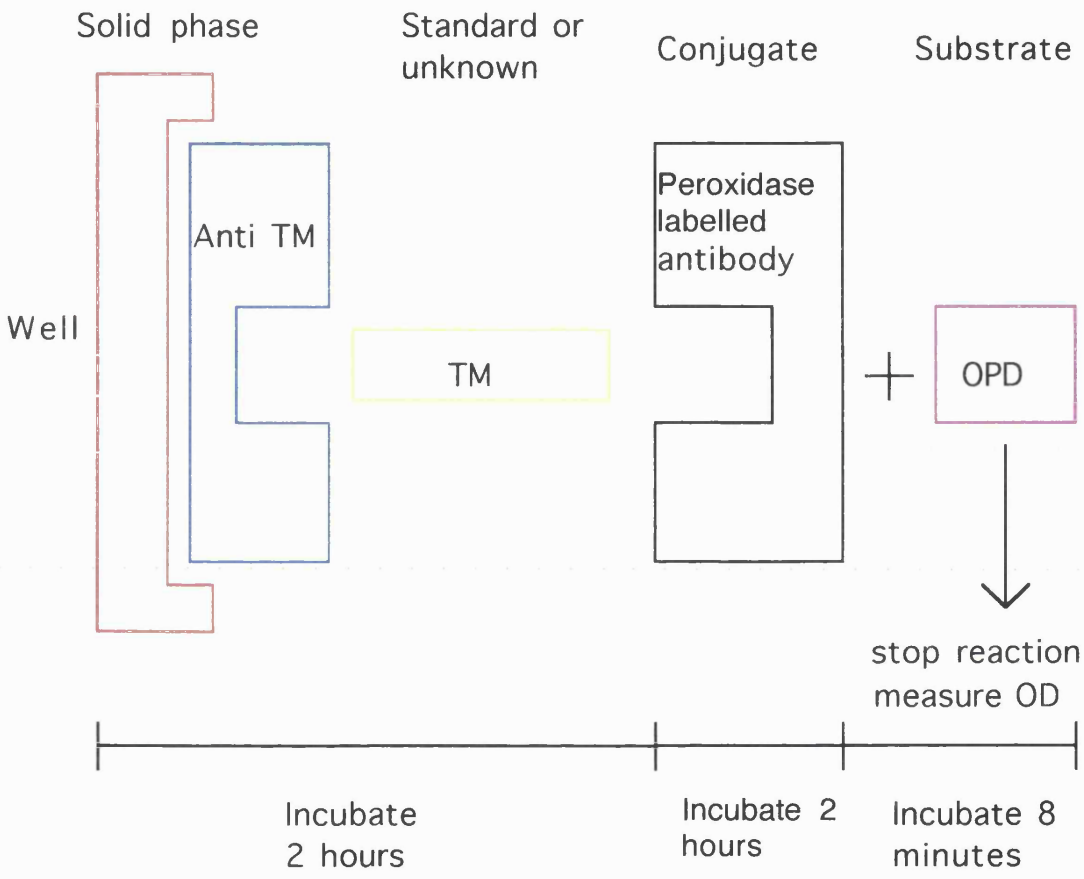


Figure 41. Schematic diagram illustrating principle of ELISA for thrombomodulin.

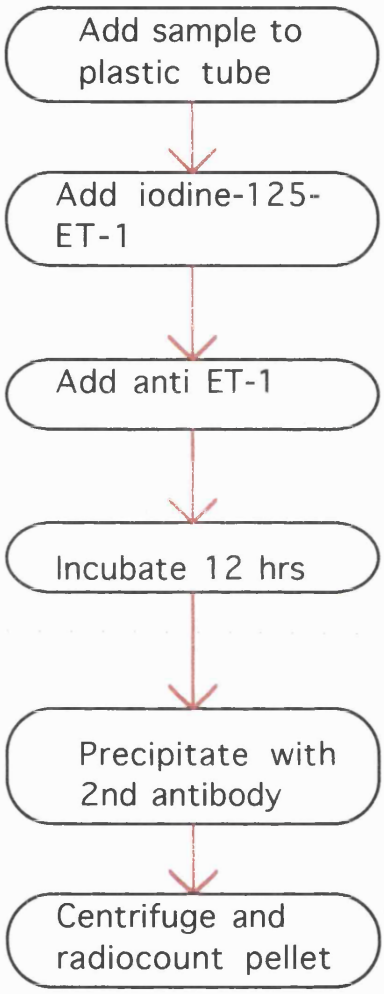


Figure 42: Summary of radioimmunoassay for endothelin-1

incubation. After three washes colour development was achieved by the addition of orthophenylene-diamine in the presence of hydrogen peroxide. The reaction was stopped with sulphuric acid. Plates were read at 492nm and a standard curve plotted using absorbance against log concentration (appendix 14).

Statistics

Values are given as means $\pm$ SD. The significance of the differences were calculated using unpaired t-tests. Values <0.05 were considered significant.

3.4 Results

The mean plasma level of ET-1 in the control group (age range 42-82, mean 59.5, 8 females and 14 males) was 2.7pg/ml, (SD=1.37, n=22). Whilst in the colorectal cancer group, those without metastases (age 55-77, 7 female and 5 male, mean 64.2) had a mean ET-1 of 3.9pg/ml, (SD=1.32, n=12) and those with liver metastases (age 46-75, 2 females and 15 males, mean 58.9) a mean ET-1 of 4.5pg/ml, (SD=1.61, n=18) as illustrated in (figure 44).

