CLINICAL MEASUREMENT AND MODULATION OF MICROREGIONAL TUMOUR BLOOD FLOW

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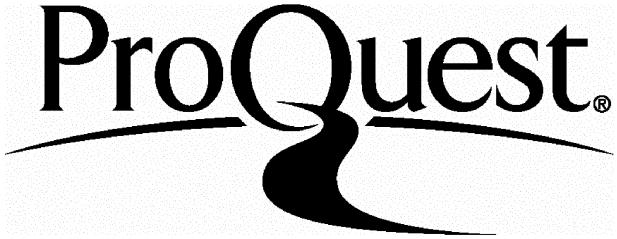

Abstract

Modification of the tumour microenvironment by improving tumour blood flow and oxygenation may be a method of enhancing cure rates. Pre-clinical studies have shown the combination of carbogen and nicotinamide can effectively radiosensitize, however, the mechanism of action for this remains unclear. Carbogen enhances tumour oxygenation primarily by increasing the amount of oxygen dissolved in blood. Animal studies suggest it may also improve tumour blood flow. In murine tumours nicotinamide reduces the occurrence of temporal fluctuations in blood flow. Commercially available laser Doppler microprobes have now made possible the direct real-time measurement of erythrocyte flux in human tumours, allowing the influence of carbogen and nicotinamide on microregional blood flow to be assessed.

Red blood cell flux was measured using the Oxford Array multiple channel laser Doppler system (Oxford Optronix, Oxford, UK). Measurements were taken for 60 minutes in thirteen patients (control group), eight also breathed carbogen for 10 minutes. A further ten patients were pre-treated with nicotinamide prior to carbogen.

Fluctuations in erythrocyte flux were a common event, 62% of sampled areas showed a change of 1.5 fold or more. Both increases and decreases were seen. Carbogen caused small, tumour dependent changes in flux with both increases and decreases. Nicotinamide did not significantly alter changes in microregional blood flow but when combined with carbogen an overall increase in flux of 22% was seen, with all but one tumour showing an increase.

The improvement in microregional perfusion seen with the combination of carbogen and nicotinamide could have important clinical implications for both radiotherapy and the delivery of systemic agents.

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CHAPTER 1

Introduction

1.1 The tumour microenvironment and treatment resistance

Over the past three decades there have been considerable advances in cancer treatment, with the advent of chemotherapy, immunotherapy, antibody and gene therapy. But despite initial enthusiasm for the various new treatments, their limitations have become apparent. In recent years we have developed better knowledge of the particular intrinsic molecular and cellular characteristics that lead to treatment resistance. Such traits include inherent radiosensitivity and gene amplification. Furthermore, response to treatment is determined by the metabolic microenvironment of an individual tumour, and it is the disorganised and inadequate vascular network, so typical of tumours, that defines the physiological properties that create this micromilieu. These properties include blood flow, oxygen and nutrient supply, energy status and tissue pH. They are all inextricably linked and interdependent upon each other and may, either directly or indirectly, influence the response to many treatment modalities, including radiation, chemotherapy, biological therapy and photodynamic therapy.

Achieving a better understanding of the pathophysiology of tumour perfusion may lead to the development of targeted strategies designed to enhance the therapeutic potential of both conventional and novel anti-cancer treatments. It is the aim of this work to help gain this understanding by characterising the dynamic nature of microregional blood flow within human tumours.

1.2 Historical perspective

It was in 1909 that Gottwald Schwarz demonstrated that if blood flow to an area was reduced, the radiation reaction of skin was diminished, enabling a higher dose to be given to deep seated tumours (Schwarz 1909). Although he did not recognise it as such, Gottwald Schwarz had described the first clinical observation of the significance of hypoxia.

In the late 1920s and early thirties, following the pioneering work of Warburg, it came to be understood that certain physiological characteristics distinguished solid tumours from normal tissue. Warburg studied tumour metabolism and noted their lower oxygen and glucose supply (Warburg 1930). He suggested that these changes led to the development of cancer, and that if the physiology of tumour cells could be rendered normal, the malignant process could be halted. Although some critics at the time felt that such physiological changes were likely to be the symptoms rather than the cause of cancer, innovative work using hyperbaric oxygen and gas mixtures was introduced.

Campbell and Cramer exposed tumour inoculated mice and rats to 60% oxygen for several weeks but saw no effect on the tumours (Campbell and Cramer 1928). It was noted, however, that using hyperbaric oxygen in animal tumours regression could be achieved by combining the oxygen with injection of copper and selenium compounds (Fischer 1927). This led to patients being exposed to hyperbaric oxygen for up to 100 hours in a compression chamber with claims of symptom relief (Auler 1927).

Fischer-Wasels in an attempt to create normal tumour metabolism used the gas mixture now known as carbogen (Fischer-Wasels 1930). The carbon dioxide was added to oxygen to stimulate the respiratory centre, counteracting oxygen deficiency. His work in animals showed that breathing 95% O_2 and 5% CO_2

doubled the oxygen concentration in both normal and tumour tissue and slowed tumour growth. Carbogen, in combination with X-ray treatment, was used by Fischer-Wasels in the clinic with some success (Figure 1.1). Patients were required to breathe carbogen for two to four hours daily and, as well as symptomatic improvement, two cures were reported in patients with inoperable stomach and oesophageal cancer.

This approach to treatment was not pursued at that time, perhaps due to the political climate or possibly because of the success of Coutard and his colleagues in improving the cure rates of radiotherapy by fractionated treatment.

Although Thomlinson and Gray are largely credited with realising the importance of oxygen to radiotherapy the pioneering work of Mottram should not be forgotten. He described in 1936 how irradiation of tar warts in mice led to enhanced damage at the edge of the tumours (Mottram 1936). This was, he postulated, because cells at the margin had a better blood supply and thus higher oxygen concentrations than the apparently more radioresistant central cells.

It was to be a further 30 years before interest in the tumour microenvironment, and in particular the importance of oxygen in radiotherapy, was rekindled by the work of Gray (Gray et al. 1953). He and Thomlinson (Thomlinson and Gray 1955) attempted to explain the presence of hypoxic cells. Using histological sections of bronchial carcinoma, they described a corded structure to the tumours, comprising a central area of necrosis surrounded by tumour cells and encased in a vascular stroma. They noted that a necrotic core of cells was not present in a cord of less than 160μm diameter and that however extensive the area of central necrosis, the rim of viable tumour cells never exceeded 180μm. From this work, the concept of chronic or diffusion limited hypoxia developed (Section 1.3.4.1), and spawned a host of treatment strategies to overcome this problem.

The limited diffusion capacity of oxygen was initially believed to be the sole reason for the presence of radiobiologically hypoxic cells, and it was only in 1979 that Brown postulated a second mechanism contributing to tumour hypoxia (Brown 1979). He suggested that transitory changes in blood flow could induce temporary regions of hypoxia. The realisation that perfusion driven events could be contributing to tumour hypoxia gave the impetus to look afresh at ways of overcoming hypoxia.

1.3 Current perspective of tumour blood flow

1.3.1 Development of tumour vasculature

Most solid tumours begin as an avascular group of cells relying on simple diffusion as a means of nutrient exchange. Growth of the tumour is only possible if an adequate blood supply is established. The development of tumour vasculature depends on the existence of pre-existing host vessels that become integrated into the tumour structure, and microvessels arising from neovascularisation of normal host vessels. The process of neovascularisation, which is rare in normal tissue, appears to be a common feature of tumours (Folkman 1990), and is stimulated by the growing tumour producing angiogenesis factors. This results in new vessels arising from venules either within, or on the edge of the tumour mass.

Some of the pre-existing host vessels incorporated into the tumour simply disappear, whilst others become distorted or obstructed. They do, however, retain some of their normal function and ability to respond to physiological and pharmacological stimuli. In contrast, new vessels are structurally and functionally abnormal. They are tortuous and thin, often composed of only a single layer of endothelial cells. In addition they may lack smooth muscle and innervation which means they cannot autoregulate (Mattsson et al. 1979), and they often contain aberrations in the structure of the constituent basement membrane and endothelial cells (Vaupel et al. 1989).

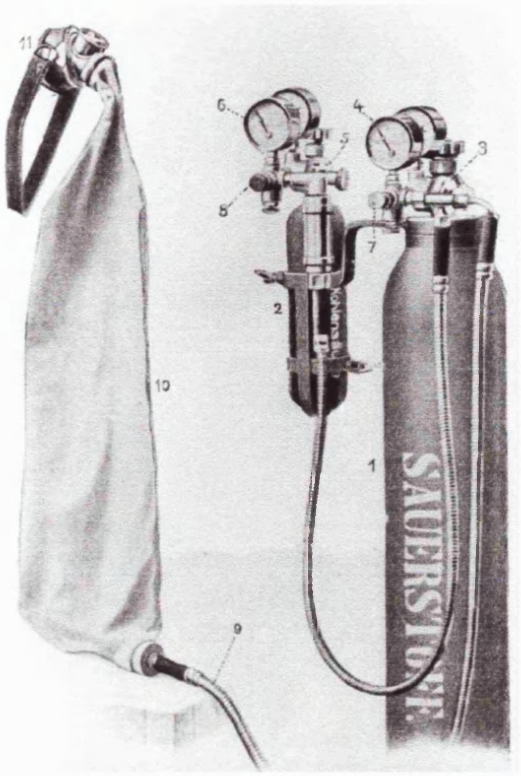


Figure 1.1 *Carbogen breathing equipment used by Fischer-Wasels in 1930*

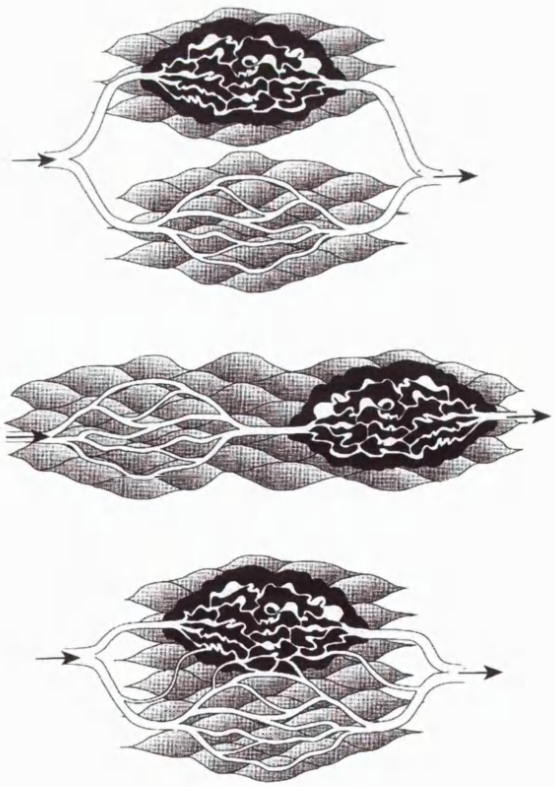


Figure 1.2 *Schematic diagram of the possible relationships of the normal to the tumour vascular bed: showing vascular beds in parallel (top), in series (middle) and a combination of the two (lower). (from Song, 1998).*

1.3.2 Vascular architecture

The tumour vasculature comprises a heterogeneous arrangement of vessels and does not conform to the standard morphology seen in normal tissue of artery to capillary bed to vein. In addition, there is considerable variation in vascular architecture between tumours that is thought to be directly related to the rate of tumour growth (Vaupel et al. 1989). Slow-growing tumours are more likely to have a better developed vascular network since angiogenesis can keep up with tumour cell growth. On the other hand, time for vascular differentiation is not available in rapidly growing tumours and thus they tend to have more rudimentary, abnormal vessels. Experimental work in human gliomas supports this theory, with slower growing tumours having a higher vascular density than those which divide more rapidly (Foltz et al. 1995).

1.3.3 Consequences of aberrant tumour vasculature

In normal tissue the vascular supply is matched to the metabolic requirements of that tissue. But in a neoplasm, the process of neovascularisation cannot keep pace with tumour growth and randomly distributed regions of altered metabolic microenvironment develop. The metabolic demands of a tumour outstrip supply and accentuate these regions of hypoxia, acidosis and nutrient depletion. This, in turn may lead to resistance to treatment.

Since many tumour vessels lack smooth muscle they are unable to respond to normal physiological stimuli. As a result, changes in tumour blood flow are largely due to alterations in systemic blood pressure and blood flow in adjacent normal tissues. The influence of these external factors in tumour blood flow is determined by the geometric relationship between the vascular beds of tumours and normal tissue. Vascular beds may be in parallel, in series or combined (Figure 1.2). If they are in parallel an increase in normal tissue blood flow leads to a decrease in tumour blood

flow. This is due to blood being shunted away from the tumour. If normal tissue blood flow is in series with that of the tumour, changes would be similar since blood flows directly from normal into tumour tissue. Many tumours contain a combination of vascular beds in parallel and series with normal tissue and the change in blood flow in such tumours depends on the contributions of each type (Song 1998).

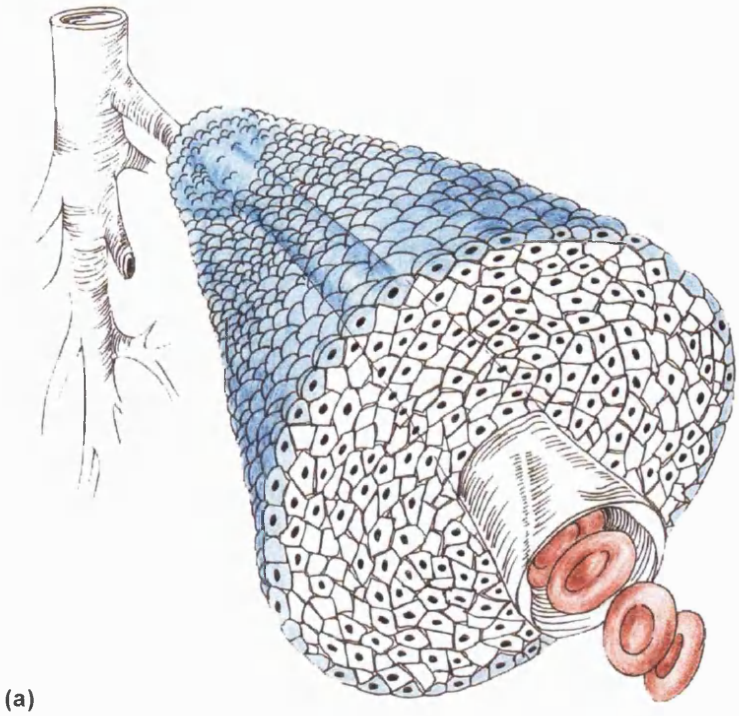
1.3.4 Relationship between tumour vasculature and oxygenation

1.3.4.1 Chronic hypoxia

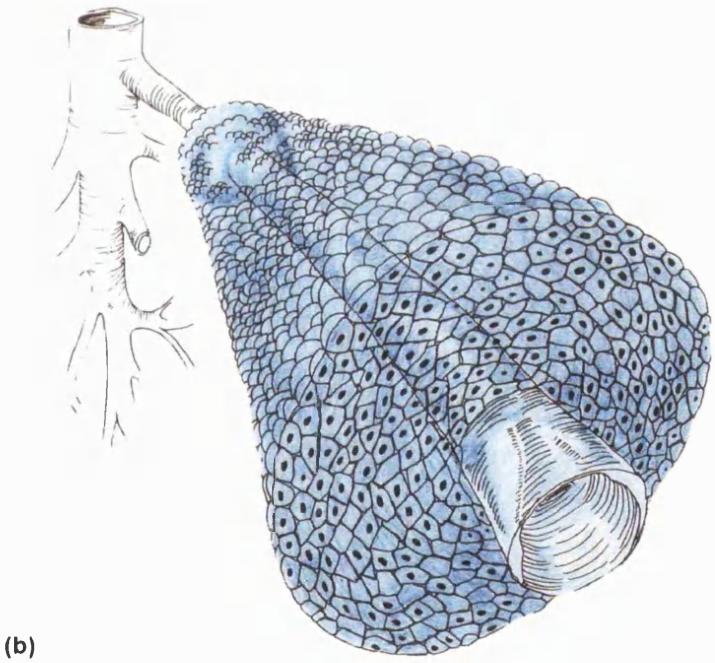
As a result of the disordered and rapid formation of tumour blood vessels large intercapillary distances develop, which lead to low oxygen tension regions between vessels (Awaad et al. 1986). This picture fits with the classic model of hypoxia (Section 1.2) based on the premise that oxygen can only diffuse a limited distance from a vessel (up to 180μm) and is utilised by those cells situated closest to the vessel. Cells beyond the diffusion limit will be anoxic and become necrotic. Adjacent to the necrotic region is a rim of cells that are on the border of the oxygen diffusion distance. They are hypoxic but receive enough oxygen and nutrients to remain viable. Such cells have chronic diffusion limited hypoxia (Figure 1.3a).

1.3.4.2 Acute hypoxia

Experimental work with the RIF-1 murine tumour, showing two distinct populations of hypoxic cells with different radiosensitivities, led to the idea that hypoxia results not simply from a paucity of nearby blood vessels, but from dynamic changes in tumour blood flow (Brown 1979). This hypothesis of vascular shut-down leading to regions of hypoxia was supported by direct evidence of temporary non-perfusion of vessels in tumours grown between transparent plates ('sandwich preparations') (Reinhold et al. 1977). Further animal studies using two fluorescent perfusion markers in conjunction with flow cytometry and histological examination confirmed



(a)



(b)

Figure 1.3 *Schematic cross-section through a tumour vessel and its surrounding cells to show:*

(a) *Mechanism of chronic hypoxia. Oxygen can diffuse a distance of about 150 µm thus the blood vessel is surrounded by aerated cells. Cells beyond the diffusion limit are necrotic but surrounding the anoxic zone will be cells that are hypoxic but viable and clonogenic.*

(b) *Mechanism of acute hypoxia. Flow in a vessel has transiently stopped leaving surrounding cells temporarily hypoxic. (from Chaplin, 1998)*

that hypoxic cells can result from dynamic perfusion changes within the tumour microcirculation (Chaplin et al. 1987; Trotter et al. 1989). These cells which are hypoxic because of such fluctuations in blood flow have a perfusion limited hypoxia and are termed acutely hypoxic (Figure 1.3b).

The duration of these reductions in perfusion may be from a few minutes to several hours and may occur for a number of reasons. These include a decrease in perfusion pressure leading to closure of part of the vascular bed, white blood cells or platelets temporarily plugging a narrow vessel, or decreased erythrocyte deformability which impairs their flow.

1.4 Therapeutic implications of impaired tumour blood flow

1.4.1. Radiotherapy

The presence of oxygen is a critical determinant of the response of tumours to radiotherapy. Hypoxic cells are relatively resistant to radiotherapy, requiring up to three times the dose of radiation that well-oxygenated cells need to achieve the same degree of cell kill. Figure 1.4 shows a curve of relative sensitivity against oxygen tension. Radiosensitivity is virtually unaffected at oxygen tensions above 30mmHg, but drops significantly at levels below 3mmHg (0.5%O_2). Cells below this value are often termed radiobiologically hypoxic to infer their relative resistance to radiation.