There was a significant difference between the cancer patients and the control group ($p=0.001$ and $p=0.02$ for patients with metastases and primary cancer respectively) There was no significant difference between the two groups of cancer patients. No correlation was found between ET-1 levels and serum CEA or tumour size.

There was no statistically significant difference in thrombomodulin level between the control group (mean=53.8ng/ml, SD=15.8) the primary colorectal cancer group (mean=52ng/ml, SD=11.76) and the metastases group (mean=64.3ng/ml, SD=22.6) showed no significant difference (figure 43).

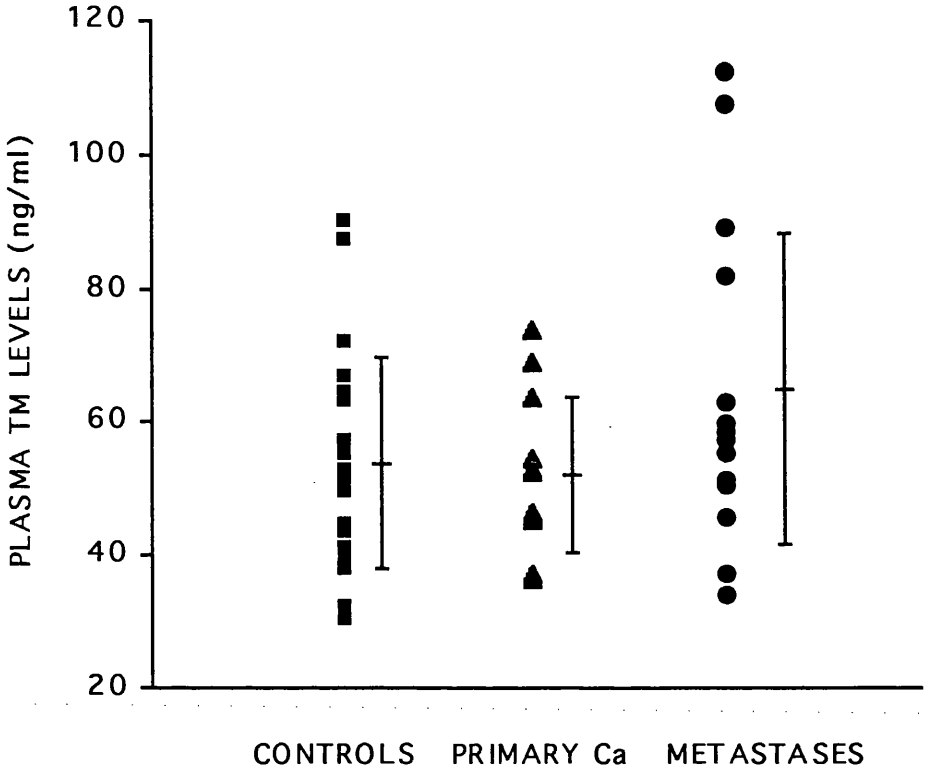


Figure 43: Levels of thrombomodulin (TM) detected in plasma from patients with or without colorectal cancer.

The Y-axis depicts TM levels (ng/ml) calculated by ELISA. The three groups of patients are shown on the X-axis: controls (n=22), patients with primary colorectal cancer (n=12) and patients with previously detected primary colorectal cancer who have now developed liver metastases and those with synchronous liver metastases. Each point corresponds to results from each patient; means and standard deviations (SD) for the three groups of patients are also shown.

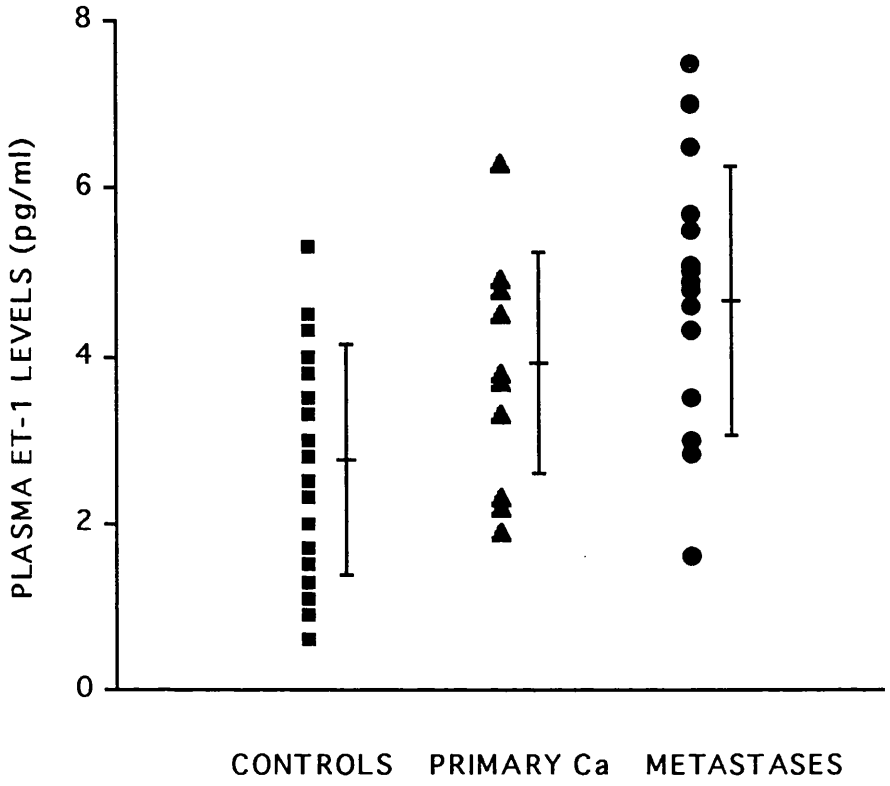


Figure 44: Levels of endothelin-1 (ET-1) detected in plasma from patients with or without colorectal cancer.

The Y-axis depicts ET-1 levels (pg/ml) calculated by radioimmunoassay . The three groups of patients are shown on the X-axis: controls (n=22), patients with primary colorectal cancer (n=12) and patients with previously detected primary colorectal cancer who have now developed liver metastases and patients with synchronous liver metastases. Each point corresponds to results from each patient; means and standard deviations (SD) for the three groups of patients are also shown.

3.5 Discussion

ET-1 is known to be produced predominantly by vascular endothelial cells (Yanagisawa et al 1988), with the precursor peptide processed to the mature form inside the cell. As well as having a local effect, a systemic action is suggested by its presence in the plasma of healthy individuals. The results of this study suggest that a number of different cell types within colorectal liver metastases produce ET-1 with a concomitant rise in plasma levels.

The presence of ET-1 within the endothelium of the normal hepatic vessels -demonstrated by immuno-electronmicroscopy - is in keeping with previous findings in other vascular beds e.g. skin and coronary circulation (Howard et al 1992, Terenghi et al 1991) and conforms to the suggested function of ET-1 as an endothelium derived vasoconstrictor. ET-1 is known to influence normal hepatocytes, bile flow and hepatic arterial and portal venous blood flow, with the liver also being one of the major sites of endothelin clearance (Bluhm et al 1993, Gandhi et al 1990). It is not however a major source of ET-1 as illustrated by its absence in the remainder of the liver cell population as demonstrated in this study.

ET-1 was present in large amounts within the cytoplasm of tumour cells and surrounding myofibroblasts in all the specimens examined, with tumour endothelial cells also stained strongly for ET-1. This elevated expression suggests ET-1 might be produced by both tumour cells and surrounding associated cells.

Previous immunohistochemical studies relating to primary colorectal cancer have demonstrated increased expression of ET-1 receptors on surrounding cells including myofibroblasts.

Furthermore studies involving tissue culture systems have previosly shown that a variety of tumour cell lines including colonic adenocarcinomas produce ET-1, which is then detectable in the culture medium (Masayoshi et al 1991).

A possible explanation for these findings may be found outside the commonly accepted role of endothelins as vasoconstrictors. ET-1 is known to be mitogenic for a variety of cell types including vascular smooth muscle, renal mesangial cells and colonic cancers (Hirak et al 1989, Simonson et al 1989, Kusuhara et al 1989, Masayoshi et al 1991). Masayoshi and colleagues demonstrated that epithelial human cancer cell lines not only produce ET-1, but also caused a rise in mitogenesis in a dose dependant manner. Given the above findings ET-1 may perform a paracrine or autocrine function in colorectal cancers with regard to tumour growth. In addition to its mitogenic effect, ET-1 is also directly and indirectly angiogenic; a process of particular importance in tumour growth. ET-1 is a direct endothelial cell mitogen and indirectly stimulates angiogenesis by causing monocytes to produce interleukin-8, a potent angiogenic agent (Giaid et al 1995, Huribal et al 1994). Therefore ET-1 may play a part in tumour growth regulation in colorectal liver metastases.

The increase in production of ET-1 by colonic cancers is mirrored in the plasma of such patients, suggesting a systemic as well as a local function. It is recognised that primary tumours may influence not only their own growth but also those of distant metastases (Prehn 1991). This appears to be achieved by the release of regulatory angiogenic and mitogenic substances (Blood and Zetter 1990). ET-1 may perform a function in this regard with increased production perhaps leading to greater metastatic growth. The use of ET-1 as a tumour marker is however unlikely to be of clinical usefulness given the degree of overlap in the three groups of patients studied and the number of other conditions independently associated with its elevation.

The increased expression of ET-1 in tumour vessel endothelial cells compared with normal hepatic vasculature - as demonstrated using immunospecific peroxidase - may suggest an up regulation of its vasoconstricting effect in addition to growth stimulation.

Systemic levels of ET-1 are elevated in a variety of pathological conditions associated with vasospasm including pregnancy induced hypertension, cardiogenic shock and myocardial infarction (Miyauchi et al 1989, Kamai et al 1990, Cernacek et

al 1989). It has been known for some time that the presence of liver metastases is associated with subtle alterations in hepatic blood flow (Hemmingway et al 1991). The hepatic perfusion index, which is the ratio of hepatic arterial flow to total liver blood flow, is elevated in such patients, even at a micrometastatic stage (Leen et al 1995) .This phenomenon is caused by splanchnic vasoconstriction and a subsequent reduction in portal inflow. Animal studies suggest that this is due to the presence of a circulating vasoconstrictor which is not one of the commonly identified physiological vasoactive agents (Warren et al 1993). Clinical studies looking at patients with liver metastases suggest that the HPI may be used as an accurate predictor of the presence of hepatic metastases (Leen et al 1996). Given that ET-1 is a potent vasoconstrictor with marked effects on the splanchnic bed and is produced by colorectal cancers, it might have a role in this phenomenon. However in this study there was no significant difference in the plasma levels of ET-1 between patients with and without liver metastases.

This study demonstrates that in colorectal liver metastases endothelial cells contain elevated levels of ET-1 and ET-1 expression is upregulated in both tumour cells and associated myofibroblasts. An elevation of ET-1 is also found in the plasma of patients with colorectal cancer with and without liver metastases. Whether or not ET-1 regulates tumour growth locally and systemically and whether its increased levels indicate progression of disease remains to be determined by future studies.