The importance of hypoxia to clinical practice is difficult to assess for several reasons. Firstly, a proportion of the hypoxic cells may be non-clonogenic and will thus not influence outcome. Secondly, during a fractionated course of radiotherapy re-oxygenation may occur, allowing cells that were previously hypoxic to become more radiosensitive (Kallman 1972). Thirdly, there are many other factors such as inherent radiosensitivity, cell kinetics, tissue pH and absolute number of clonogens that are all important in determining the success or failure of radiotherapy to achieve local control. However, a recent meta-analysis by Overgaard of 82 randomised

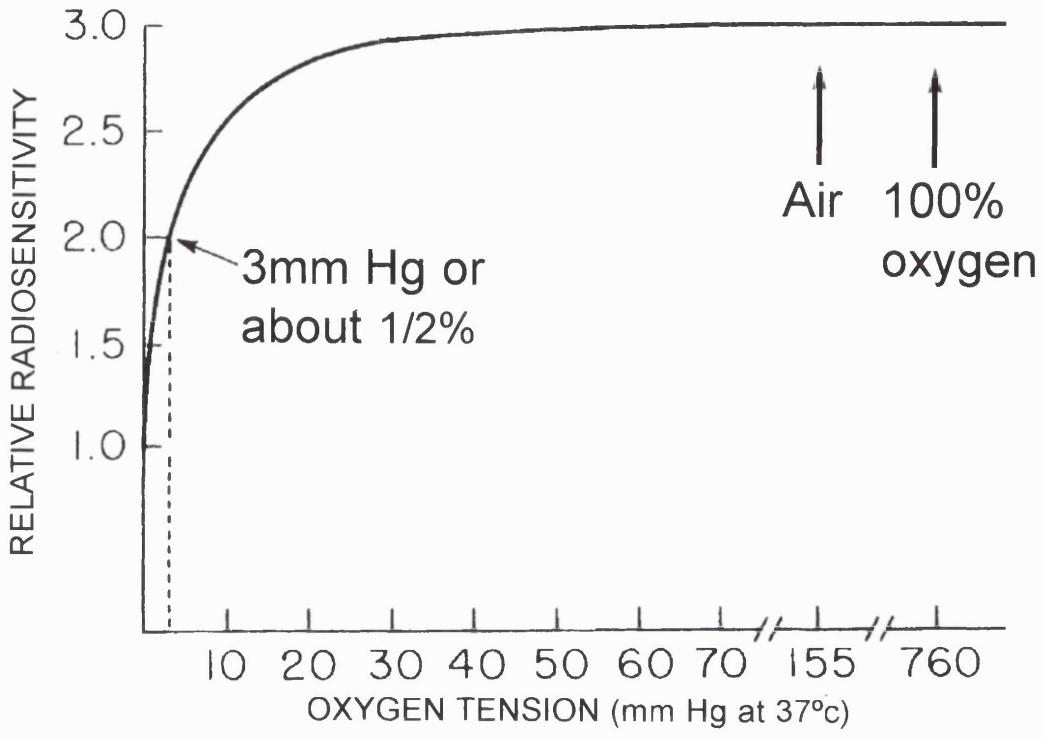


Figure 1.4 *Curve to show dependence of radiosensitivity on oxygen concentration (from Hall,1978). When oxygen tension is about 3mHg (0.5% O_2) a sensitivity of halfway between full oxygentaion and anoxia occurs.*

controlled trials using hypoxia modifying agents, provides strong clinical evidence for the importance of hypoxic cells in influencing the outcome of a fractionated radiotherapy regimen (Overgaard and Horsman 1996; Overgaard and Horsman 1997). Over 10,000 patients were treated in trials using hyperbaric oxygen (29 trials), hypoxic cell sensitizers (52 trials), carbogen or oxygen breathing (3 trials), or blood transfusion (1 trial). An overall improvement in local control of 5% (51% versus 46%, $p<0.0001$) was found. Although, when analysed according to site, only head and neck tumours showed a significant improvement (7%, $p<0.0001$) in local control.

1.4.2. Chemotherapy

Tumour blood flow is also closely linked with resistance to drug treatment. Perhaps the most obvious way in which this occurs is through the delivery of drugs to tumour cells; a heterogeneous vascular network with many cells distant from vessels will lead to a reduced inhomogenous delivery of systemically administered agents. In this situation the ability of drugs to ultimately reach their target cells will depend also upon their lipophilicity and metabolic stability.

The potency of certain cytotoxic drugs is dependent on the proliferative activity of the cells i.e. whether they are cycling and their position in the cell cycle. Experimental work using a DNA binding fluorescent marker has shown that cells distant from vessels have a reduced proliferation rate (Minchinton et al. 1990) which would render them less sensitive to many agents.

Hypoxia has also been shown to protect cells from the effect of certain cytotoxic drugs that require oxygen for maximal activity. In vitro studies with a range of anti-cancer agents such as adriamycin, bleomycin, etoposide, and taxol have shown their cytotoxic activity to be affected by oxygen concentration, with hypoxic cells more resistant than oxic cells (Teicher 1994).

An alternative approach has been to exploit the presence of hypoxic cells and use drugs which are cytologically active under low oxygen tension. There are three main class of agents that are preferentially toxic to hypoxic cells. They are aromatic nitro compounds, N-oxides such as tirapazemine and quinones, such as mitomycin-C and porphyromycin. Their main clinical role is in combination with other treatment that will target the surviving aerobic cell population.

1.4.3 Biological therapies

As with any systemically given compound, biological treatments will rely on a good blood supply to reach their target cells in adequate concentrations. Moreover, oxygen levels can limit the effectiveness of certain cytokines. For instance, by changing the oxygen tension of the cellular environment from 12% to 4% the anti-proliferative effect of Interferon-γ is reduced (Aune and Pogue 1989). In addition, the induction of lymphokine activated killer (LAK) cells by interleukin-2 (IL-2) is directly related to oxygen tension. The IL-2 activated effector-to-target-cell ratio had to be increased by 3-fold if the effector cells were incubated with IL-2 at 5% instead of 20% oxygen, and by a factor 20 if the incubation was carried out at 2% oxygen (Ishizaka et al. 1992). Similarly, when sarcoma-F cells are incubated in a hypoxic environment a 50 fold increase in resistance to the anti tumour activity of tumour necrosis factor (TNF) occurs (Sampson and Chaplin 1994; Lynch et al. 1995).

1.4.4 Photodynamic therapy

Photodynamic therapy (PDT) combines the systemic administration of a photosensitising agent, typically a porphyrin bound compound, that is then activated within the tumour using light (Pass 1993). This causes production of oxygen free radicals that can directly lead to cell death or cause indirect cell kill by damage to tumour endothelium. Hence oxygen enhances the tumouricidal action of PDT.

The oxygen requirements appear similar to those of ionising radiation. Oxygen tensions above 38 mm Hg are ideally necessary to achieve the best effect and oxygen tensions of 7 mm Hg or below give half maximum sensitivity. Hypoxia is thus a critical factor in determining the success of PDT, and since oxygen is consumed during the treatment, additional hypoxia can be induced which would further reduce the effectiveness of therapy.

1.5 Overcoming tumour hypoxia and perfusion limitation

1.5.1 Hyperbaric oxygen

Working on the hypothesis of Warburg, that improving tumour oxygen levels could promote a cure (Warburg 1930), Auler used hyperbaric oxygen in cancer patients (Auler 1927). He reported some amelioration in symptoms but no tumour regression. Many years later, Churchill-Davidson, combining radiation with hyperbaric oxygen, was the first to show an improvement in response with this approach (Churchill-Davidson et al. 1955). This work led to a series of randomised controlled trials that, when reviewed in Overgaard's meta-analysis, showed a 9% (62% versus 53%, $p<0.0001$) improvement in local tumour control (Overgaard and Horsman 1996; Overgaard and Horsman 1997).

This method of treatment was, however, not widely adopted and is no longer used for radiotherapy. This is probably due to the necessity for special equipment, the difficulty in applying certain radiotherapy techniques, the risk of fits, the morbidity of treatment and the discovery of simpler methods to overcome hypoxia, namely hypoxic cell sensitisers.

1.5.2 Haemoglobin concentration

The haemoglobin level will determine the amount of oxygen that can be transported to the tissues and thus anaemic patients may have lower oxygen levels within tissues

which could influence the response to treatment. There are many clinical studies that have looked at the question of whether haemoglobin levels influences the outcome of radiotherapy. In a review, 39 studies with over 14000 patients showed an effect of haemoglobin, this compare with 12 studies with 2800 patients in which haemoglobin could not be related to outcome (Grau and Overgaard 1998). There are however, few published randomised controlled trials looking at the use of blood transfusion in anaemic patients. In a prospective study in carcinoma of the cervix, patients were randomised into two groups. The control arm, who were not transfused unless their haemoglobin dropped below 10g/dl and the experimental group who received blood transfusions to maintain their haemoglobin above 12.5g/dl. A significantly improved tumour control rate was found in low haemoglobin patients transfused prior to radiotherapy (Bush 1986). In a more recent trial in head and neck cancer (DAHANCA 5) low haemoglobin levels predicted for poorer local control but was not improved for those patients who were given blood transfusion (Overgaard et al. 1998). This is being further evaluated in the DAHANCA 7 study which is currently underway.

1.5.3 Perfluorochemical emulsions

These compounds are able to carry large amounts of oxygen from well-oxygenated regions to be released in an area of low oxygen tension. Fluosol is a perfluorochemical originally designed as a blood substitute that has been used with oxygen in conjunction with radiotherapy in patients with head and neck, lung and brain tumours (Rose et al. 1986; Lustig et al. 1990; Evans et al. 1993). Animal studies have suggested that perfluorochemical emulsions combined with oxygen or carbogen can improve the therapeutic response to chemotherapeutic drugs (Teicher 1994).

Further progress with these agents has been slow due to concerns over drug safety, but new formulations have been developed which may be suitable for clinical use.

1.5.4 Carbogen

Observations that hyperbaric oxygen decreased blood flow in normal tissue (Bird and Telfer 1965) and animal tumours (Kruuv et al. 1967) led to the addition of carbon dioxide to atmospheric oxygen to overcome the vaso-constrictive effect of O_2. Animal studies using carbogen (95%O_2, 5%CO_2) showed increased tumour oxygenation and blood flow compared with O_2 alone (Kruuv et al. 1967), and improved cure rates when carbogen was breathed during radiation (Du Sault 1963; Inch et al. 1966). The radiosensitising action of carbogen is thought to be due to better tumour oxygenation, which results from an increase in dissolved oxygen within the blood and a shift of the haemoglobin dissociation curve to the right. This latter effect, that decreases the oxygen affinity of haemoglobin allowing oxygen to be given up more readily, is probably of more importance in areas of lower oxygen tension.

Clinical studies of carbogen inhalation during radical radiotherapy were undertaken, but ultimately proved disappointing (Keresteci and Rider 1978; Rubin et al. 1979). The failure of these early randomised controlled trials to show a benefit may, at least in part, be explained by the use of a pre-irradiation breathing time (PIBT) of up to 90 minutes and poor delivery systems. Animal studies have since suggested that the PIBT can be a critical factor in determining the degree of tumour sensitisation by carbogen (Siemann et al. 1977; Chaplin et al. 1993). This has also been shown in human tumours where direct measurement of oxygen tension using polargraphic electrodes have confirmed the existence of time dependent changes in pO_2 during carbogen breathing. Falk demonstrated that carbogen induces only a transitory rise in pO_2 in human tumours reaching a maximum within 8-12 minutes which falls after 18 minutes (Falk et al. 1992).

Rojas showed in animal models that using a shorter PIBT and clinically relevant fraction sizes, enhancement ratios of up to 1.6 could be achieved with carbogen

(Rojas 1991). This work led to renewed interest in carbogen and clinical studies using this approach were undertaken (Dische et al. 1992).

Carbogen in conjunction with perfluorochemical emulsions and various chemotherapy agents has been used in animal tumours. Drugs such as adriamycin, cyclophosphamide and BCNU all show increased tumour growth delay suggesting an enhanced anti-tumour effect with this technique (Teicher 1994). A recent study has shown that breathing carbogen alone can lead to enhanced uptake and cytotoxicity of ifosfamide (Rodrigues et al. 1997). This observation lends more weight to the suggestion that carbogen may also be inducing important blood flow changes (Honess and Bleehen 1995), which will be further discussed in chapter four.

1.5.5 Vasoactive Agents

1.5.5.1 Angiotensin II and calcium antagonists

The evidence that regions of hypoxia could result from intermittent interruption of tumour blood flow (acute hypoxia) led to the realisation that hypoxia could never be completely overcome using techniques such as breathing high oxygen content gases or correcting anaemia. These approaches are directed at those cells existing in an environment of chronic hypoxia. This prompted the use of vasoactive agents to provide short-term, selective increases in tumour blood flow, hence improving oxygenation and drug delivery. The differences in vascular structure and function between normal and tumour tissue, and in particular the lack of smooth muscle in many tumour vessels, allows these vasoactive drugs to be tumour specific. It is likely that these drugs act not directly on tumour vasculature but by altering systemic blood pressure and resistance in normal vessels.

Angiotensin II (ATII) is a vasoconstrictor peptide that by preferentially constricting normal tissue vasculature can cause a relative increase in tumour blood flow (Suzuki et al. 1981; Tozer and Shaffi 1993). Systemic infusion of ATII can increase the

efficacy of chemotherapy and reduce the regions of hypoxia (Suzuki et al. 1981; Trotter et al. 1991). However, its effect on blood flow is unpredictable with reports of both increases and decreases in tumour blood flow (Jirtle 1988; Tozer et al. 1996). The variation in tumour response to ATII has limited its usefulness as a clinical agent and accentuates the need to understand the reasons behind why individual tumours respond differently.

Calcium channel blockers inhibit the transmembrane flux of calcium, preventing contraction of vascular smooth muscle. In addition, they increase red cell deformability, reducing blood viscosity and improve flow. Flunarizine has been shown in murine tumours to increase blood flow, oxygen availability and reduce the radiobiologically hypoxic fraction (Wood and Hirst 1989). Variable changes in blood flow and radiosensitisation are seen with verapamil, diltiazem and nisoldipine, although at higher doses they cause reduced tumour blood flow and increased radioresistance (Wood and Hirst 1989).

Modification of blood flow with these agents has been largely unsuccessful, with no constancy in results. This may be in part due to the finding that blood flow changes induced by these drugs are dependent on the blood pressure and the proportion of normal vessels within a tumour (Vaupel and Menke 1989). In addition, the doses required for some of these agents to be effective would give rise to unacceptable clinical toxicity.

1.5.5.2 Nicotinamide

Nicotinamide, the amide form of vitamin B3, is a drug that has been widely prescribed for the treatment of schizophrenia and skin disorders safely using daily doses of up to six grams (Zackheim et al. 1981). Its potential as a radiosensitising agent was recognised in 1970 (Calcutt et al. 1970) but perhaps due to the advent of

more promising radiosensitisers, namely the nitroimidazoles, it remained on the shelf for over a decade.

Interest in nicotinamide as a potential radiosensitiser was renewed in the late 1980s and experimental work showed that in murine tumour models it acted as a tumour specific radiosensitiser (Horsman et al. 1987). Additional studies have demonstrated that using clinically relevant 2 Gy fraction sizes enhancement ratios of between 1.2 and 1.7 can be achieved (Rojas 1992). The radiosensitising effect is thought to be related to its ability to enhance tumour perfusion and oxygenation by reducing the temporary occlusion of microregional vessels within a tumour (Chaplin et al. 1990). There is also some evidence that nicotinamide may have direct anti-tumour activity in transplanted tumours, but this is only apparent at high doses and considerable toxicity (Horsman 1995).

The effect of nicotinamide on acute hypoxia led to the suggestion of combining it with a treatment that might influence chronic hypoxia. The addition of carbogen breathing to nicotinamide was shown to improve response to irradiation in a range of tumours (Chaplin et al. 1991; Kjellen et al. 1991). Further animal studies have shown that irradiating with clinically relevant 2 Gy fractions gives rise to an enhancement ratio of up to 2.1, with greater therapeutic gains relative to normal tissues such as kidney, lung and skin than seen with other radiosensitisers (Rojas 1992).

The encouraging experimental data from this strategy of targeting both diffusion and perfusion limited hypoxia has led to the introduction of carbogen and nicotinamide into the clinic in combination with radiotherapy (Kaanders et al. 1995; van der Maazen et al. 1995; Hoskin et al. 1997; Saunders et al. 1997). Nausea, vomiting and headaches are the chief side effects of nicotinamide, with some groups reporting only a 50% compliance rate due to these symptoms (Hoskin et al. 1997;

Saunders et al. 1997). However, initial tumour control rates are encouraging and further studies are on-going.

1.5.5.3 Pentoxifylline

The toxicity associated with nicotinamide has led to interest in other vasoactive compounds. One candidate is pentoxifylline, an orally active drug that is in clinical use for the treatment of peripheral vascular and cerebrovascular disease. It improves microcirculatory blood flow and diminishes thrombus formation by increasing red cell deformability, reducing blood viscosity, and decreasing platelet adhesion and aggregation. Several murine tumours have shown evidence of radiosensitisation with pentoxifylline which is believed to be due to the concurrent increase in tumour oxygenation and perfusion (Honess et al. 1993; Lee et al. 1993; Song et al. 1994). Oxygenation studies with pentoxifylline in human tumours have been undertaken and show promising findings with both good patient tolerance and increases in tumour pO_2 (Laurence et al. 1996).

1.6 Clinical methods of measuring human tumour blood flow

In order to develop treatment strategies to manipulate tumour blood flow, techniques to measure human tumour perfusion are necessary. Table 1.1 summarises clinical approaches to tumour blood flow measurement. Thermal washout techniques have been widely used for both superficial and deep-seated pelvic tumours (Samulski et al. 1987; Lagendijk et al. 1988; Feldmann et al. 1993). The problem with thermal clearance is that the technique itself can lead to errors in blood flow estimation. This happens first, because temperature modulation of the tissue may lead to changes in blood flow and second, due to conductive heat loss occurring as a result of the temperature gradients that exist between different sites of the tissue.

Isotope clearance techniques include wash-in and wash out methods. Wash-in methods involve administering a tracer that is delivered to the tissue via the blood. Several methods use this approach including ^{201}Th scans, SPET, and Positron emission tomography (PET) scans (Beaney et al. 1984; Nishizawa et al. 1991; Wilson et al. 1992; Rowell et al. 1993). They are minimally invasive (requiring injection only) but are limited by the need for special equipment and poor spatial resolution.

An example of a wash-out method includes ^{133}Xe clearance which has been widely used in animal studies. The radionuclide is injected directly into the tumour and its clearance from the lesion provides a measure of tumour blood flow. The major drawbacks of this technique are that only superficial lesions can be studied and it is invasive. However, results of human tumour blood flow using this method (Mantalya et al. 1988) do correlate well with data using other techniques.

Dynamic CT and MRI imaging methods have been employed using injected gadolinium as a contrast medium (Feldmann et al. 1993). The main advantages of these techniques are that spatial resolution is good and they are readily available in many clinical departments. They involve, however, intravenous injection of contrast media which, because of the risk of anaphylaxis with repeated use, means they are not really suitable for serial measurements.