As mentioned ET-1 elicits its actions via 2 receptors A and B under normal physiological conditions. Within the framework of carcinogenesis and tumour growth regulation it is suggested that the ETA receptor is more important (Nelson et al 1996, Economos et al 1992). These studies pertain to prostate and ovarian cancers, as yet no similar studies have been performed using colorectal cancers. Two key points need to be answered prior to contemplating the clinical application of ET-1 receptor antagonists. Firstly which receptor, if any, is upregulated in colorectal cancers and secondly does application of the appropriate antagonist lead to a reduction in cell turnover in vitro.

Once these questions have been answered then the next question is route of administration. Prophylactic therapy aimed at reducing the development of colorectal liver metastases at the time of resection of the primary tumour would dictate use of the portal vein whilst treatment of established hepatic metastases might be given via the hepatic artery.

Since the MC28 tumour when grown in Hooded Lister rats produces tumours with similar morphological and ET-1 characteristics to human colorectal liver metastases, this might be the most appropriate model in which to assess the effect of ET-1 receptor antagonists.

Conclusion of thesis

Summary of findings

1. Infusion of vasoconstrictors via the hepatic artery in non-tumour bearing animals led to reductions in hepatic blood flow which varied both in duration of action and maximal effect. This reduction in blood flow was coupled with a rise in systemic blood pressure which lasted throughout the infusion.

2. None of the agents studied produced reductions in liver blood flow throughout the entire infusion period.

3. When the 20% BP rise dose was considered seperately the agent which produced the greatest overall reduction in blood flow over the 30 minute period in non-tumour bearing animals was vasopressin.

4. In tumour bearing animals HAI of the optimum doses of vasopressors again led to variable reductions in both liver and tumour blood flow. None of the agents produced increases in tumour blood flow.

5. Over the entire infusion period none of the agents produced a significant change in the T/N blood flow ratio. However over the period in which the agents produced changes in liver blood flow all the agents apart from l-NAME produced improvements in the T/N ratio ($p<0.05$), with NA having the greatest effect.

6. When radiolabelled 5FU was administered in conjunction with HAI vasopressors, none of the agents produced changes in drug uptake by the liver. However NA produced significant improvements in uptake of 5FU by the tumour ($p<0.05$).

7. The plasma levels of ET-1 were elevated in patients with colorectal cancer, with and without liver metastases. This elevation was not due to tissue trauma, as might occur with tumour expansion, as demonstrated by the similar ($p>0.05$) TM levels in all three groups of patients.

8. In the normal liver the only cells which stained weakly positive for ET-1 using immunoelectronmicroscopy were the endothelial cells. Within the tumour the tumour cells, fibroblasts and endothelial cells all stained strongly positive for ET-1.

Blood vessels within colorectal liver metastases have been demonstrated to differ from normal hepatic vessels both in their morphology and innervation. Such differences may allow manipulation of the blood flow within the liver of patients with colorectal liver metastases to improve drug delivery to the tumour.

Using an animal model, HAI vasoconstrictor agents, including ET-1, have been shown to produce variable reductions in liver blood flow. However none of the agents produced effects lasting the duration of the infusion. Those doses of agent which produced a 20% rise in systemic blood flow were then infused via the hepatic artery in animals with intra-hepatic MC28 tumours.

All the agents, excluding L-NAME, produced elevations in the T/N ratio, although only noradrenaline produced improvements in uptake of 5FU by the tumour. Given that none of the agents produced improvements in total tumour blood flow, alterations in drug uptake by the tumour must be due to mechanisms other than simple changes in blood flow. Changes in pressure gradients within the tumour and adjacent liver may account for the alterations seen.

Vasoconstrictor agents, especially noradrenaline, may have a role in improving drug delivery in regional chemotherapy. However their short durations of action preclude their use as a continuous infusion. This leaves two possible clinical uses. Firstly they may be infused with bolus therapy, administered during the peak effect of the agent.

Secondly if the refractory period of the agent could be calculated then the vasoconstrictor could be re-administered during a continuous infusion. In this instance the agent could be infused at time intervals depending on the refractory period of the agent in an attempt to limit the effect of the escape phenomenon.

Although ET-1 did not improve drug uptake when administered with HAI it may possess other actions which might be modulated in an attempt to treat tumour growth from another perspective. In addition to its actions as a vasoconstrictor ET-1 may play a role as both a angiogenic and mitogenic agent in the natural history of colorectal cancer.

It has been demonstrated in this study that a variety of cell types within colorectal liver metastases produce ET-1, with the adjacent liver producing minimal amounts. It has also been shown that the plasma levels of ET-1 are elevated in patients with colorectal cancer, with and without liver metastases. This elevation is due to increased production rather than release from damaged tissue.

These findings suggest that ET-1 actions are both local and systemic and may play a part in the regulation of tumour development. The fact that adjacent fibroblasts produce ET-1 suggests that these cells may play a role in controlling tumour growth. The presence of ET-1 within endothelial cells in the tumour in such high concentrations (compared to those within the normal liver) may suggest a role for ET-1 in the regulation of tumour angiogenesis or perhaps as a controller of tumour blood flow.

The elevation in systemic plasma levels raises the posibility that ET-1 may play a role in the metastatic potential of a tumour. Its secretion into the circulation may promote distant tumour growth, especially given its known actions on angiogenesis and tumour mitogenesis. Although the plasma levels are too low to induce obvious changes in vasomotor tone, the elevated levels may be sufficient to alter the response of blood vessels to stimuli. Such changes in feeding vessels may improve blood flow to the tumour whilst shifting blood away from other structures.