Non-invasive MRI imaging techniques are being developed which allow qualitative assessment of tumour perfusion. This new technology has the advantage of using readily available equipment and being acceptable to both clinicians and patients, many of who are already having frequent MRI scans. Functional MRI is an example of this which, by using deoxygenated red blood cells as an endogenous contrast agent (BOLD contrast - Blood Oxygen Level Dependent), can detect changes deoxyhaemoglobin concentration which can then be related to tumour oxygenation and perfusion. An increase in oxygen content causes a decrease in deoxyhaemoglobin concentration and if blood flow increases, but there is no

proportional change in oxygen uptake by the tumour, deoxyhaemoglobin decreases (Robinson et al. 1995; Griffiths et al. 1997; Robinson et al. 1998). Functional MRI has been used to study the perfusion of a range of head and neck and pelvic tumours before and during a course of radiotherapy looking in particular at response to the hypoxia modifying agent, carbogen (Griffiths et al. 1997). The main limitation of this technique is differentiating between the influence of blood flow change and altered oxygenation on the MRI signal.

Another example is EPISTAR MR imaging where alternating radio frequency is applied to arterial spins. This has been applied to perfusion imaging of brain tumours and has shown good correlation with SPECT (Gaa et al. 1996).

All of the above techniques for measuring tumour perfusion give a measure of gross changes occurring either within the whole tumour or a part of it. However, alterations in the tumour microenvironment can lead to changes in sensitivity to cancer treatment. The laser Doppler microprobes allow study of blood flow changes at the microvascular level.

The probes are very fine being only 300μm in diameter allowing measurement of a sampling volume in the region of $10^{-2}mm^3$. Chaplin has estimated that in the carcinoma NT tumour approximately 5 capillaries would be present in such a sample volume (Chaplin and Hill 1995). This is only a rough guide and would certainly vary between different tumours. The excellent resolution, the ability to provide real-time monitoring of microvascular fluctuations, combined with reproducibility have made this the gold standard in animal work for measuring dynamic perfusion changes. Human studies have shown that although invasive, it is well tolerated (Pigott et al. 1996). Further details of the laser Doppler system and its use will be described in Chapter 2.

1.7 Aims

There were three objectives to this work. The first was to develop a safe, well tolerated, reproducible clinical technique for measuring real time changes in microregional human tumour perfusion using a commercially available laser Doppler system. Secondly, it aimed to assess the influence of the hypoxic cell sensitiser, carbogen, on microregional blood flow. The final objective was to measure the effect of the vascular modifier nicotinamide alone and in combination with carbogen breathing on microregional perfusion.

Technique	*Invasive*	*Quantitative*	*Limitations*	*References*
Isotope clearance				
SPET & ^{99}Tc	yes	yes		Rowell 1993
^{133}Xe, ^{41}Ar, ^{85}Kr	yes	yes	poor resolution	Mäntylä, 1979, 1988
^{201}Th	yes	no	poor spatial resolution	Nishizawa 1991
Thermal clearance	yes	yes	superficial tumours, temp alteration affects blood flow	Samulski, 1987 Lagendjik,1988 Feldmann 1992,
PET	yes	yes	limited spatial resolution, tracers have short half life only measure snapshot	Beaney, 1984 Wilson, 1992
Dynamic CT scan	minimally	yes	hypersensititvity with contrast media	Feldmann,1993
Dynamic MRI	minimally	no	hypersensititvity with contrast media	Feldmann,1993,
Functional MRI	no	no	difficult to differentiate oxygenation from perfusion changes	Griffiths,1997 Gaa, 1996
Laser Doppler	yes	no	invasive, superficial tumours only	Pigott,1996

Table 1.1 Different methods of clinically measuring human tumour blood flow

CHAPTER 2

Materials and Methods

2.1 Laser Doppler Flowmetry

2.1.1 Introduction

Laser Doppler flowmetry allows continuous quantitative measurement of tissue blood flow at the microvascular level. It operates on the principle that light reflected from moving objects (erythrocytes) within the tissue will show a change in frequency related to the velocity of the particle. Devices for such measurements are commercially available and we used the Oxford Array multi-channel Laser Doppler system (Oxford Optronix, Oxford UK).

2.1.2 Instrument Design

This is a system incorporating up to 12 cylindrical probes which are able to simultaneously measure blood flow in discrete sites at a sampling rate of 20Hz. A laser diode of wavelength 780nm is coupled to the probes which contain optical fibres (130μm in diameter) delivering and collecting light to the tissue. Each probe measures 25mm in length and 300μm in diameter and has an estimated sampling volume of 0.01mm^3 (Figure 2.1) (Chaplin and Hill 1995).

2.1.3 Theory

The basic principle of laser Doppler flowmetry is the determination of the velocity of a particle, in this case, a red blood cell (RBC) from the change in frequency of light waves reflected off the particle.

The laser light is reflected, absorbed and scattered within the tissue. Longer wavelengths are more deeply penetrating since less light is absorbed and scattered. Light of wavelength between 600 and 1200nm falls into the range termed the "therapeutic window," which allows deeper penetration into tissue due to lower scattering and absorption coefficients (Oberg 1990).

When incident light interacts with a stationary cell the scattered, secondary radiation has the same frequency as that of the incident light. If, however, the incident light hits a moving cell, such as an erythrocyte, the scattered light will differ in frequency. This shift in frequency caused by the motion of the particle relative to the observer is known as the Doppler shift, first described eponymously in 1842.

The magnitude of frequency change is given by

$$\Delta f = (v/c)V_O$$

Where v is the velocity of the source relative to the observer, c is the velocity of the light, and V_O is the unshifted frequency. For measuring the microcirculation the probe now acts as the observer and red blood cells (RBCs) as independent light sources (Amois et al. 1988; Oberg 1990). Thus if c and V_O are known v can be calculated.

In practice, the light from photons interacting with many red blood cells results in a broad distribution of Doppler shifted frequencies. Shifted and unshifted light is returned to photodetectors and, using well-described signal processing algorithms (Bonner and Nossal 1981), a mean frequency is derived which is proportional to the mean velocity of the RBC.

The number of Doppler shifts per photon is proportional to erythrocyte concentration (or blood volume) and can be calculated mathematically from the signal generated (Haumschild 1986).

The product of blood velocity and volume is directly proportional to RBC flux (or blood flow) and provides a relative measure of blood flow, expressed as relative laser Doppler flow or flux (LDF).

2.1.5 Backscatter

An additional signal, the backscatter, is also recorded which is proportional to the total amount of light detected by the probe. This can be used as a monitor of probe movement, since any change in position of the probe results in an altered probe/tissue interface and thus causes a precipitous change in the backscatter signal.

2.1.6 Biological Zero

A residual signal is generated in tissues even where the arterial supply has been completely occluded, this is known as the biological zero (BZ). This value is believed to be important and many groups have recommended that it is subtracted from all observations (Wahlberg et al. 1991; Abbot and Beck 1993; Colantuoni et al. 1993). The BZ has been shown to arise from signals that are not flow related. It was initially thought that the BZ was due to Brownian motion of cells in front of the probe. This theory is based mainly on work performed by Caspary using a heated probe on the skin of normal subjects, patients with peripheral vascular disease and cadavers (Caspary et al. 1988). It was noted that the BZ increased with increasing temperature suggesting that Brownian motion was responsible for this non flow related value. BZ is also thought to be influenced by the oscillatory motion of arteriolar vessels. This effect has been observed in animal studies following

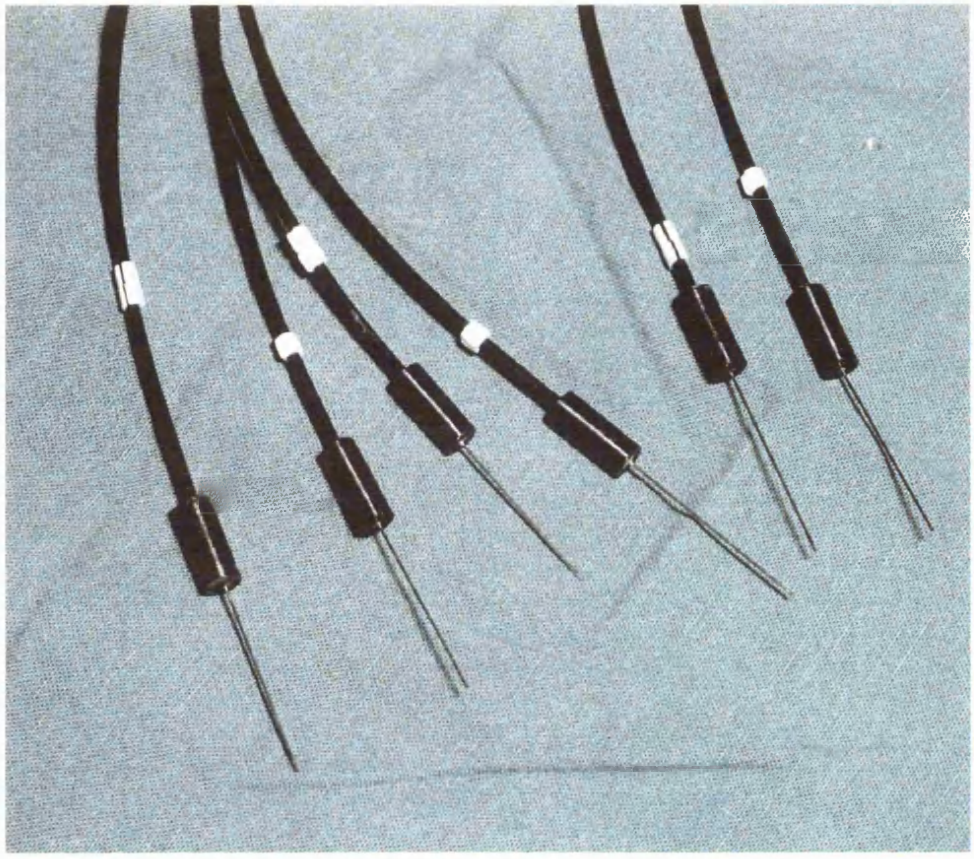


Figure 2.1 *Photograph of the six custom-made microprobes*

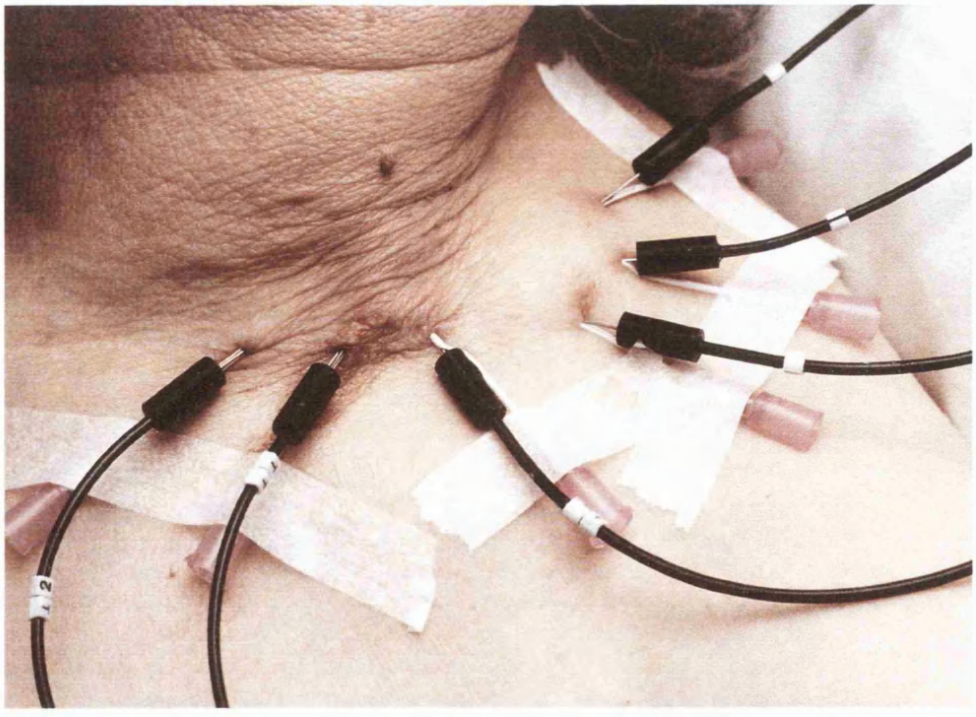


Figure 2.2 *Close-up of the probes within a tumour*

occlusion of the arterial supply and appears to be distinct from heart and respiratory rate (Colantuoni et al. 1993)

There is considerable variation in the BZ between individual subjects and in different tissues. In well-perfused tissue, such as gut the BZ approaches zero (Ahn et al. 1985), whereas, in normal skin it is 15% of the resting flow signal. This compares with values of up to 80% seen in patients with severe peripheral vascular disease (Caspary et al. 1988).

Estimating the BZ in a tumour is more complex. Extrapolation from normal tissue data may be misleading since tumour vasculature can be organised in a haphazard manner and is not under the same regulatory control as normal blood vessels. Chaplin and Hill have estimated the BZ in animal tumour models by measuring flux after the death of an animal. This has given figures of a mean value of 30% (range 4-77%) of resting LDF levels which they then subtracted from all LDF readings (Chaplin and Hill 1995). This may slightly underestimate the BZ since oscillation of blood vessels is not taken into account. However the 30% value does lie within the range noted in other series looking at normal animal tissue. Although some caution is necessary when extrapolating animal data to humans, this value of 30% does approximate to BZ figures noted in human tissues by other investigators (Wahlberg et al. 1991; Abbot and Beck 1993) and has been used in our human tumour studies.

2.2 Experimental Set-Up

2.2.1 Patient preparation

Patients were positioned comfortably either on a bed or in a chair and asked to remain as still as possible for the duration of the readings. Insertion of the probes was carried out using an aseptic technique. The skin overlying the tumour was cleaned using chlorhexidine solution and, in order to minimise discomfort for the patient, a bleb of subcutaneous lignocaine (1%) was used to anaesthetise the skin

before the probes were inserted. Since the probe tip lies about 25mm from the insertion site it was felt lignocaine would be unlikely to affect the readings.

2.2.2 Insertion of laser Doppler probes

The probes were sterilised by soaking in Asep solution (Gallen Ltd, Craigoven, UK) for 10 minutes prior to use. They are blunt ended and to facilitate entry into the lesion they were introduced through a 20 gauge cannula. This was done by making a nick in the sleeve of the cannula which was then was inserted into the skin overlying the tumour. The probe was then advanced down the plastic sleeve, and lodged within the tumour (Figure 2.2).

A small amount of bleeding inevitably occurs after insertion of the probes and was found to cease within a matter of minutes. To account for this an equilibration period of 5 to 10 minutes was allowed prior to measurements commencing. If bleeding persisted the probe was removed and gentle pressure applied.

2.2.3 Monitoring blood flow

Once the probes were positioned, the back scatter of each trace was examined, and if there was evidence of probe instability (seen by precipitous alterations in back scatter readings) the relevant probe was gently repositioned. Readings were then taken for up to one hour (Figures 2.3 and 2.4) and any patient movement noted so that if the movement led to an apparent change in laser Doppler flow it could be discounted.

Following this, either carbogen breathing was started, or the probes were removed and a pressure dressing applied over the puncture sites as necessary.

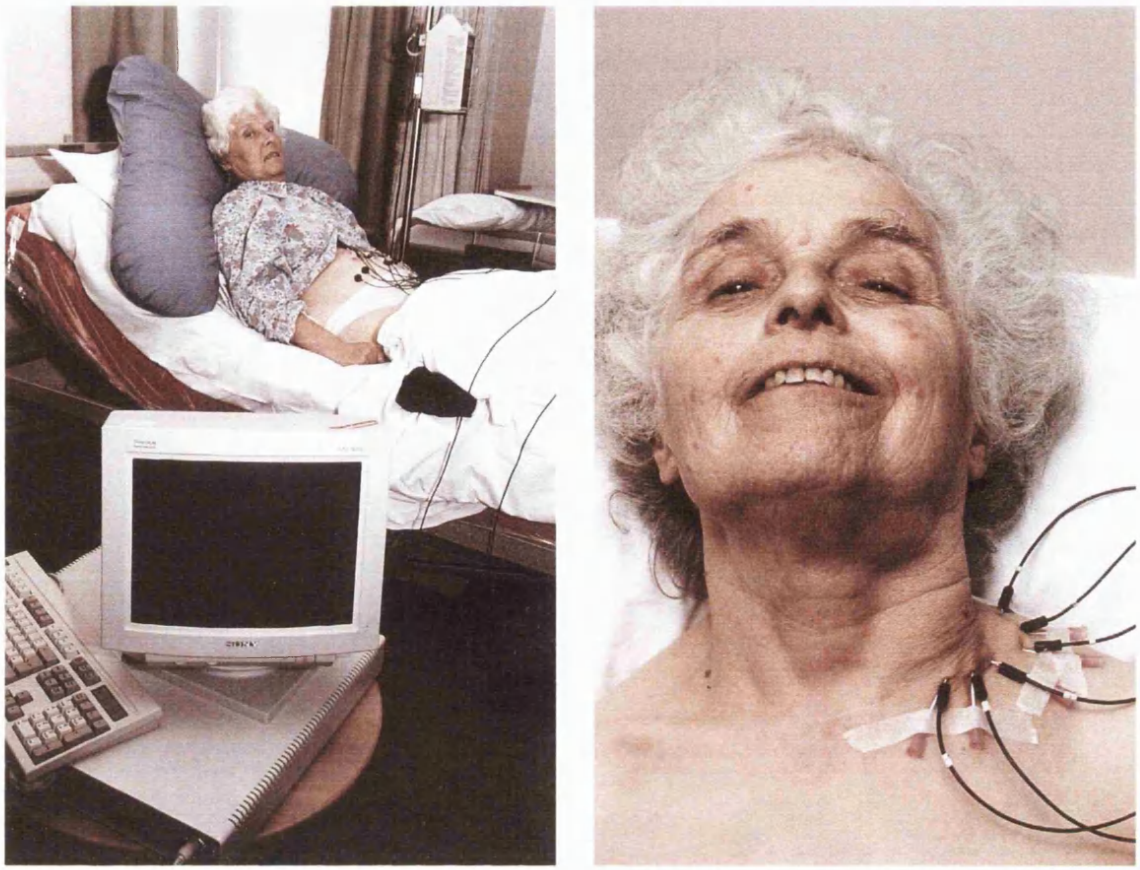


Figure 2.3 *Patients resting in bed. The probes are in-situ and LDF readings are underway*

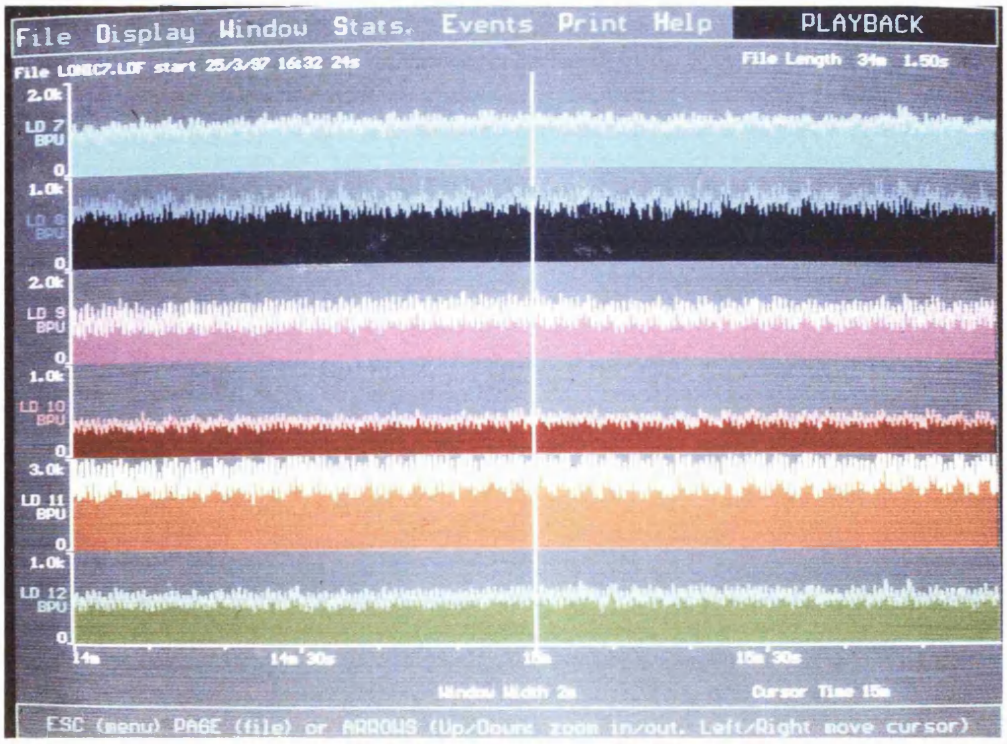


Figure 2.4 *Close-up of computer screen showing LDF measurements as they are taken*

2.3 Carbogen breathing

2.3.1 Carbogen in the clinic

A technique for carbogen breathing was developed at Mount Vernon Hospital for use during radiation (Dische et al. 1992) and a similar method continues to be used. A close-fitting face mask (Intersurgical, UK) covers both the nose and mouth and carbogen (95% oxygen and 5% carbon dioxide) is delivered at a flow rate of 10-30 litres/min through a closed system using a one-way valve and a 3 litre reservoir breathing bag (Figure 2.5). Carbogen is started as soon as the patient lies on the treatment couch, and with an interval to onset of radiotherapy of two to four minutes and treatment time of about five minutes, most patients breathe carbogen for between eight and ten minutes.

2.3.2 Carbogen with laser Doppler

Patients breathed carbogen using the system as described in Section 2.3.1 for 10 minutes. This timing was selected so that blood flow could be recorded over a period approximating to the treatment time in the clinical setting.

Measurements were then continued for a further 10 minutes with the patient breathing room air. The probes were then removed and a pressure dressing applied.

2.4 Nicotinamide Administration

2.4.1 Nicotinamide in the clinic

Nicotinamide is being used in combination with carbogen breathing and accelerated radiotherapy in phase II clinical trials (Zackrisson et al. 1994; Hoskin et al. 1997; Saunders et al. 1997). Pharmacokinetic data has shown a dose of 80 mg/Kg of nicotinamide can achieve plasma concentrations of 0.7-1.0μmol/ml (Stratford et al. 1992; Horsman et al. 1993; Hoskin et al. 1995), similar levels in animal studies have shown an enhancement ratio of up to 2.1 when combined with carbogen (Rojas et al. 1993). Since radiosensitisation with nicotinamide is maximal at its peak plasma

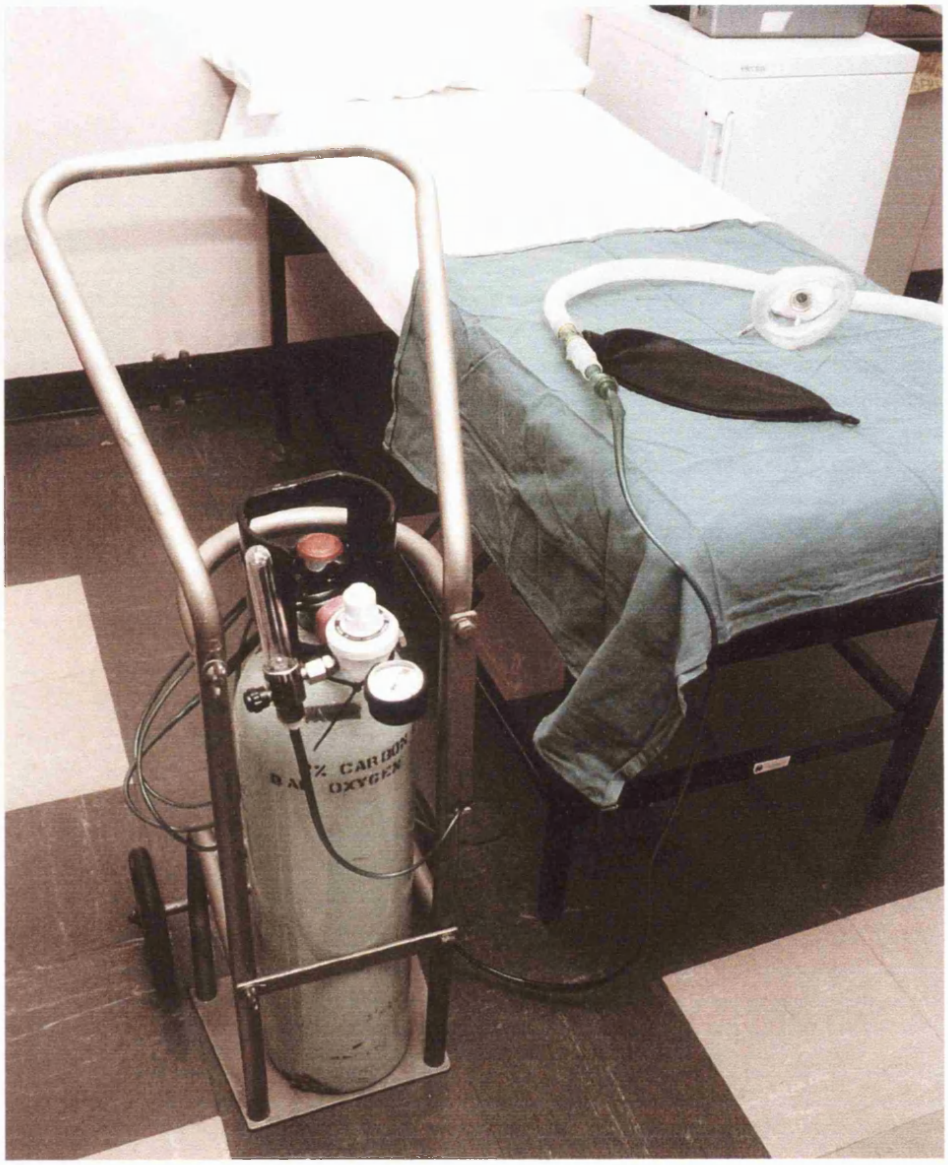


Figure 2.5 *Carbogen breathing equipment*

concentration, which occurs between one and two hours after taking the drug (Hoskin et al. 1995), radiotherapy is given two hours after administration of an oral tablet formulation of nicotinamide (80mg/Kg).

2.4.2 Nicotinamide with laser Doppler

The administration of nicotinamide was carried out as described in Section 2.4.1. Salivary or plasma levels of nicotinamide were assayed at 15-20 minute intervals during the study period.

Laser Doppler measurements began approximately 60 minutes following nicotinamide and were continued for a further 60 minutes before breathing carbogen using the technique described in Section 2.3.2. Thus the carbogen breathing was designed to coincide with the approximate time of radiotherapy in the clinical situation (Hoskin et al. 1997; Saunders et al. 1997).

2.5 Patients and Tumour Types

Since the probes measure 25mm in length only superficial tumours could be assessed. In addition, since patients were requested to remain as motionless as possible sites on the back and close to the jaw were not studied.

Tumours that have been investigated form a diverse group and include primary breast carcinomas, primary and secondary lymph node cancers and metastatic skin nodules from a variety of different primary cancers (Sections 3.3, 4.3, 5.3).

Approval for these studies was given by Local Ethics Committee and written informed consent was obtained from all patients prior to measurements being taken.

2.6 Data Analysis

2.6.1 Producing traces

Twenty readings per second are recorded from every probe and an average of the 2400 readings acquired over each two minute interval was calculated for each probe. On the basis of animal data (Chaplin and Hill 1995; Hill and Chaplin 1995) a correction factor was applied to all of the readings to allow for the biological zero (Section 2.1.4). This was calculated for each set of readings as 30% of the final two minute average of the observation period and subtracted from each two minute average within that particular lead.

Average laser Doppler flux (LDF) readings, expressed in arbitrary units were plotted against time and compared with the original recorded data, which included both LDF and backscatter readings.

2.6.1 Excluding traces

Periods of blood flow fluctuation were carefully examined with the backscatter data. If abrupt changes in the backscatter were seen coinciding with an alteration in LDF, these changes were considered to be movement artefacts and excluded from final analysis (Sections 3.2 and 3.3). Where fluctuations in the backscatter were observed throughout the study period the whole trace was excluded since this implied that the particular probe was not firmly lodged within the tumour.

CHAPTER 3

Detecting temporal changes in microregional tumour perfusion

3.1 Introduction

Characterising the nature of the human tumour microenvironment is one of the first steps necessary towards developing strategies to exploit the therapeutic potential of both conventional and novel anticancer treatments. It was the advent of the Eppendorf pO_2 histograph into the clinic that readily allowed the demonstration of hypoxic cells within human tumours (Gatenby et al. 1988; Vaupel et al. 1991; Hockel et al. 1993), and showed the heterogeneity of hypoxic regions both within and between tumours. It is now understood that such areas of hypoxia arise from both diffusion limitations, with cells farthest from blood vessels being relatively starved of oxygen and nutrients, and perfusion limitations. Dynamic microcirculatory fluctuations within tumours were postulated to be the cause of these perfusion driven changes in oxygenation (Brown 1979) and this was confirmed in a range of experimental tumours using fluorescent vascular markers (Chaplin et al. 1987).

It is thought that within a tumour, cells may be under hypoxic conditions which are both diffusion and perfusion limited. If cure is to be achieved both populations must be targeted.

Defining the importance of perfusion limited hypoxia within human tumours has, until recently, not been possible since these experimental techniques are not suitable for clinical use. The development of a multi-channel laser Doppler system with customised probes has allowed for the first time the *in-vivo* study in murine tumours of microregional blood flow, giving real time spatial information on blood flow

fluctuations in a tumour, without the injection of perfusion markers. Although minimally invasive the system is suitable for clinical use with the ability to provide new information on temporal variations in the microvascular perfusion of human tumours.

3.2 Aim

The aim of this study was to apply the technique of laser Doppler flowmetry to human tumours and determine the presence and nature of temporal variations in microregional perfusion.

3.3 Patients and Methods

3.3.1 Patients

A total of thirteen patients, six males and seven females, with histologically proven advanced malignancy were studied. Laser Doppler flux measurements on patients 1 to 8 were carried out by Dr Katherine Pigott (Pigott et al. 1996). Tumour characteristics of all patients are shown in table 3.1. Due to the length of the laser Doppler probes (25mm), tumours suitable for study were limited to those which were readily accessible, such as skin deposits or superficial lymph nodes.

3.3.2 Laser Doppler flowmetry

Relative changes in microvascular perfusion were measured using the Oxford Array multi-channel laser Doppler system (Oxford Optronix, Oxford UK) as described in section 2.1.

3.2.3 Experimental Set-Up

Patients were positioned and probes inserted as described in Section 2.2

3.3.4 Measuring microvascular blood flow

Blood flow measurements were taken for 60 minutes with the patients lying as still as possible to limit movement artefact in the traces. Two patients (numbers 4 and 12) with multiple sites of disease were studied on two separate occasions

3.3.5 Data Analysis

Traces for individual probes were generated as described in Section 2.6. The final plots of red blood cell flux together with the original recorded data were examined and any traces showing evidence of patient movement or probe movement excluded from analysis.

3.4 **Results**

3.4.1 Toxicity

Patients tolerated the procedure well and numbers 4 and 12, with multiple sites of disease, agreed to be studied for a second time. Insertion of the microprobes sometimes gave rise to a mild sensation of pressure, but once lodged within the tumour, all patients became oblivious to the probes such that some individuals were even able to sleep. Removal of the probes was painless and an adhesive dressing over the puncture sites was all that was required.

Patient no	*Tumour site & size*		*Primary tumour*	*Histology*
1	breast	10cm	breast	ductal adenocarcinoma
2	skin	7cm	lung	adenocarcinoma
3	breast	8cm	breast	ductal adenocarcinoma
4a	node (R groin)	4cm	penis	squamous cell carcinoma
4b	node (L groin)	8cm		
5	node (L groin)	10cm	lymph node	NHL (low grade)
6	breast	10cm	breast	ductal adenocarcinoma
7	peritoneum	4cm	pancreas	adenocarcinoma
8	breast	10cm	breast	ductal adenocarcinoma
9	node (neck)	3.5cm	lymph gland	NHL (high grade)
10	node (neck)	3cm	lymph gland	NHL (high grade)
11	node (neck)	6cm	supraglottis	squamous cell carcinoma
12a	peritoneum	3cm	colon	adenocarcinoma
12b	peritoneum	3.5cm		
13	node (R groin)	4cm	skin	NHL (high grade)

Table 3.1 *Tumour characteristics in control patients*

3.4.2 Analysing traces

Up to six traces of laser Doppler flux (LDF) were obtained from each tumour. However, in patients 10, 11 and 12 only five of the six probes were functional and in patient 13 only four could be used, after which a new set was acquired.

Eighty-four separate microregions were sampled, and each blood flow trace and its respective backscatter reading carefully studied for evidence of patient or probe movement. A total of 11 traces were excluded due to movement of the probe. An example of traces that were excluded is shown in Figure 3.1, where the LDF from five sampled microregions in patient 10 are shown. Each point is the average of 2400 readings generated over a two minute period. All of the areas sampled, except lead 2, show erratic traces that are mirrored by irregular backscatter readings, suggesting instability of the probe position. Figure 3.2 illustrates the LDF and backscatter from leads 2 and 6. The backscatter in lead 2 remains constant over the 60 minute observation period while the LDF readings alter with time. By contrast, in lead 6 both the backscatter and LDF initially show large fluctuations, suggesting the probe is not firmly lodged within tumour. This probe was gently manipulated and after 40 minutes the backscatter stabilised, indicating the probe became fixed in the tumour. This allowed trace 6 to be used for analysis in the subsequent carbogen breathing period (Chapter 4).

Certain fluctuations seen in traces are transitory and do not necessarily reflect an alteration in the position of the probe. They may be caused by abrupt patient movement, such as coughing or sneezing or, in the case of abdominal lesions, gut peristalsis. The times such movements occurred were noted and providing the appropriate backscatter and LDF readings returned to a constant level that particular fluctuation was excluded.

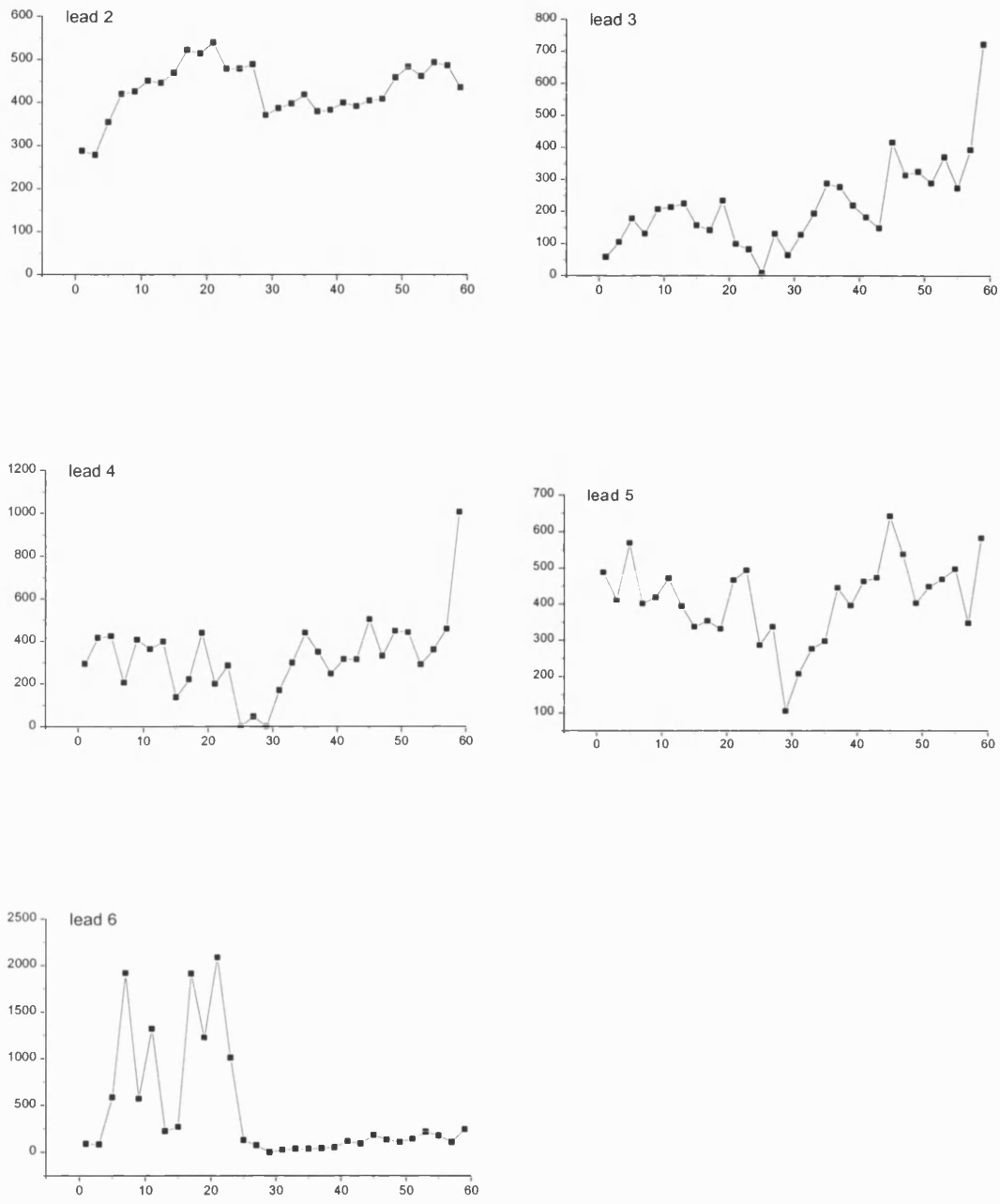


Figure 3.1 *Traces from 5 microregions in patient 10 showing change in laser Doppler flow over 60 minutes. All traces except that of lead 2 were excluded due to a combination of patient and probe movement. (In all traces laser Doppler flow in arbitrary units is plotted on the y axis against time in minutes on the x axis).*