Given the recent availability of ET-1 receptor antagonists the possibility of exploiting these findings in a clinical setting maybe possible. However prior to contemplating the use of antagonists to ET-1 in patients with colorectal cancer a number of questions need to be answered;

a)Are ET-1 receptors upregulated in colorectal cancers both in specimens and tissue culture?

b) Which ET-1 receptor is responsible for its effects on tumour growth?

c) Does application of ET-1 receptor antagonists lead to reduction of tumour load in appropriate animal models and in vitro?

d) Does ET-1 expression by colorectal tumours correlate with survival?

e) By what route should ET-1 receptor antagonists be given?

These five points should form the basis of further studies with regard to ET-1 and colorectal cancer.

Knowledge of the actions of vasopressors on blood flow and development of colorectal liver metastases may allow improvements in treating patients both in prophylaxis and established tumours.

Appendices

Appendix 1.

Results of intra-hepatic artery infusion of vasopressin on liver blood flow and systemic blood pressure in non-tumour bearing animals.

Dose vasopressin (mcg/kg/min)	Duration of effect (mins)	Max % flux drop	% area change	Max % BP change
0.6	9.6	28.1	11.0	12.0
	10.2	24.3	9.0	14.5
	10.4	32.0	4.0	10.3
	12.3	34.1	5.4	9.2
	11.8	29.6	7.2	10.4
	12.1	27.6	6.4	11.6
0.8	12.6	30.1	17.0	15.2
	13.1	34.6	14.0	16.7
	11.2	29.4	9.8	19.4
	13.4	36.3	10.7	17.4
	12.8	32.4	12.1	17.1
	13.2	30.6	11.2	14.8
1.0	14.1	40.3	16.0	23.4
	10.5	46.1	16.3	18.7
	11.4	38.2	12.3	23.1
	13.2	33.1	10.5	19.3
	12.8	34.8	13.4	18.9
	13.8	30.6	11.8	20.8
1.2	14.1	35.1	18.4	28.1
	12.6	44.1	10.3	34.3
	13.4	49.1	10.4	30.1
	14.4	37.4	17.6	37.3
	13.9	38.7	15.4	29.4
	13.4	39.4	14.8	31.7

Appendix 2.

Results of intra-hepatic arterial infusion of noradrenaline on liver blood flow and systemic blood pressure in non-tumour bearing animals.

Dose of noradrenaline (mcg/kg/min)	Duration of effect (mins)	Max % flux drop	% area change	Max % BP change
2.5	2.3	25.1	2.5	10.1
	2.6	31.2	2.8	14.6
	1.6	20.7	1.1	17.1
	1.6	24.8	1.6	12.3
	2.1	23.6	2.1	13.4
	1.7	22.6	2.8	12.1
5.0	3.2	32.2	3.6	17.4
	2.4	25.1	1.7	19.4
	2.2	28.1	1.9	22.4
	1.6	30.4	3.1	24.1
	2.1	31.6	3.5	21.3
	3.1	29.6	2.6	19.7
7.5	3.1	54.1	3.0	31.4
	2.1	46.7	1.9	42.1
	3.4	41.1	2.8	28.7
	2.5	59.2	2.8	33.4
	2.6	43.6	3.1	27.6
	2.3	39.4	2.6	24.1
10.0	3.0	42.1	3.5	47.1
	3.5	55.6	2.9	58.3
	2.1	67.8	3.4	60.2
	2.6	51.2	3.8	62.1
	3.2	54.3	2.8	51.2
	2.5	41.6	2.4	41.8

Appendix 3.

Results of intra-hepatic arterial infusion of angiotensin-II on liver blood flow and systemic blood pressure in non-tumour bearing animals.

Dose of angiotensin-II (mcg/kg/min)	Duration of effect (mins)	Max % flux drop	% area change	Max % BP change
0.2	5.1	28.1	5.0	12.0
	4.3	22.4	3.3	9.0
	3.2	33.4	4.6	13.0
	3.5	19.8	1.8	10.0
	4.1	24.5	2.1	8.0
	4.3	25.6	3.2	9.4
0.3	5.3	34.6	2.8	14.7
	4.5	30.2	3.3	17.1
	6.1	39.6	4.0	19.4
	3.5	42.7	3.8	21.7
	4.3	35.8	3.1	16.4
	4.5	33.1	2.9	16.8
0.35	4.1	40.0	3.0	19.1
	6.0	44.6	6.5	22.7
	5.0	53.1	5.6	18.6
	4.5	40.7	3.8	23.8
	5.2	42.1	4.1	21.6
	4.4	41.7	3.4	22.1
0.45	6.2	52.0	3.7	38.7
	5.3	58.0	7.1	40.1
	4.6	49.1	5.8	33.1
	6.3	50.6	6.4	43.1
	6.1	57.4	6.9	41.6
	6.4	47.8	6.8	34.8

Appendix 4.

Results of intra-hepatic arterial infusion of l-NAME on liver blood flow and systemic blood pressure in non-tumour bearing animals.

Dose of l-NAME (mg/kg/min)	Duration of effect (mins)	Max % flux drop	% area change	Max % BP rise
0.5	0	0	0	6.4
	0	0	0	5.0
	0	0	0	6.1
	0	0	0	4.2
	0	0	0	7.0
	0	0	0	6.8
1.0	24.3	5.1	5.6	11.0
	26.1	4.2	2.3	15.1
	23.5	4.8	4.1	8.0
	25.1	3.7	3.4	10.3
	26.4	4.7	4.7	12.1
	24.8	3.9	3.8	13.4
1.5	22.4	6.1	4.8	19.1
	27.1	5.6	7.1	22.1
	24.2	4.8	10.2	15.8
	25.2	4.7	6.7	18.7
	26.1	5.2	6.1	16.4
	23.8	4.6	8.1	19.8
2.0	24.35	7.4	9.4	25.1
	27.1	6.6	8.7	29.4
	26.2	5.2	8.4	30.2
	25.1	5.8	10.7	24.8
	24.9	6.2	7.4	27.4
	23.7	5.8	8.7	28.1

Appendix 5.