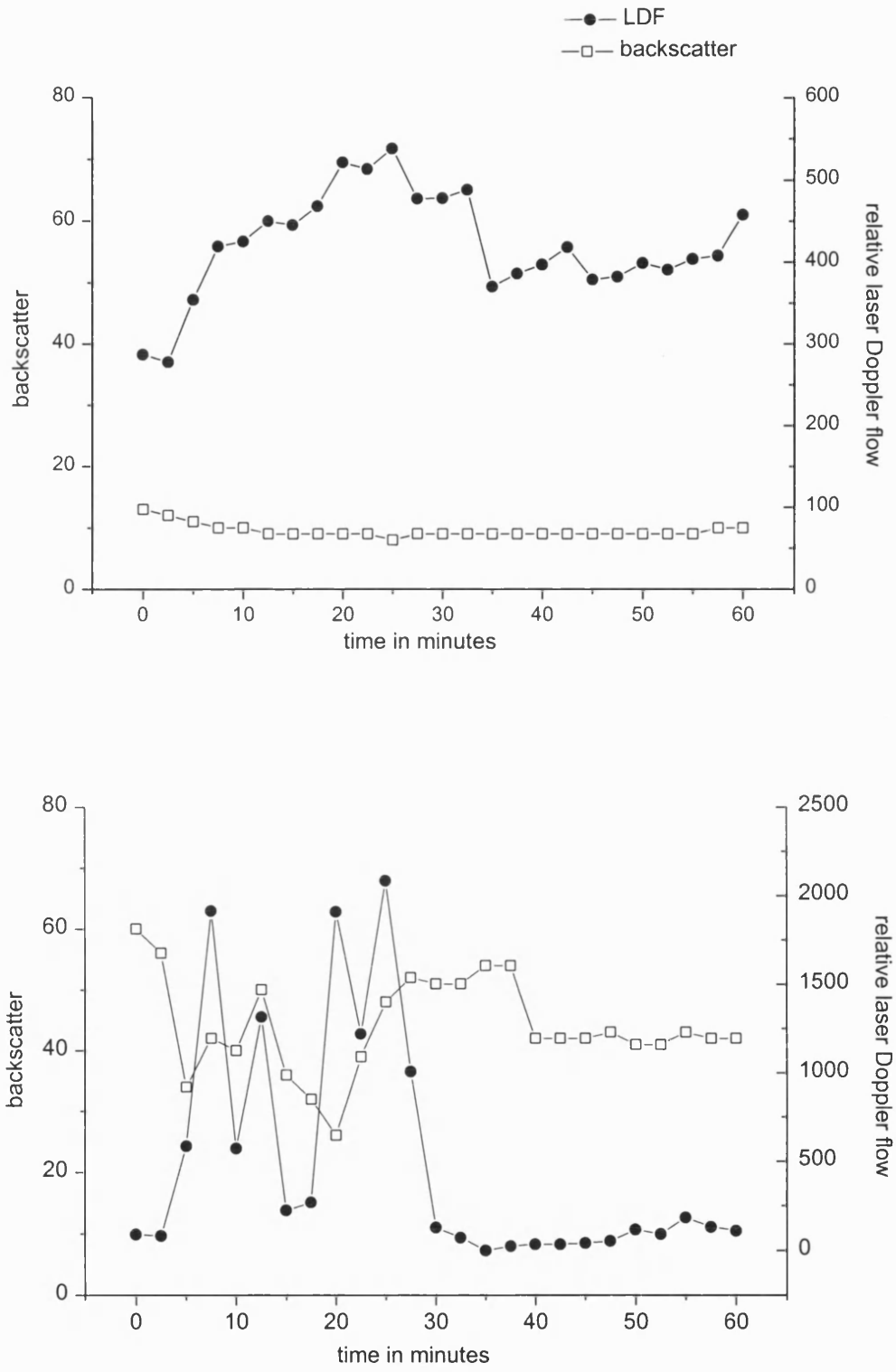


Figure 3.2 *Traces from lead 2 (top) and lead 6 (bottom) in patient 10 showing change in laser Doppler flow and backscatter.*

Figure 3.3 shows a plot of data from patient 12a illustrating the possible effect of patient movement. At eight minutes there is a sharp increase in the LDF readings which by 12 minutes has fallen to its original value. The fluctuations in LDF, however, corresponded with a sudden movement of the patient and were therefore not considered to be 'real' changes. The probe position is unlikely to have been disturbed since the LDF rapidly reverts to its initial level, this is borne out by a stable backscatter readings.

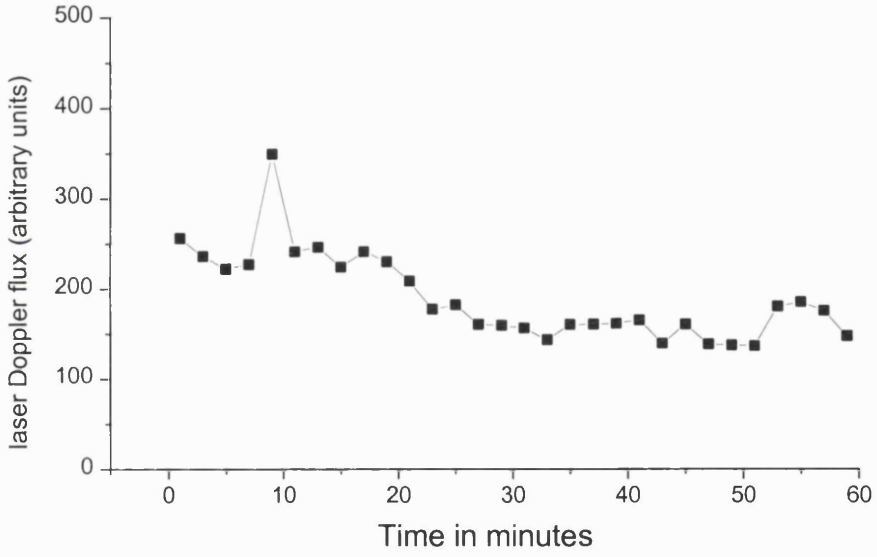


Figure 3.3 *A trace of laser Doppler flux measurements from patient 12a illustrating apparent blood flow changes between 8 and 10 minutes which were due to patient movement*

3.4.3 Incidence of blood flow changes

3.4.3.1 Individual tumours

As an example of the variation of blood flow changes seen in one tumour, five traces from patient 6 are shown in figure 3.4. Temporal fluctuations in perfusion can be seen to occur independently within individual microregions of the same tumour.

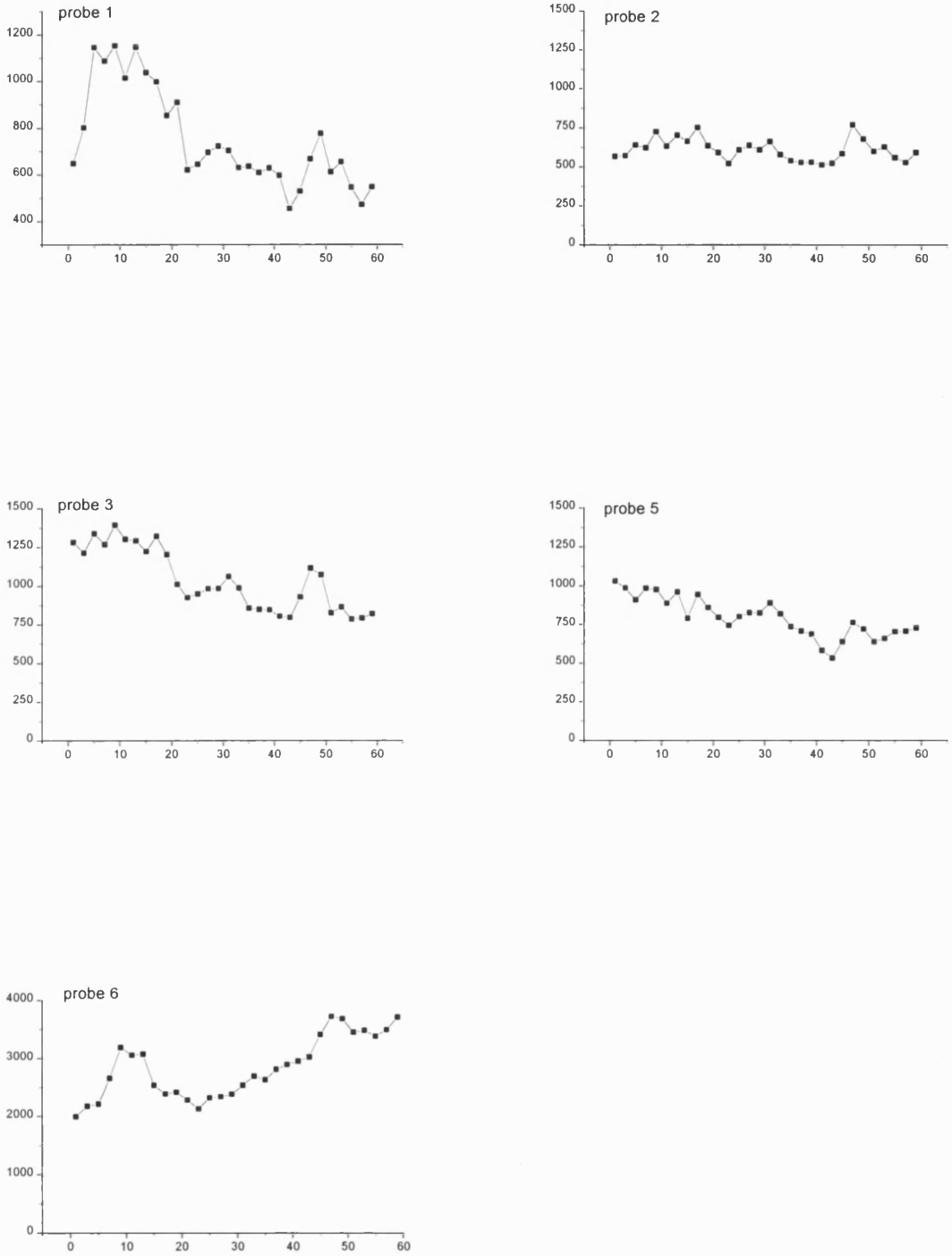


Figure 3.4 *laser Doppler flow in 5 microregions in patient 6 over a 60 minute observation period. (In all traces laser Doppler flow in arbitrary units is plotted on the y axis against time in minutes on the x axis).*

The plots of data from leads 1, 3, 5 and 6 show changes of different magnitude and direction while trace 2 remains unchanged (trace 4 excluded due to probe movement). In addition traces one and six demonstrate more than one change during the 60 minute observation period.

3.4.3.1 All tumours

Perfusion changes for all 15 tumours are summarised in table 3.2. A variation in blood flow by a factor of 1.5 or greater was noted in almost two thirds of all microregions sampled with a total 81 fluctuations seen (46 increases, 35 decreases).

Factor of change	***Number of traces showing change (n=73)***	***Number and direction of changes***	
≥1.5	45 (62%)	46 ↑	35 ↓
≥2	20 (27%)	17 ↑	13 ↓

Table 3.2 *Changes in microregional blood flow during the 60 minute observation period in 15 tumours*

The complete data acquired from all of the tumours studied is shown graphically in figure 3.5. The two minute averages for each of the 73 traces have been combined and expressed as a mean ± standard error of the mean. Overall, there appears to be a slight increase in perfusion over the 60 minute observation period, although considerable variation both within and between individual tumours is seen.

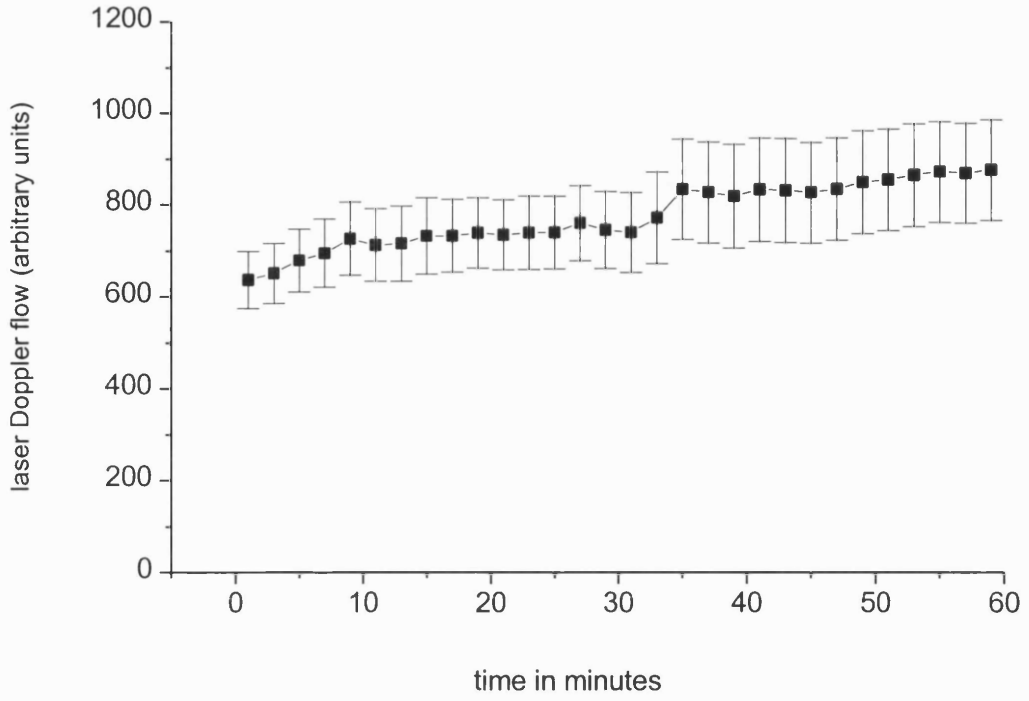


Figure 3.6 *Laser Doppler flow in 73 traces from 15 tumours over a 1 hour observation period (mean ±s.e.m.)*

3.4.4 Kinetics of blood flow changes

The continuous monitoring of tumour perfusion provides information on the speed and duration of the fluctuations seen. Kinetic data for changes in blood flow by a factor of 1.5 or more (from minimum to maximum or vice versa) are displayed in Table 3.3.

Rate of blood flow change	***Number of changes***
≤10 minutes	33 (41%)
≤ 20 minutes	56 (69%)

Table 3.3 *Kinetics of change in microregional blood flow in 15 tumours*

Although some traces showed a very slow rate of change, more than two thirds (69%) of changes occurred within 20 minutes, with the median time for a change to occur being 14 minutes with a range of 4 to 58 minutes. The initial perfusion trend was reversed in 17 traces, in other words an increase in blood flow was followed by a period of decreased perfusion or vice versa. In seven of the sampled microregions more than one reversal was seen during the observation period.

3.5 Discussion

These findings establish that laser Doppler microprobes are a practicable means of providing spatial information of real-time changes in microregional perfusion in human tumours. Additionally, it confirms the results of animal studies by demonstrating that variations in human tumour perfusion also occur frequently and can be reversible. During the observation period perfusion either increased or decreased by a factor of 1.5 or more in 62% of microregions sampled with two-fold changes seen in 27% of traces.

If just one blood vessel was responsible for the flow in a region, the LDF readings would be easy to interpret in terms of delivery of oxygen, nutrients and also systemic anti-cancer agents; a two-fold decrease would lead to a halving in the delivery of these substrates. Without histological examination of the tissue it is impossible to accurately determine the number of blood vessels within a sampled volume. If, however, it is assumed that in the nominal sampling volume of $10^{-2}mm^3$ eight capillaries are present, then a two-fold decrease in LDF could reflect an equal flow decrease in all eight capillaries, complete cessation of flow in four capillaries, or a flow decrease in a proportion of capillaries. The first possibility would lead to a homogeneous increase in the proportion of hypoxic cells, whereas the latter two would result in localised foci of hypoxic cells. The heterogeneity shown within an individual tumour in terms of blood flow from this study and pO_2 levels in other human studies would favour the second and third explanations.

The overall trend for perfusion to increase in the tumours studied over the observation period is of some concern and might imply that vessels were haemorrhaging in front of the probe. If, however, RBC flux for individual tumours is studied, 10 tumours show an elevation in LDF and 3 a reduction. Of the tumours displaying an overall rise in flow six of them also show decreases in LDF over the

observation period. This would suggest bleeding in front of the probes is not the cause of increasing LDF.

This trend for an increase in RBC flux may simply be a chance observation. All but three of the tumours show both increases and decreases in blood flow during the 60 minutes and if the study period was extended further reversals of flow might occur thus altering the overall picture.

This work establishes the existence of potentially reversible temporal microregional fluctuations in human tumour perfusion. It provides the basis upon which to evaluate the effectiveness of strategies used to enhance tumour perfusion.

CHAPTER 4

The influence of carbogen on microregional perfusion

4.1 Introduction

Although the first reports of the use of gas mixtures, including carbogen in combination with radiation, for cancer treatment were as early as 1930 (Fischer-Wasels 1930; Argyll Campbell 1931), it was not until the work of Gray (Gray et al. 1953), which highlighted the importance of hypoxic cells in determining the response to radiotherapy, that clinical trials of hypoxia modifying agents were undertaken. Reports that hyperbaric O_2 decreased blood flow to normal tissue led to the addition of carbon dioxide to atmospheric oxygen in order to overcome the vaso-constrictive effect of O_2. Animal studies using carbogen (95% O_2, 5%CO_2) showed increased oxygenation and blood flow compared with O_2 alone (Inch et al. 1966; Kruuv et al. 1967), and clinical studies of carbogen breathing during radiation were undertaken (Keresteci and Rider 1978; Rubin et al. 1979).

The failure of these early randomised controlled trials with carbogen to show a benefit may, at least in part, be explained by the use of pre-breathing times of up to 90 minutes. Animal studies have suggested the pre-irradiation breathing time can be a critical factor in determining the degree of tumour sensitisation by carbogen (Siemann et al. 1977; Chaplin et al. 1993). Indeed, direct measurements of human tumour oxygenation using polargraphic electrodes have confirmed the existence of time dependent changes in pO_2 during carbogen breathing. Falk et al demonstrated that carbogen induces only a transitory rise in pO_2 in human tumours reaching a maximum within 8-12 minutes and falling after 18 minutes (Falk et al. 1992). As a result of animal work showing enhancement ratios of between 1.3 and 1.6 with

fractionated radiotherapy (Rojas 1991) carbogen is being re-appraised in clinical studies.

Carbogen sensitisation is thought primarily to affect diffusion limited hypoxia by increasing the amount of dissolved O_2 in the blood. Recent animal work, however, using $^{86}RbCl$ in RIF-1 tumours has suggested that carbogen induced increases in tumour blood flow may be a more important component of enhanced tumour oxygenation than was previously thought (Honess and Bleehen 1995). If this is the case for human tumours, then carbogen breathing may be a useful adjunct to not only radiotherapy but also drug therapies, by improving the tumour delivery of systemically administered agents.

4.2 Aim

The aim of this study was to determine the effect of carbogen breathing on modulating microvascular perfusion in human tumours.

4.3 Patients and Methods

4.3.1 Patients

Eight patients were studied, six male and two female, with a median age of 63 years (range 46 to 86), all with histologically proven advanced malignancy. Tumour characteristics are shown in table 4.1. Two patients (numbers 6 and 7) with multiple sites of disease were studied on two separate occasions.

4.3.2 Laser Doppler flowmetry

Relative changes in microvascular perfusion were measured as described in Section 2.1.

Patient no	Tumour site & size		Primary tumour	Histology
1	peritoneum	4cm	pancreas	adenocarcinoma
2	breast	10cm	breast	ductal adenocarcinoma
3	node (neck)	3.5cm	lymph gland	NHL (high grade)
4	node (neck)	3cm	lymph gland	NHL (high grade)
5	node (neck)	6cm	supraglottis	squamous cell carcinoma
6a	peritoneum	3cm	colon	adenocarcinoma
6b	peritoneum	3.5cm		
7a	node (R groin)	4cm	skin	NHL (high grade)
7b	node (L groin)	3cm		
8	node (axillary)	3cm	skin	melanoma

Table 4 1 *Tumour characteristics of patients studied*

4.3.3 Experimental Set-Up

Patients were positioned and probes inserted as previously described in Chapter 2.2

4.3.4 Measuring microvascular blood flow

Eight of the patients were monitored for 60 minutes before breathing carbogen (these measurements are described in Chapter 3). The remaining two patients (7 and 8) after a period of equilibration, had baseline measurements taken for 10 minutes before carbogen breathing.

4.3.5 Carbogen Breathing

Following acquisition of baseline readings, a 10 minute period of carbogen breathing was commenced as described in Chapter 2, Section 2.3

Measurements were continued for a further 10 minutes after the cessation of carbogen breathing with the patient breathing room air.

4.3.6 Data Analysis

Traces for individual probes were generated as described in Section 2.6. Flow during carbogen breathing was related to a baseline value which was a single mean calculated from the measurements recorded over the 10 minute period prior to breathing carbogen, and was designated as the flow at time zero. Blood flow averages at five and ten minutes after starting carbogen breathing were compared with the time zero value using a paired two-tailed t-test where $p<0.05$ was considered to be significant.

4.4 **Results**

4.4.1 Toxicity and analysis of traces

In all patients the probes were well tolerated for the observation period. Blood flow measurements were analysed in a total of 50 leads in 10 tumours on 8 patients. Patients number 6, with multiple skin metastasis, and number 7, with bilateral inguinal lymphadenopathy were assessed on 2 separate occasions.

Carbogen breathing was tolerated without difficulty in all patients and thus the 10 minute carbogen breathing period could be analysed in all 50 traces (7 were excluded due to movement artefact). However, 2 patients were not evaluable for the entire 10 minute post carbogen observation period; patient number 1 had only a 4 minute post-carbogen observation period, and patient number 4 dislodged all of the

probes on movement 3 minutes after cessation of carbogen. This allowed analysis of the whole carbogen and post carbogen breathing time in 39 traces from 8 tumours (5 traces were excluded due to movement).

4.4.2 Individual tumours

Data recorded from each of the 10 tumours was analysed separately. The aim was first to acquire an overall view of the carbogen effect on blood flow in a particular tumour and then, to study variation of flux in individual microregions. Figure 4.1, shows complete data from three individual tumours, and demonstrates the variation in response to breathing carbogen between different tumours.

Table 4.2 shows the relative blood flow changes of the 10 tumours at five and ten minutes. These times were selected since they approximate to the timing of irradiation in clinical practice (Dische et al. 1992; Hoskin et al. 1997; Saunders et al. 1997) and would provide an indication of the effect of carbogen on blood flow during treatment.

After breathing carbogen for five minutes, five tumours showed an LDF increase of 10% or greater when compared with the pre-breathing value. Two tumours showed a decrease and 3 no overall change. After 10 minutes of carbogen breathing, blood flow was unchanged in four tumours, increased in four (1, 4, 6a, 6b) and decreased in two (3 and 7a). For seven of the tumours relative changes seen with carbogen were similar at both 5 and 10 minutes. This was not the case in three tumours. In patient 5 a small drop in relative flow at 10 minutes altered the response to a 'no change', in tumour 6a relative LDF increased further to become a significant change and in tumour 8, flow decreased to its pre-carbogen value.

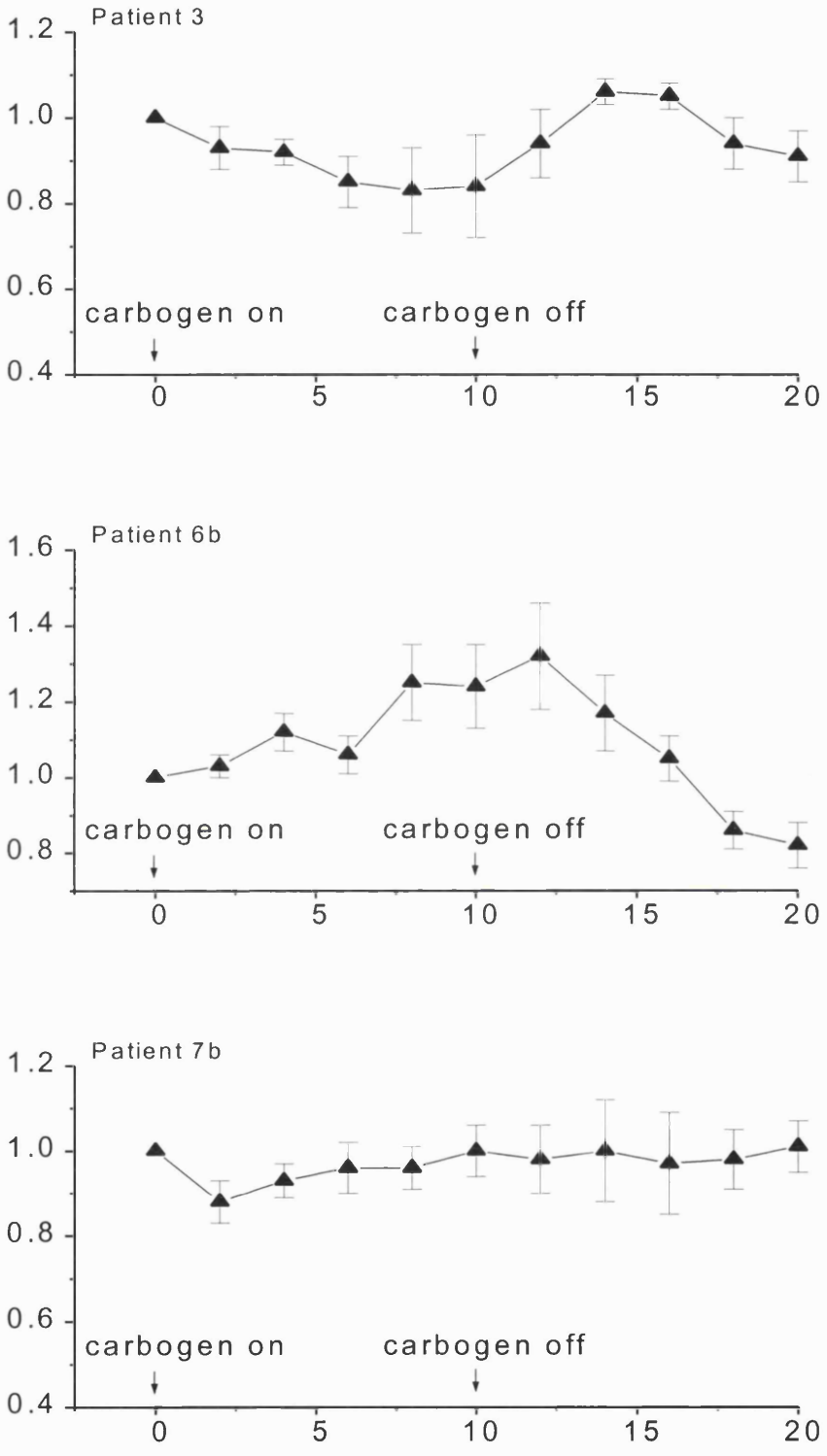


Figure 4.1 *Relative change in mean laser Doppler flow (±sem) against time in minutes on the x axis in three individual tumours showing variation in response to carbogen inhalation. Patient 3 shows an overall decrease, Patient 6a, an increase, and Patient 7b, no change.*

Patient no	*Relative LDF at 5 min*	*Relative LDF at 10 min*
1[a]	1.13 ± 0.05	1.13 ± 0.05
2	1.01 ± 0.11	0.94 ± 0.09
3	0.85 ± 0.06	0.84 ± 0.12
4[b]	2.24 ± 0.13	2.47 ± 0.67
5	1.10 ± 0.12	1.08 ± 0.16
6A	1.14 ± 0.20	1.21 ± 0.23
6B	1.06 ± 0.05	1.24 ± 0.11
7A	0.81 ± 0.18	0.79 ± 0.20
7B	0.96 ± 0.06	1.00 ± 0.06
8	1.23 ± 0.28	1.00 ± 0.05

Analysis of ten minute carbogen breathing period only because; [a]only 4 minute post carbogen observation period & [b]all probes dislodged 3 minutes into post-carbogen observation period.

Table 4.2 *Change in whole tumour relative laser Doppler flux after 5 and 10 minutes of carbogen breathing*

Variation of erythrocyte flux and response to carbogen in different microregions of a single tumour, which showed no overall change in blood flow, are illustrated in Figure 4.2. Laser Doppler flux in five areas from patient 2 are shown (one trace was excluded due to excessive probe movement). The individual traces show marked heterogeneity between the five microregions not only in terms of magnitude but also the direction of change in blood flow.

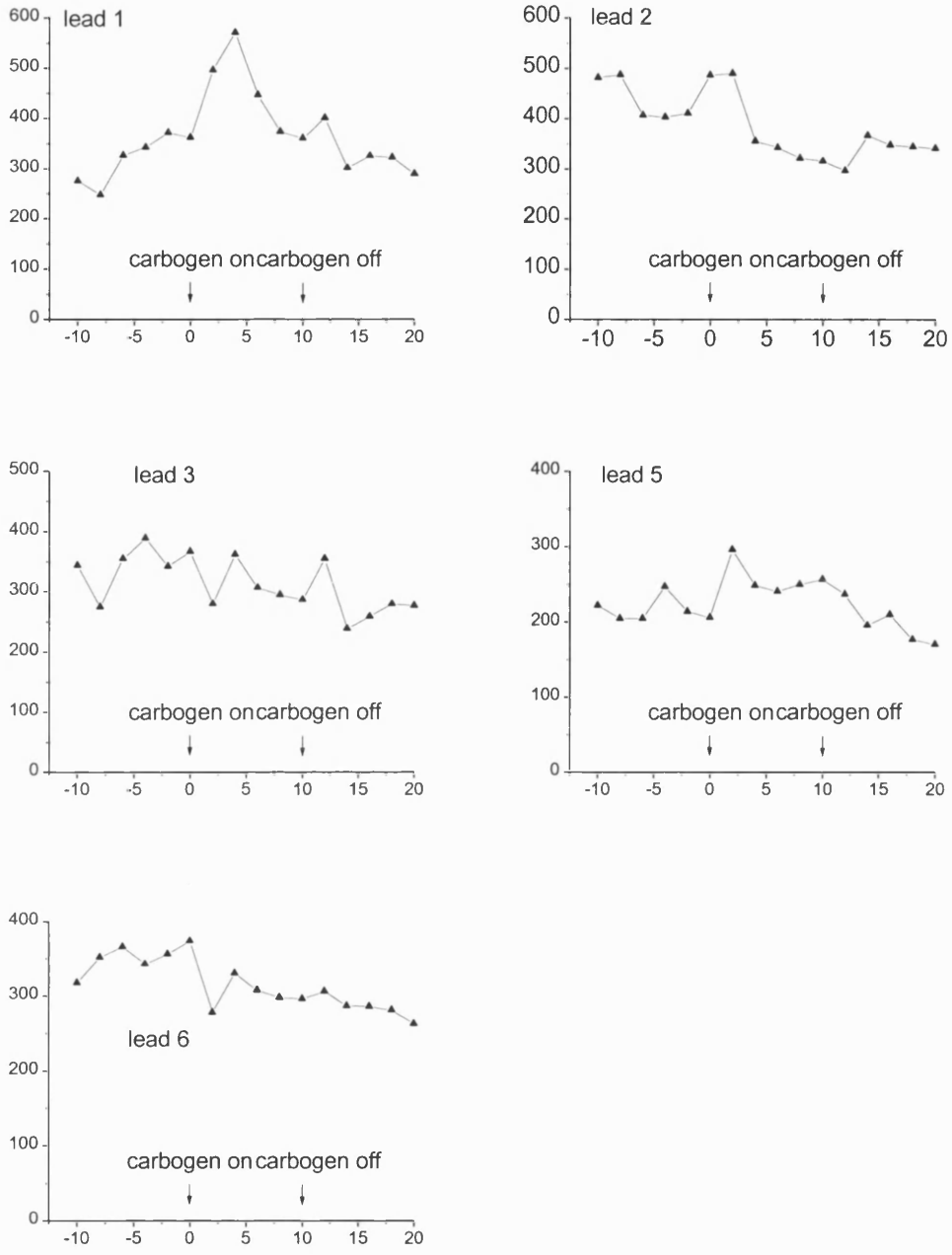


Figure 4.2 *Laser Doppler flux in 5 microregions of tumour 2, showing changes occurring during carbogen breathing. (In all traces laser Doppler flow in arbitrary units is plotted on the y axis against time in minutes on the x axis).*

4.4.3 All tumours

A summary of the effect of carbogen on RBC flux in all of the individual traces is shown in Table 4.3. At five minutes 60% of traces demonstrated a change in blood flow (37% increased, 23% decreased) and at 10 minutes 68% had altered (42% increased, 26% decreased). The magnitude of change was modest, however, with only 33% at five minutes and 42% at 10 minutes varying by 25% or more.

Factor of Change	***No of traces showing change at***			
	5 min		***10 min***	
	increases	**decreases**	**increases**	**decreases**
≥1.1	16 (37%)	10 (23%)	18 (42%)	11 (26%)
≥1.25	8 (19%)	6 (14%)	11 (26%)	7 (16%)
≥1.5	5 (12%)	1 (2%)	6 (14%)	2 (5%)
≥2	3 (7%)	0	2 (5%)	0
Total number of traces showing change ≥1.1	26 (60%)		29 (68%)	

Table 4.3 *Changes in microregional flux in all sampled areas at 5 and 10 minutes after the commencement of carbogen breathing*

Figure 4.3 shows complete data from these 8 tumours (34 traces). The values are expressed as a mean ± standard error of the mean. The data indicates that breathing carbogen does not influence RBC flux. As the patients switch to air breathing there is a marginal increase in flux which then falls. Statistical analysis of relative blood flow at 5 and 10 minutes did not show a significant variation from the pre-carbogen value (p=0.94, p=0.90 respectively)

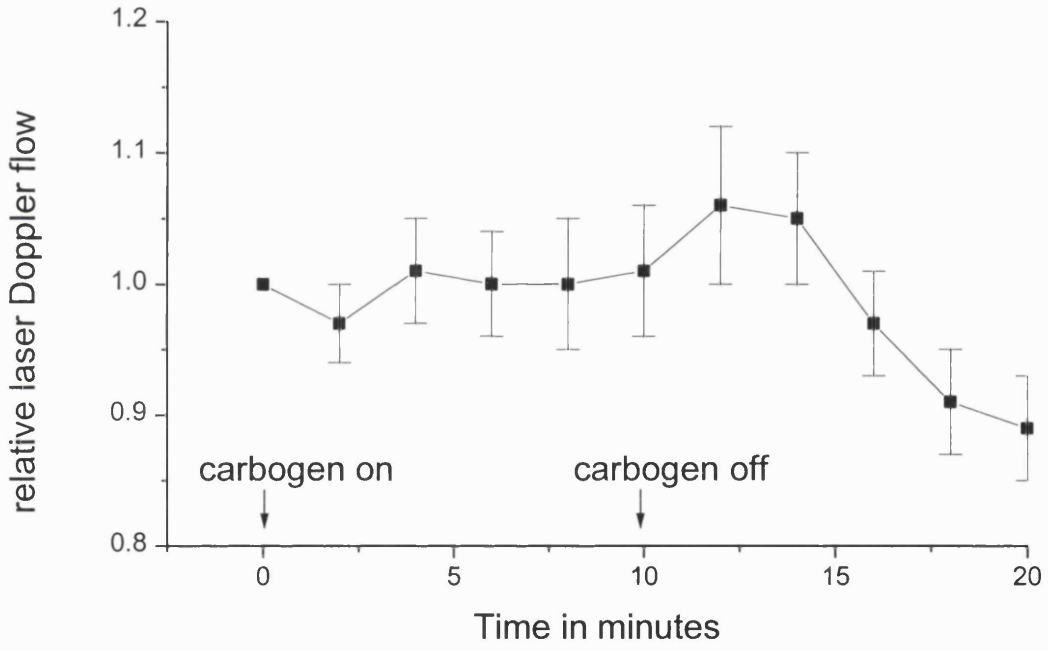


Figure 4.3 *Relative change in mean blood flow (±SEM) from 8 tumours during and after 10 minutes of carbogen breathing*

4.5 Discussion

These data confirm the presence of transient fluctuations in microregional blood flow consistent with model of acute hypoxia, but no consistent effect of carbogen on blood flow has been demonstrated. The results demonstrate that while individual fluctuation occur within the sampled microregions, overall perfusion of human tumours remains essentially unchanged during carbogen breathing. Extrapolation of RBC flux variation in microregions to be representative of perfusion fluctuations in the entire tumour may be misleading. But since the positioning of the probes is a random procedure we have assumed that the sampled areas are representative of adjacent regions within the tumour.

It confirms previous animal studies (Chaplin et al. 1993; Siemann et al. 1994) showing that the response of individual tumours to carbogen can vary and even where increases in flow are seen during carbogen breathing these are relatively small, with only two tumours in this series showing a fluctuation of more than 25%. Overall change in blood flow in a particular tumour was obtained by averaging readings from up to six probes inserted into that tumour. Since each probe has a sampling volume of$10^{-2}mm^3$ containing only a few capillaries, information is only available about blood flow changes in selected microregions of the tumour. However, since fluctuations in flow in discrete microregions have been implicated in radiation resistance, through the existence of perfusion limited hypoxia (Chaplin et al. 1987), investigation of flow changes on this level are potentially important.

At five minutes into carbogen breathing one-third of all sampled microregions show a variation in flow of at least 25% with an approximately equal number of increases and decreases and, in the small percentage of regions which display a variation of greater than 50%, increases outnumber decreases by a factor of 5 to 1. This indicates that carbogen may have a limited role in modulating perfusion limited hypoxia. It should be noted, however, that random fluctuations of more than 50% over a 60

minute period can be seen in both human (Chapter 3) and animal tumours (Hill and Chaplin 1995) suggesting that quite large random variations in blood flow may occur without the introduction of any other agents in such a system.

Although the data may offer some credence to the hypothesis that the carbon dioxide content abolishes vasoconstriction provoked by the high levels of oxygen, it suggests that the radiosensitising action of carbogen lies primarily in its ability to increase the oxygen carrying capacity of blood. This supports the introduction of agents such as nicotinamide with carbogen breathing to allow eradication of both diffusion and perfusion limited hypoxia.

CHAPTER 5

The effect of nicotinamide alone and with carbogen on microregional perfusion

5.1 Introduction

Nicotinamide is a drug that has been widely used in a variety of medical conditions, including psoriasis, pellagra and schizophrenia. Its use in cancer treatment stemmed from initial reports showing tumour specific radiosensitisation in murine tumours (Jonsson et al. 1985; Horsman et al. 1987). The mechanism of action bringing about this enhancement of radiosensitivity remains unclear. Although it was initially suggested that radiosensitisation was due to interference with repair of radiation-induced DNA damage (Ben-Hur et al. 1985), further experimental work has shown that nicotinamide could improve tumour oxygenation by reducing microregional fluctuations in blood flow (Horsman et al. 1989; Chaplin et al. 1990).

The enhancing effect of nicotinamide on tumour control by radiation is modest, with an enhancement ratio of between 1.2 and 1.7 (Rojas 1992). When nicotinamide is used with carbogen, however, enhancement ratios of 1.75 to 1.9 are consistently seen (Kjellen et al. 1991). Such an effect might be expected by the combining of these agents which are believed to overcome chronic diffusion limited hypoxia (carbogen) and acute perfusion related hypoxia (nicotinamide). Encouraging pre-clinical studies have led to clinical trials evaluating carbogen and nicotinamide in combination with radiotherapy (van der Maazen et al. 1995; Hoskin et al. 1997; Saunders et al. 1997).