Results of intra-hepatic arterial infusion of ET-1 on liver blood flow and systemic blood pressure in non-tumour bearing animals.

Dose of ET-1 (mcg/kg/min)	Duration of action (mins)	Max % flux drop	% area change	Max % BP rise
0.17	15.0	14.2	3.5	11.0
	19.1	10.5	4.4	12.5
	15.3	12.6	4.2	8.3
	14.3	13.1	2.7	13.2
	17.3	14.6	1.9	7.1
	18.5	14.1	3.0	9.6
0.33	16.0	19.0	3.7	15.0
	16.4	21.4	6.2	17.0
	15.4	16.3	7.2	15.8
	23.1	17.1	11.3	16.7
	19.1	16.5	4.6	14.1
	18.4	17.8	5.1	18.0
0.5	19.3	26.4	8.1	16.1
	19.6	21.1	9.1	21.7
	21.0	18.7	5.6	18.6
	22.4	24.1	7.4	20.6
	24.5	19.6	6.8	17.5
	20.3	22.4	6.1	18.7
0.66	19.7	25.2	13.2	29.1
	24.6	27.8	9.8	31.6
	23.4	21.7	11.1	36.4
	18.6	20.8	10.6	21.7
	23.1	22.4	12.1	26.4
	25.1	26.1	13.2	28.1

Appendix 6.

% changes in the T/N ratio over the whole 30 min hepatic arterial infusion period for the agents studied.

Saline	AG-II	NA	VAS	ET-1	l-NAME
0.56	1.5	4.0	3.0	6.9	3.0
0.25	1.9	4.3	2.5	3.1	1.7
0.58	1.2	2.9	1.2	4.6	1.2
0.67	1.3	3.5	2.8	2.9	2.4
0.43	1.5	3.2	3.0	8.8	1.3
0.76	1.8	2.8	3.4	5.3	2.0

Appendix 7.

% changes in the T/N ratio over the period of effect of the hepatic arterially infused agents studied.

Saline	AG-II	NA	VAS	ET-1	l-NAME
2.2	10.1	35.0	7.6	17.1	3.2
1.2	14.2	40.1	7.4	11.7	2.1
1.7	8.0	29.3	3.3	14.1	1.8
2.3	9.2	35.4	6.9	10.4	2.8
1.4	10.7	38.2	8.6	22.2	1.9
2.6	7.8	27.3	5.9	7.2	2.4

Appendix 8.

Results of uptake of [^{3}H]-5FU by normal liver after hepatic arterial infusion of the various agents (cpm/g).

Saline	AG-II	NA	VAS	ET-1	l-NAME
42.2	9.1	34.6	13.5	4.7	15.6
9.7	10.0	10.8	39.2	6.9	4.0
49.3	14.1	6.1	14.2	15.5	13.1
24.1	6.6	9.8	13.0	7.3	15.4
3.9	10.5	10.8	1.7	6.1	13.3
3.1	21.0	13.2	13.4	14.8	20.9

Appendix 9.

Results of uptake of [^{3}H]-5FU by tumour after hepatic arterial infusion of the various agents (cpm/g).

Saline	AG-II	NA	VAS	ET-1	l-NAME
6.8	4.9	36.2	10.4	3.8	6.6
7.2	3.2	19.5	9.8	10.8	2.5
9.2	14.1	27.3	7.8	39.7	2.3
4.2	2.8	9.2	8.1	26.2	4.5
1.9	1.1	25.7	1.7	3.8	6.8
1.0	4.8	13.9	3.5	10.9	3.2

Appendix 10

Details of patients in the control group undergoing measurement of plasma levels of ET-1 and TM.

Initials	Age/sex	Operation	ET-1 plasma levels (pg/ml)	TM plasma levels (ng/ml)
WT	50/M	Inguinal hernia	4.0	40.2
AS	64/M	Inguinal hernia	1.1	41.2
RC	65/M	Incisional hernia	0.9	43.5
AI	71/F	Periumbilical hernia	4.5	30.3
MM	61/M	Inguinal hernia	3.8	53.1
MT	52/M	Inguinal hernia	4.3	52.2
PL	54/M	Lipoma	5.3	63.5
AS	49/F	Benign breast lumpectomy	3.0	44.8
JF	61/M	Epididymal cystectomy	2.5	87.6
DK	60/M	Inguinal hernia	1.5	67.1
TH	73/M	Inguinal hernia	0.6	41.1
DB	64/F	Benign breast lumpectomy	2.8	37.9
AL	51/F	Haemmoroid	3.0	64.7
AB	46/M	Benign anal stricturoplasty	3.5	57.4
JV	69/F	Lap choly	1.3	50.7
HM	42/F	Lap choly	2.0	32.4
RU	45/M	Inguinal hernia	2.3	90.4
MF	67/F	Benign breast lumpectomy	1.7	55.4
IB	65/F	Inguinal hernia	0.9	57.2
EM	63/M	Inguinal hernia	3.3	51.1
LP	56/F	Benign breast lumpectomy	4.5	49.8
AZ	58/F	Subtotal thyroidectomy Benign	3.8	72.3

Appendix 11.