5.2 Aim

The aim of this study was, using the laser Doppler probes, to assess the effect on microregional blood flow of nicotinamide alone and in combination with carbogen. In addition the results are compared with control patients in chapters 3 and 4 who were not pre-treated with nicotinamide.

5.3 Patients and Methods

5.3.1 Patients

Ten patients were studied, seven male and three female, with histologically proven advanced malignancy. Tumour characteristics are shown in Table 5.1.

5.3.2 Laser Doppler flowmetry

Relative changes in microvascular perfusion were measured as described in section 2.1.

5.3.3 Administration of nicotinamide

Nicotinamide was given in a dose of 80mg/Kg using a fast release oral preparation. This dose which is used in clinical practice can be tolerated by patients and achieves plasma levels ≥700μmol in most patients (Hoskin et al. 1995; Hoskin et al. 1997; Saunders et al. 1997).

Salivary or plasma levels of nicotinamide were measured by high performance liquid chromatography (Stratford and Dennis 1992) at 15 to 20 minute intervals during the study period.

Once nicotinamide had been given, patients waited 60 minutes before laser Doppler flow readings began. This timing was chosen in order to conform with current

clinical practice where patients receive radiotherapy and concomitant carbogen two hours after nicotinamide (Hoskin et al. 1997; Saunders et al. 1997). This is based upon experimental evidence that suggests radiosensitisation is maximal when radiation is given at peak plasma nicotinamide levels occurring between 0.5 and 3 hours after administration (Horsman et al. 1993)

5.3.4 Carbogen Breathing

Carbogen breathing was carried out using the system described in Chapter 2.2.3

5.3.5 Experimental Set-Up

Patients were positioned and probes inserted as described in Chapter 2.2

5.3.6 Measuring microvascular blood flow

Once the probes had been inserted and the patients were comfortable, laser Doppler flow readings began. After 60 minutes of 'baseline' measurements, a 10 minute carbogen breathing period commenced, and finally, an additional 10 minutes of LDF readings were acquired with the patient breathing room air.

5.3.7 Data Analysis

Traces for individual probes were generated as described in Section 2.6. Plots were studied and analysed as detailed in Sections 2.6 and 4.2.6.

Case	*Tumour site and size*		*Primary tumour*	*Histology*
1	node (SCF)	3cm	lung	squamous cell carcinoma
2	skin (abdomen)	3cm	colon	adenocarcinoma
3	node (neck)	6cm	pharynx	squamous cell carcinoma
4	skin (thigh)	5cm	skin	melanoma
5	breast	7cm	breast	adenocarcinoma
6[a]	skin (abdomen)	3cm	colon	adenocarcinoma
7[b]	skin (arm)	3cm	lung	squamous cell carcinoma
8	skin (flank)	3cm	kidney	transitional cell carcinoma
9	node (axilla)	5cm	lymph gland	NHL (low grade)
10	node (groin)	4cm	lymph gland	NHL (low grade)

[a]excluded from 60 min observation

[b]unable to breathe carbogen

Table 5.1 *Tumour characteristics in patients given nicotinamide*

5.4 Results

5.4.1 Toxicity

Several patients reported mild symptoms of light-headedness, but otherwise nicotinamide was tolerated with the minimum of toxicity. This group of patients seemed to have more difficulty with carbogen breathing than the control patients (Chapter 4). Patient 7 was unable to tolerate the face mask and did not breathe carbogen at all, and patient 10 commented the smell of the carbogen was nauseating but was able to complete the gas breathing period. Two patients (1 and 3) breathed carbogen for only eight minutes. This was because their tidal volume increased to such an extent that it exceeded the amount of carbogen being delivered (15 litres per

minute). Following this, two cylinders of carbogen were used making available up to 30 litres of gas per minute.

5.4.2 Analysing traces

Therapeutic levels of nicotinamide (≥700μmol) were achieved in all except patient 6, in whom levels were slow to rise and were just sub-optimal at the time of carbogen breathing. Table 5.2 details plasma nicotinamide concentrations 60 minutes after administration and a further 60 minutes later, at the time of carbogen breathing.

Case	***nicotinamide level nmol/ml at***	
	60 minutes	***start of carbogen***
1	1112	1022
2	2024	1452
3	1595	1054
4	1832	834
5	3217	1768
6	378	680
7	1364	1246
8	1849	1546
9	721	1676
10	1078	913

Table 5.2 *Plasma nicotinamide levels measured 60 minutes after administration and at the start of carbogen breathing*

The six data plots of patient 6 were excluded from the 60-minute observation period but included for analysis of carbogen breathing. The five traces acquired from patient 7 could only be analysed with respect to the 60-minute observation since he was unable to breathe carbogen. Nine patients were therefore eligible for analysis of both the baseline and carbogen measurements from a total of 52 sampled microregions were sampled for the baseline and carbogen breathing period. Seven traces from the baseline group and six from the carbogen group were excluded due to probe or patient movement.

Patient 5 moved abruptly after 40 minutes of observation, and although the probes were not dislodged the backscatter indicated that the probes had moved. All traces showed a corresponding large fluctuation in LDF which rapidly stabilised, the measurements acquired over the 40 minutes were used in the baseline analysis which might slightly underestimate the number of blood flow fluctuations seen.

5.4.3 Incidence of blood flow changes with nicotinamide

5.4.3.1 Individual tumours

As has already been shown in the preceding two chapters, heterogeneity of perfusion changes were seen within sampled microregions of the same tumour and between individual tumours. Figure 5.1 shows traces from 5 microregions sampled in patient 10. LDF in trace 4 remains steady whilst the other traces show several changes in LDF occurring at different rates and times during the hour.

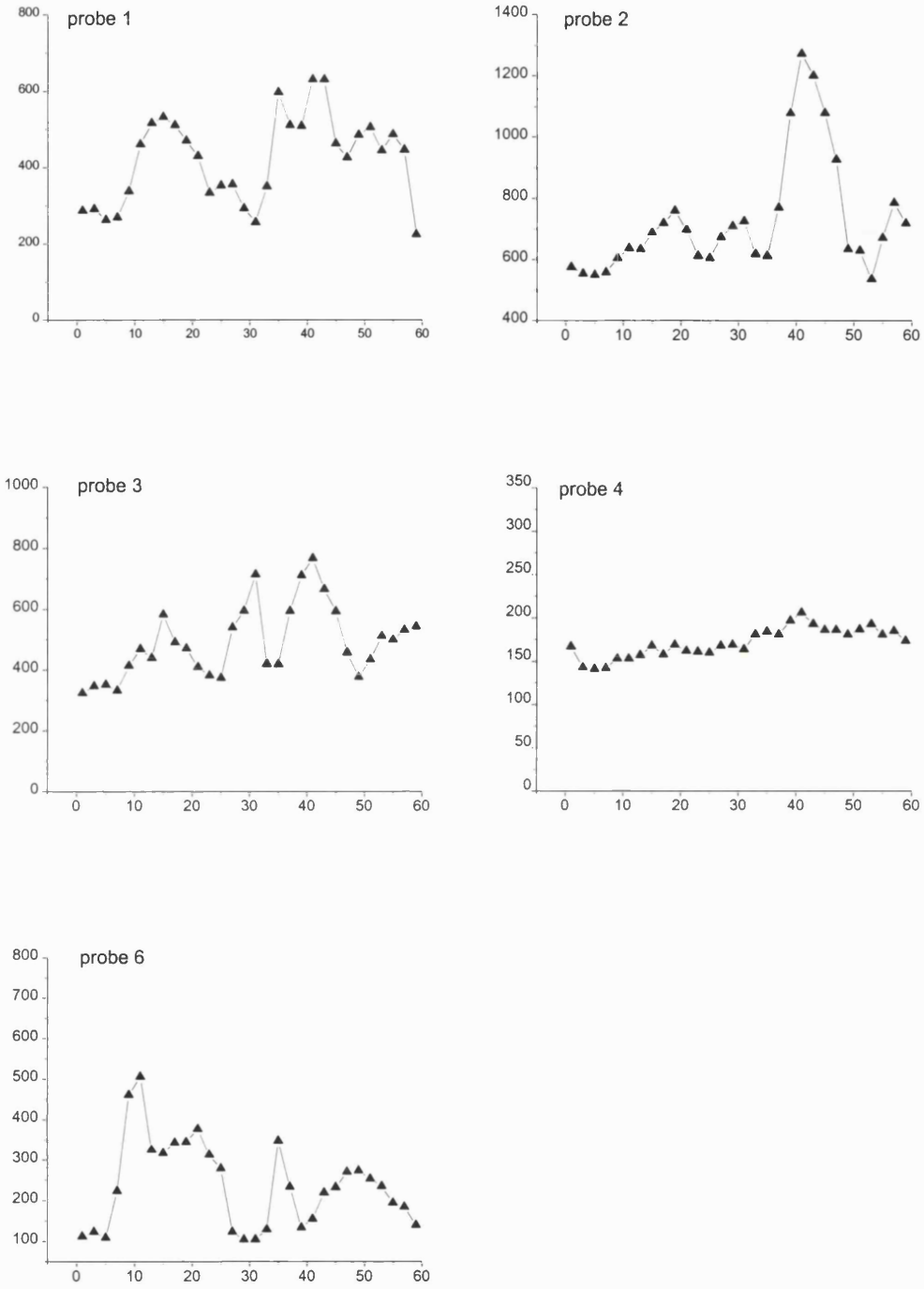


Figure 5.1 *Laser Doppler flux measured in 5 discrete microregions in the tumour of patient 10 over a 60 minute period 1 hour following oral nicotinamide. (All graphs show laser Doppler flow in arbitrary units on the y axis against time in minutes on the x axis)..*

5.4.3.2 All tumours

Perfusion changes for all nine tumours are summarised in table 5.3. Temporal fluctuations in perfusion by a factor of 1.5 or more occurred in over two thirds of all microregions sampled and 13 traces (29%) showed changes of two fold or more. In 12 traces initial changes in flow were subsequently reversed.

Factor of change	***Number of traces showing change (n=45)***	***Number and direction of changes***
≥1.5	31 (69%)	30 ↑ 19 ↓
≥2	13 (29%)	14 ↑ 0 ↓

Table 5.3 *Changes in microregional blood flow during 60 minute observation periods in 9 tumours pre treated with nicotinamide*

The complete data from the hour observation period from all 45 sampled microregions is shown in figure 5.2. Each point is a single mean calculated from each two minute average in the 45 traces and expressed as mean laser Doppler flow ± standard error of the mean (sem). It can be seen that overall blood flow within this group of tumours remains constant.

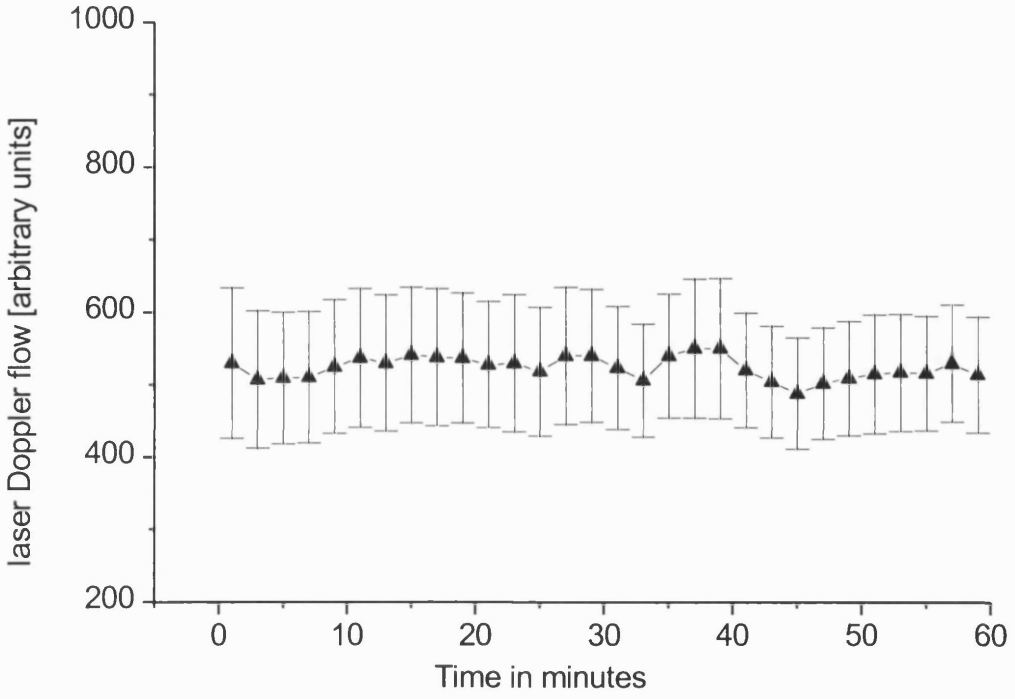


Figure 5.2 *Mean laser Doppler flow ± sem over 60 minutes from 45 microregions in 9 tumours pre treated with nicotinamide*

5.4.4 Kinetics of blood flow changes

The majority of changes in blood flow occurred within a 20 minute period, with the median time for fluctuation 12 minutes (range four to sixty minutes). The initial perfusion trend was reversed in 12 traces with more than one reversal in 4 leads.

Rate of blood flow change	***Number of changes (49 total)***
≤10 minutes	27 (55%)
≤ 20minutes	34 (69%)

Table 5.4 Kinetics of change in microregional blood flow in 9 tumours

5.4.5 Carbogen breathing

5.4.5.1 Individual tumours

Table 5.5 shows relative blood flow changes after five and ten minutes of carbogen breathing. Five tumours showed more than a 10% increase in flow after 5 minutes of carbogen breathing and in 4 tumours flow was essentially unchanged with less than a 10% variation from pre-carbogen values. Following 10 minutes of carbogen, flow had increased in 6 tumours and was unchanged in three.

Patient no	*Relative LDF at 5 min*	*Relative LDF at 10 min*
1[a]	1.16 ± 0.11	1.18 ± 0.05
2	1.02 ± 0.04	1.10 ± 0.04
3[a]	1.05 ± 0.07	0.92 ± 0.05
4	1.09 ± 0.07	1.16 ± 0.09
5	1.17 ± 0.12	1.26 ± 0.14
6[b]	1.18 ± 0.06	1.25 ± 0.07
8	1.11 ± 0.03	1.07 ± 0.03
9	0.97 ± 0.19	0.96 ± 0.19
10	1.77 ± 0.39	1.77 ± 0.39

[a]completed only 8 minutes of carbogen breathing

[b]plasma nicotinamide just below 'therapeutic' levels

Patient 7 excluded because unable to breathe carbogen

Table 5.5 *Relative change in laser Doppler flow in all sampled microregions of each tumour after 5 and 10 minutes of carbogen breathing*

5.4.5.2 *Individual traces*

A summary of the effect of carbogen on RBC flux in individual traces is shown in table 5.6. This once again illustrates the heterogeneity of blood flow within sampled microregions. At five minutes 51% of traces demonstrate a variation in flow of 10% or greater (42% increased and 9% decreased). The changes are sustained after 10 minutes but are modest with only a quarter of sampled microregions varying by 25% or more.

Factor of Change	***No of traces showing change at***			
	5 min		***[a]10 min***	
	increases	*decreases*	*increases*	*decreases*
≥1.1	19 (42%)	4 (9%)	17 (46%)	2 (5%)
≥1.25	10 (22%)	1 (2%)	8 (22%)	2 (5%)
≥1.5	3 (6%)	0	6 (16%)	2 (5%)
≥2	2 (4%)	0	2 (5%)	0
Total number of traces showing change ≥1.1	23 (51%)		19 (51%)	

[a]2 patients completed only 8 minutes of carbogen breathing

Table 5.6 *Changes in microregional flux in all sampled areas at 5 and 10 minutes after the commencement of carbogen breathing*

5.4.5.3 *All tumours*

Figure 5.3 shows the relative blood flow changes in nine patients (46 traces) as a result of carbogen breathing. Values are expressed as a mean ± SEM. The data indicate that overall blood flow increased with carbogen breathing appearing to plateau after eight minutes. Analysis after five and ten minutes of carbogen showed blood flow to have increased by 17% and 22% relative to the pre-carbogen value. This reaches statistical significance at $p<0.004$ and $p<0.001$ respectively.

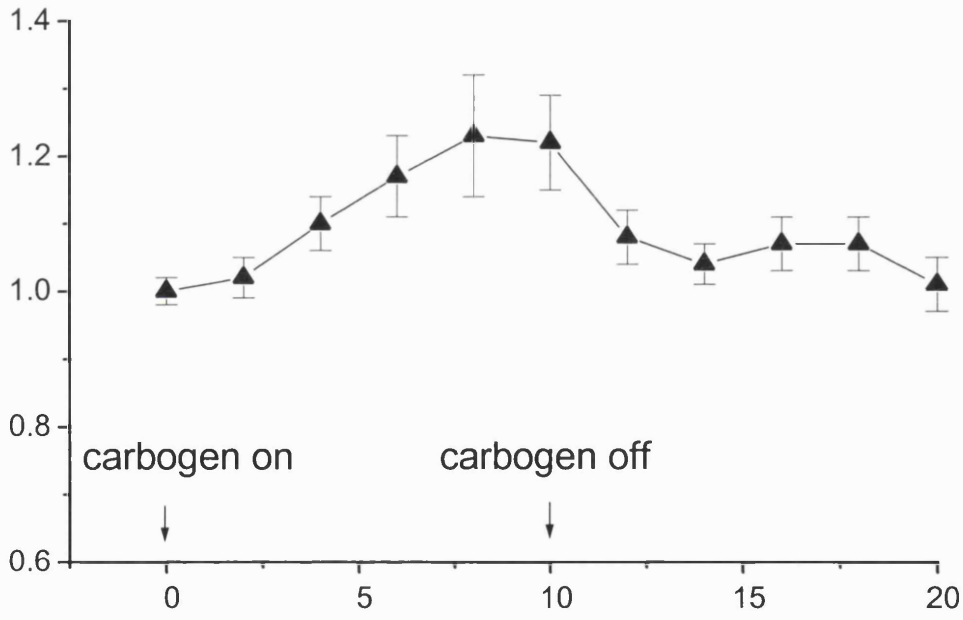


Figure 5.4 *Relative change in laser Doppler flow ±SEM during and after carbogen breathing*

5.5 Discussion

The effect of nicotinamide in improving tumour oxygenation is thought to be due to a reduction in the transient closure of microregional blood vessels which leads to a more homogenous microregional blood flow (Chaplin et al. 1990). The mechanism of action for this has not been fully elucidated, but may in part be due to an influence on white blood cells which show decreased filterability under hypoxic conditions. Nicotinamide can increase leukocyte filterability (Honess et al. 1996), possibly by down regulation of leukocyte adhesion molecules (Hiromatsu et al. 1992).

The proposed effect of nicotinamide on microregional blood flow might lead us to expect that its administration would lead to a rise in blood flow, or at least an alteration in the ratio of cyclical blood flow fluctuations with fewer decreases occurring. These data, however, show a similar result to random blood flow fluctuations seen in the group of untreated control patients (Chapter 3) in whom 62% of traces changed by a factor of 1.5 or more (compared with 69% in this series), and 27% by a factor of 2 (29% in this series), with increases in blood flow outnumbering decreases in both groups (Table 5.7).