Details of patients with primary colorectal cancer with no evidence of liver metastases undergoing measurement of plasma ET-1 and TM levels.

Initials	Age/sex	Operation	Dukes stage	ET-1 plasma levels (pg/ml)	TM plasma levels (ng/ml)	CEA plasma levels
AB	75/F	Rt Hemi	A	4.5	46.0	<1
MP	58/F	Lt Hemi	B	3.3	53.1	1
MT	55/F	Subtotal colectomy	B	4.8	45.4	<1
BR	60/F	Ant Res	B	4.5	37.1	1
NH	80/F	Ant Res	C	1.9	45.7	2
RG	77/M	A-P Res	B	2.2	46.7	1
NF	68/M	Ant Res	B	3.7	69.0	43
BM	65/F	Subtotal colectomy	C	3.8	52.5	3
TS	59/M	Rt Hemi	A	4.9	73.9	2
PM	63/M	A-P Res	B	4.9	54.5	3
JS	66/F	Ant Res	C	2.3	36.6	<1
LL	67/M	Rt Hemi	B	6.3	63.8	16

Appendix 12.

Details of patients with colorectal liver metastases undergoing measurement of plasma ET-1 and TM levels.

Initials	Age/sex	Primary resection/ time to Biopsy	Hepatic load	Primary histo	ET-1 plasma levels (pg/ml)	TM plasma levels (ng/ml)	CEA plasma levels
DN	75/M	Ant Res Sync	Bilateral >3 per lobe	Dukes C	6.5	58.7	720
CT	55/M	Ant Res Sync Bx during HAC 1/12 later	Bilateral >3 per lobe	Dukes B	4.8	45.8	51
CS	50/M	Rt Hemi Sync	Bilateral >3 per lobe	Dukes C	7.5	107.7	1650
JM	48/F	3years prior-Ant Res Lt Hep	x2 left lobe	Dukes C	4.3	34.0	<1
CT	52/M	Advanced Ca rectum +bilateral Sync liver mets	Bilateral >3 per lobe	NO specimen	5.7	63.0	44
EB	57/M	2 years prior Ant Res	Bilateral >3 per lobe	Dukes C	3.0	37.1	22
GB	61/M	10/12 prior Rt Hemi Metac mets	Bilateral >3 per lobe	Dukes C	1.6	59.6	12
PG	59/M	2 years prior Lt Hemi Metac mets Rt Hep	x 1 Rt lobe met	Dukes B	3.5	57.5	4
MD	57/M	14/12 Ant Res Metac mets HAC	Bilat >3 each lobe	Dukes C	3.0	81.7	8
RK	54/F	Rt Hemi+ Hickman Sync mets	Bilat >3 each lobe	Dukes C	4.6	89.0	54

Initials	Age/sex	Primary resection/ time to Biopsy	Hepatic load	Primary histo	ET-1 plasma levels (pg/ml)	TM plasma levels (ng/ml)	CEA plasma levels
WL	69/M	Sig Col+ HAC Sync mets	Bilat >3 per lobe	Dukes C	5.0	55.4	41
MS	51/F	Sig Col 3yr prior Metac mets Liver Bx	x 1 Met Rt lobe	Dukes B	5.5	51.4	8
WG	71/M	Rt Hemi Sync mets	Bilat >3 per lobe	Dukes C	7.0	81.9	11
PT	53/M	Ant Res +chemo 10/12 prior Sync mets Rt Hep	x2 Mets Rt lobe	Dukes C	4.6	51.5	4
BC	64/M	Rt Hemi 10 yrs prior Metac met Rt Hep	x1 Met Rt lobe	Dukes B	5.1	50.4	6
DM	66/M	Rt Hemi 3yrs prior Metac mets Hickman	Lt lobe replaced by tumour	Dukes C	2.8	57.3	7
CS	62/F	Lt Hemi 2yrs prior Metac mets Hickman	Bilat >3 per lobe	Dukes C	4.9	112.5	3

Appendix 13

Construction of standard curve for endothelin-1 assay

Standard curve:

The standard curve is generated using endothelin standards prepared at the time of each assay. Generation of the curve is as follows;

1. Calculate the average counts (cpm) for each pair of assay tubes.

2. Subtract the average of the NSB(non-specific binding) tubes from every other count to obtain correct cpm.

3.Calculate the percent maximum binding (%Bo/T) if desired, by dividing the corrected cpm of the zero standard (Bo) by the average cpm of the total count tubes (TC).

4. Calculate the percent %B/Bo for each standard point by dividing the average corrected counts for each standard tube by the average corrected counts for the zero standard (Bo).

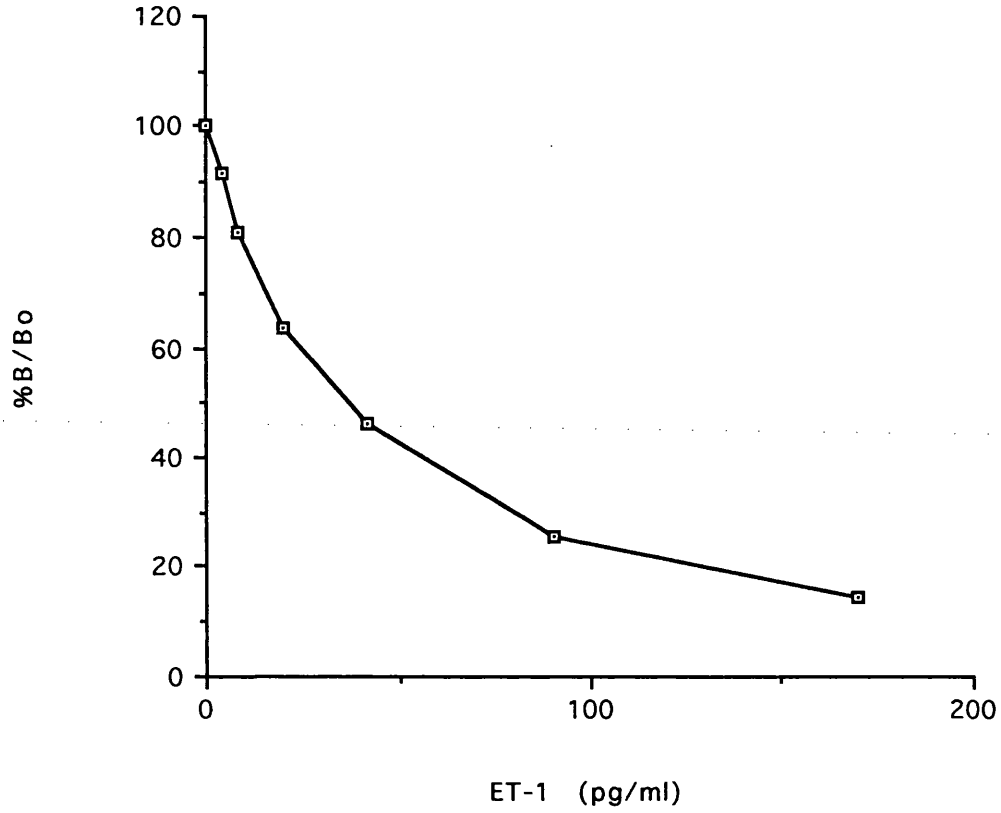
5.Prepare the standard curveby plotting %B/Bo on the ordinate against the standard concentrations on the abscissa using semi-log graph paper.

Sample calculation:

1. Calculate the % B/Bo for each sample by dividing the average corrected counts for each sample by the average corrected counts of the zero standard.

2. Read sample value off the standard curve in pg/ml.

3. To obtain final result, divide sample value read from the curve by a factor of 4.



Standard curve for calculation of plasma level of ET-1. ET-1 (pg/ml) on the X axis and %B/Bo on the Y axis. Refer to previous section to convert raw data into final value.

Appendix 14

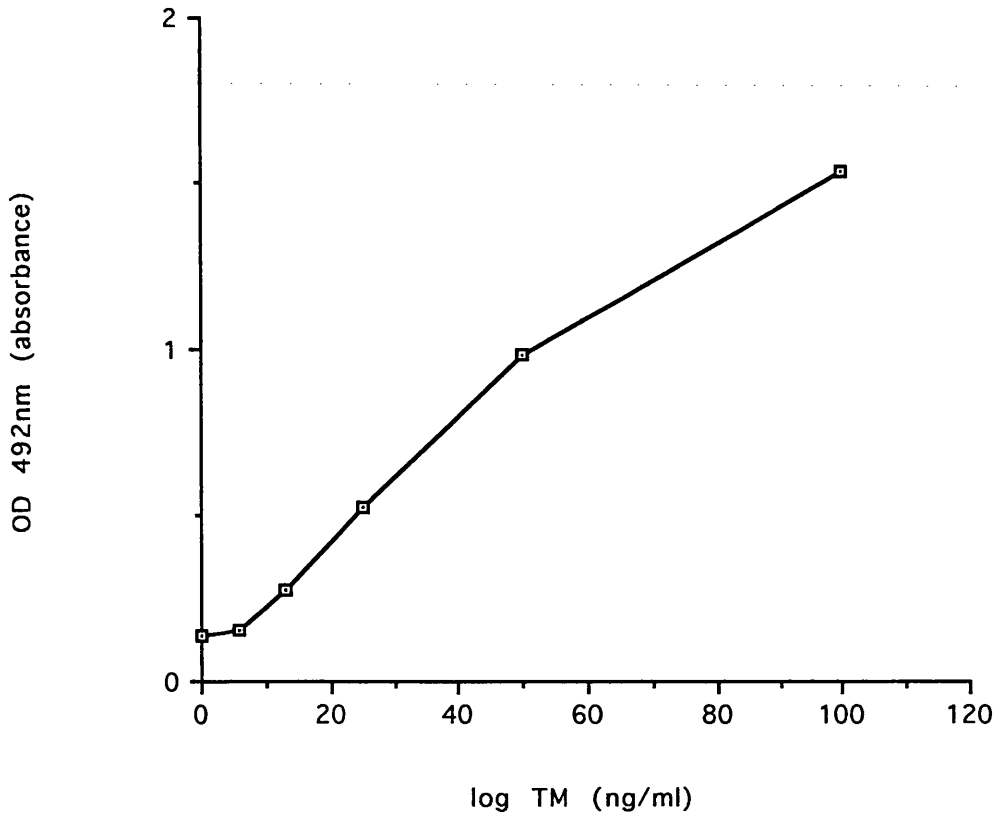
Construction of the standard curve for thrombomodulin assay

Each kit is supplied with a series of standard thrombomodulin calibrators.

1.Plot the log concentration of the thrombomodulin calibrators (ng/ml) on the X axis and the corresponding log optical density values on the Y axis.

2. Interpolate the concentrations of thrombomodulin of test solution on the calibration curve to determine their values.

3. To obtain the thrombomodulin concentration of the tested sample multiply the value obtained by the dilution factor of 10.



Standard curve for calculating plasma thrombomodulin levels. Optical density values at 492nm(absorbance) on the Y axis and log TM (ng/ml) on the X axis.

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