	Control (81 changes)	Nicotinamide (49 changes)
% increases	57%	61%
% decreases	43%	39%

Table 5.7 *Percentage of increases and decreases (≥1.5) in microregional LDF during the 60 minute observation period in control and nicotinamide patients*

Thus, nicotinamide, at the doses administered in the clinic, does not appear to prevent transient fluctuations in human tumour microregional blood flow. This finding is consistent with animal work using laser Doppler probes showing doses of 250mg/Kg do not affect microregional RBC flux, although at a dose of 500mg/Kg, nicotinamide reduces the number of decreases measured (Hill and Chaplin, 1995). This is consistent with findings of earlier studies using fluorescent perfusion markers in experimental tumours, that suggested high dose nicotinamide reduced or eliminated such blood flow instability (Chaplin et al. 1990). It should be emphasised, however, that these studies have used much higher doses of nicotinamide that cannot be achieved in man without unacceptable toxicity (Hoskin et al. 1995).

These results demonstrate that, together, carbogen and nicotinamide give rise to in an increase in blood flow. The overall increase of 22% may seem modest, but such a global view ignores the spatial heterogeneity of both blood flow and oxygenation known to exist in tumours. If increases were occurring where capillary flow has either ceased or is severely reduced, in other words where microregional perfusion is most compromised, they could bring important benefits for drug delivery and the eradication of hypoxia.

Why the combination of carbogen and nicotinamide increases tumour blood flow by over 20% while individually their effect is minimal remains unclear. It may involve a complex interaction between tumour cells and tumour vasculature. Tumours produce a variety of vasoactive compounds with different actions. For example, nitric oxide, released by endothelial cells which line blood vessels causes vasodilatation thereby increasing blood flow. Whereas endothelin, a peptide

produced by microvascular endothelium is a potent vasoconstrictor. These substances appear to work in opposition to maintain vascular tone with each being able to modify the activity and production of the other. The production of both endothelin and nitric oxide may vary considerably between different tumour types and appears to be independently altered by carbon dioxide and nicotinamide. Endothelin synthesis is decreased with increasing CO2 concentrations which in brain tissue leads to vasodilatation(Yoshimoto et al. 1991). Nicotinamide, however, inhibits nitric oxide synthase (NOS) (Pellat-Deceunynck et al. 1994), and animal work has shown that inhibition of NOS using nitro-L-arginine analogues can reduce tumour blood flow (Horsman et al. 1996).

Thus, a selective alteration in local production of such vasoregulatory compounds that might, for example, prevent vasoconstriction of vessels within a tumour, may be a possible explanation for the effects on tumour perfusion seen in our study with the combination of carbogen and nicotinamide.

This work confirms that tumour perfusion can be increased with the combination of carbogen and nicotinamide. Clearly, consistent increases are necessary if the use of nicotinamide and carbogen is to become routine. These initial studies, however, provide a basis for further investigation of a wider range of tumours and the evaluation of other agents that might offer larger and more dependable improvements in tumour perfusion.

Chapter 6

Concluding Discussion

The spatial and temporal variations that appear to characterise microregional human tumour blood flow impact on virtually all therapeutic options available. The delivery and distribution within a tumour of systemic anti-cancer agents such as drugs, antibodies and gene products may be compromised. In addition, these microcirculatory fluctuations can alter the tumour microenvironment resulting in acidosis, substrate depletion and regional hypoxia. Cells within areas of under perfusion and reduced oxygenation are capable not only of survival but proliferation.

The hypoxia that develops within a tumour exerts an influence at both molecular and cellular levels. Genes affecting angiogenesis, metastatic behaviour and repair may be induced by low pO_2, and, on the cellular level, the presence of oxygen is key to the success of radiation and photodynamic therapy as well as biological and certain cytotoxic drug treatments.

Directly modifying tumour blood flow has not, so far, been particularly successful in the clinic. Laboratory work has identified agents which by exploiting the differences between normal and tumour vasculature can selectively alter tumour blood flow. An example is angiotensin II, a vasoconstrictor peptide, which causes relative increases in tumour perfusion. Inconsistency in results, however, complicates its potential clinical application. The converse approach has been to use agents such as hydralazine that decrease tumour blood flow in conjunction with bioreductive drugs (Chaplin 1989). Laboratory and human studies, however, showed that a significant and selective drop in blood flow could only occur when systemic blood pressure

decreased by at least 15%. Clearly, such reductions in blood pressure would lead to unacceptably high risks for routine clinical practice.

Historically the most widely used and arguably most successful clinical approach to modification of the tumour milieu has been to use hyperoxic gas inhalation aimed primarily at improving tissue oxygenation. Breathing 100% oxygen, or 95% oxygen and 5% carbon dioxide, targets chronically hypoxic cells, reducing the fraction of hypoxic regions (Falk et al. 1992) and improving clinical response (Overgaard 1995). Reversible fluctuations in tumour microcirculation, however, lead to acutely hypoxic cells and a strategy aimed at improving tissue oxygenation without also targeting this population will always have this limitation.

The aim of this work has been to characterise changes that occur spontaneously in human tumour microcirculation and to assess how these are affected by both breathing carbogen gas and taking oral nicotinamide alone and in combination with carbogen.

The multi-channel laser Doppler system has been widely used in animal tumours and has become the benchmark for measurement of microcirculatory dynamics. This work shows that laser Doppler microprobes can be readily used in human tumours, providing excellent resolution down to capillary level. In addition the technique developed for clinical use has proved to be reproducible and, despite being invasive, especially well tolerated.

Spontaneous random fluctuations in microregional red blood cell flux by a factor of at least 1.5 occur frequently in tumours (62% of regions sampled) and over two thirds of these changes occur within 20 minutes. All of the results show a preponderance of increases in RBC flux. There are two possible explanations for this. First, vessels could be haemorrhaging in front of the probes. Some bleeding is inevitable due to the invasive nature of the procedure, but it would be unlikely to

persist for the 60 minute observation period. Second, and perhaps more likely, is the method of analysing changes in flux. The change was calculated as a percentage of the previous peak or trough value. If, for example, LDF rose from 100 to 150 and then fell back to 100, LDF would increase by a factor of 1.5 and decrease by 1.3 and therefore this would be considered to be a significant increase but not a significant decrease.

Blood flow changes in response to breathing carbogen appear to be tumour dependent with both increases and decreases seen. Understanding the biological reasons for differing tumour responses to carbogen may help in the selection of patients who would benefit from such modulation.

The addition of nicotinamide at a dose of 80mg/kg had little or no effect on the random fluctuations in microregional perfusion. But the combination of nicotinamide with carbogen inhalation inhibits decreases in blood flow with an average 20% increase in red blood cell flux seen. Even if no increase in flux was found, simply the elimination of blood flow decreases could be of therapeutic importance, allowing a more homogenous distribution of drugs and fewer cells to become acutely hypoxic.

Each microprobe measures a sampling volume of only $0.01mm^3$ giving information about real time blood flow changes in a discrete region. The random positioning of the probes in a tumour and the differing laser Doppler flux readings noted between sampled areas is assumed to reflect the heterogeneity of blood flow within a tumour. This would be compatible with the large degree of variation seen in tumour pO_2 when measured using the Eppendorf pO_2 histograph (Falk et al. 1992).

The measurements from individual probes within a tumour have been combined to provide a global picture of tumour blood flow. But such extrapolation may not be an accurate depiction of tumour perfusion as a whole. For example a two-fold flow

increase in one microregion combined with a two-fold decrease in another would, using this method, imply no change in tumour blood flow, whereas the tumour vasculature is in a constant dynamic state. The description of a global view of tumour blood flow, however, remains valid since ultimately response to treatment is based on the tumour as a whole and not of its individual parts.

One of the limitations of the laser Doppler system is that the technique is necessarily invasive and although subcutaneous lignocaine was used to limit discomfort, the probes required introduction through a cannula. The short probe length meant that only superficial tumours could be studied, and since they were inserted randomly without the use of imaging, there was no method of verifying the position of the probes once in the tumour. The use of either magnetic resonance imaging or CT scanning to insert the cannulae may be helpful in ensuring accurate positioning within a tumour, avoiding macroscopically necrotic areas and comparing tumour blood flow measurements with other techniques such as functional MRI.

Perhaps the most difficult problem to solve is that of patient movement. The probes can, with only a cough or sneeze, be dislodged and because real time fluctuations were measured, patients had to remain completely still for up to 90 minutes. This is a difficult proposition for any individual and in particular one with advanced cancer. Ensuring optimum pain control and a comfortable environment is essential but the tenacity, goodwill and humour shown by the patients studied proved to be the key to achieving such reliable results.

The finding that pre-treatment with nicotinamide can improve microregional blood flow in tumours during carbogen breathing is an important clinically relevant finding and justifies further study. The nausea and vomiting associated with 80mg/kg has limited its clinical use in some centres (Hoskin et al. 1997). This has prompted radiosensitisation studies with lower doses. Initial results in the sarcoma F tumour suggest enhancement ratios of carbogen plus nicotinamide are maintained

with lower plasma nicotinamide levels (Chaplin et al. 1998). The effect on tumour blood flow with carbogen breathing after pre-treatment with nicotinamide at more tolerable lower doses needs evaluation as a potential therapeutic modifier.

The interaction of nicotinamide with carbogen inhalation to enhance microregional blood flow when nicotinamide alone has little or no effect, warrants study into the mechanisms of this interaction. The endogenous vasoactive compounds endothelin and nitric oxide regulate tumour vascular tone by a complex interaction that is not yet fully understood. Their production is modified by the presence of oxygen, carbon dioxide and nicotinamide and in addition varies between tumour types (Chaplin et al. 1998). Correlating nicotinamide and carbogen-driven tumour blood flow and oxygenation changes with endothelin and nitric oxide levels will help characterise the relevance of these compounds to tumour perfusion.

The relationship between fluctuations in microregional perfusion to tumour pO_2 needs to be defined and the recent development of a probe designed to measure dynamic changes and oxygen tension will allow greater insight into the interaction of these parameters.

To conclude, this research shows that real-time fluctuations in human tumour blood flow are measurable, and the combination of nicotinamide and carbogen lead to increases in microregional blood flow. Such a finding has the potential to enhance both the response to radiotherapy treatment and the delivery of systemic anti-cancer agents. Further work, studying a wider range of tumours may confirm tumour types that respond to such manipulation and allow treatment to be individually tailored.

References

Abbot N C, Beck J S (1993) Biological zero in laser Doppler measurements in normal, ischaemic and inflamed human skin. Int J Microcirc Clin Exp 12:89-98.

Ahn H, Lindhagen J, Nilsson G E, Salerud G, Jodal M, Lundgren O (1985) Evaluation of laser Doppler Flowmetry in the assessment of intestinal blood flow in the cat. Gastroenterology 88:951-7.

Amois H I, Goffman T E, Komaki R, Cox J D (1988) Acute radiation effects on cutaneous microvasculature: Evaluation with a laser Doppler perfusion monitor. Radiology 169:557-60.

Argyll Campbell J (1931) The use of gas mixtures in cancer therapy. Cancer Review 6:289-302.

Auler H (1927) Zeits für Krebsforsch. 25:357.

Aune T M, Pogue S (1989) Inhibition of tumour cell growth by Interferon gamma is mediated by two distinct mechanisms, dependent on tumour oxygenation, induction of tryptophan degradation and depletion of intracellular nicotinamide adenine dinucleotide. J Clin Invest 84:863-75.

Awaad H, Naggar M, Mocktar N, Barsoum M (1986) Intercapillary distance measurement as an indicator of hypoxia in carcinoma of the cervix uteri. Int J Radiat Oncol Biol Phys 12:1329-33.

Beaney R P, Lammertsma A A, Jones T, McKenzie C G, Halnan K E (1984) Positron emission tomography for in-vivo measurements of regional blood flow, oxygen utilisation and blood volume in patients with breast carcinoma. Lancet 1:131-4.

Ben-Hur E, Chen C C, Elkind M (1985) Inhibitors of poly-(adenosine diphospharibose)synthetase, examination of radiation response in Chinese hamster cells. Cancer Res 45:2123-7.

Bird A D, Telfer A B M (1965) Effect of hyperbaric oxygen on limb circulation. Lancet 1:335-56.

Bonner R F, Nossal R (1981) Model for laser Doppler measurements of blood flow tissue. Appl Opt 20:2097-107.

Brown J M (1979) Evidence for acutely hypoxic cells in mouse tumours, and a possible mechanism of reoxygenation. Br J Radiol 52:650-6.

Bush R S (1986) The significance of anaemia in clinical radiation therapy. Int. J. Radiat. Oncol. Biol. Phys. 12:2047-50.

Calcutt G, Ting S M, Preece A W (1970) Tissue NAD levels and the response to irradiation or cytotoxic drugs. Br J Cancer 24:380-8.

Campbell J A, Cramer W (1928) . Lancet 1:828.

Caspary L, Creutzig A, K A (1988) Biological zero in laser Doppler fluxmetry. Int J Microcirc Clin Exp 3:367-71.

Chaplin D (1989) Hydralazine induced tumour hypoxia; A potential target for cancer therapy. J Natl Cancer Inst 81:618-22.

Chaplin D J, Hill S A (1995) Temporal heterogeneity in microregional erythrocyte flux in experimental solid tumours. Br J Cancer 71:1210-3.

Chaplin D J, Hill S A, Bell K M, Tozer G (1998) Modification of tumour blood flow: Current status and future directions. Semin Rad Oncol 8:151-63.

Chaplin D J, Horsman M R, Aoki D S (1991) Nicotinamide, Fluosol DA and Carbogen: a strategy to reoxygenate acutely and chronically hypoxic cells in vivo. Br J Cancer 63:109-13.

Chaplin D J, Horsman M R, Siemann D W (1993) Further evaluation of nicotinamide and carbogen as a strategy to reoxygenate hypoxic cells in vivo: importance of nicotinamide dose and pre-irradiation breathing time. Br J Cancer 68:269-73.

Chaplin D J, Horsman M R, Trotter M J (1990) Effect of nicotinamide on the microregional heterogeneity of oxygen delivery within a murine tumor. J Natl Cancer Inst 82:672-6.

Chaplin D J, Olive P L, Durand R E (1987) Intermittent blood flow in a murine tumor: radiobiological effects. Cancer Res 47:597-601.

Churchill-Davidson I, Sanger C, Thomlinson R H (1955) High-pressure oxygen and radiotherapy. Lancet 1:1091-5.

Colantuoni A, Bertuglia S, Intaglietta M (1993) Biological zero of laser Doppler fluxmetry: microcirculatory correlates in the hamster cheek pouch during flow and no flow conditions. Int J Microcirc Clin Exp 13:125-36.

Dische S, Rojas A, Rugg T, Hong A, Michael B D (1992) Carbogen breathing: a system for use in man. Br J Radiol 65:87-90.

Du Sault L A (1963) The effect of oxygen on the response of spontaneous tumours in mice to radiotherapy. Br J Radiol 36:749-54.

Evans R G, Kimler B F, Morantz R A, Batnitzky S (1993) Lack of complications in long-term survivors after treatment with fluosol® and oxygen breathing as an adjuvant to radiation therapy for high-grade brain tumours. Int J Radiat Oncol Biol Phys 26:649-52.

Falk S J, Ward R, Bleehen N M (1992) The influence of carbogen breathing on tumour tissue oxygenation in man evaluated by computerised p02 histography. Br J Cancer 66:919-24.

Feldmann H J, Sievers K, Fuller J, Molls M, Lohr E (1993) Evaluation of tumor blood perfusion by dynamic MRI and CT in patients undergoing thermoradiotherapy. Eur J Radiol 16:224-9.

Fischer A (1927) Zeits für Krebsforsch. 24:528 and 63.

Fischer-Wasels B (1930). Die ergebnisse der gasbehandlung bösartiger geschwülste beim menschen. In: Frankfurter Zeitschrift für Pathologie. J. F. Bergmann, Munich,39 p.231-53.

Folkman J (1990) What is the evidence that tumours are angiogenesis dependent? J Natl Cancer Inst 82:4-6.

Foltz R M, McLendon R E, Friedman H S, Dodge R K, Bigner D D, Dewhirst M W (1995) A pial window model for the intracranial study of human glioma microvascular function. Neurosurgery 36:976-84.

Gaa J, Warach S, Wen P, Thangaraj V, Wielopolski P, Edelman R R (1996) Non-invasive perfusion imaging of human brain tumors with EPISTAR. Eur Radiol 6:518-22.

Gatenby R A, Kessler H B, Rosenblum J S (1988) Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of therapy. Int J Radiat Oncol Biol Phys 14:831-8.

Grau C, Overgaard J (1998). Significance of hemoglobin concentration for treatment outcome. In: Molls M and Vaupel P (eds) Blood perfusion and microenvironment of human tumours. Implications for clinical radiooncology. Springer, p.101-12.

Gray L H, Conger A D, Ebert M (1953) The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Br J Radiol. 26:638-48.

Griffiths J R, Taylor N J, Howe F A, Saunders M, Robinson S, Hoskin P, Powell M, Thoumine M, Caine L, Baddeley H (1997) The response of human tumours to carbogen breathing, monitored by gradient-recalled echo magnetic resonance imaging. Int J Radiat Oncol Biol Phys 39:697-701.

Hall E J (1988). Radiobiology for the radiologist. J. B. Lipincott, Philadelphia.

Haumschild D J (1986). Microvascular blood flow measurement by laser Doppler flowmetry. TSI Incorporated, St Paul, Minnesota.

Hill S A, Chaplin D J (1995) The effect of nicotinamide on microregional blood flow within tumours assessed using laser Doppler probes. Acta Oncol 34:401-4.

Hiromatsu Y, Sato M, Yamada K, Nonaka K (1992) Nicotinamide and 3-aminobenzamide inhibit recombinant human interferon-gamma-induced HLA-DR antigen expression, but not HLA-A, B, C antigen expression, on cultured human thyroid cells. Clin Endocrinol Oxf 36:91-5.

Hockel M, Knoop C, Schlenger K, Vorndran B, Baubmann E, Mitze M, Knapstein P, Vaupel P (1993) Intratumoural pO_2 predicts survival in advanced cancer of the uterine cervix. Radiother Oncol 26:45-50.

Honess D, Kitamoto Y, Rampling M, Bleehen N (1996) Nicotinamide and pentoxifylline increase leucocyte filterability: a possible mechanism for reduction of acute hypoxia. Br J Cancer 74 Suppl:236-40.

Honess D J, Bleehen N M (1995) Perfusion changes in the RIF-1 tumour and normal tissues after carbogen and nicotinamide, individually and combined. Br J Cancer 71:1175-80.

Honess D J, Dennis I F, Bleehen N M (1993) Pentoxifylline: its pharmacokinetics and ability to improve tumour perfusion and radiosensitivity in mice. Radiother Oncol 28:208-18.

Horsman M, Chaplin D, Hill S (1996) Effect of nitro-L-arginine on blood flow, oxygenation and the activity of hypoxic cell cytotoxins in murine tumours. Br J Cancer 74:168-71.

Horsman M R (1995) Nicotinamide and other benzamide analogs as agents for overcoming hypoxic cell radiation resistance in tumours. A review. Acta Oncol 34:571-87.

Horsman M R, Chaplin D J, Brown J M (1987) Radiosensitization by nicotinamide in vivo: a greater enhancement of tumor damage compared to that of normal tissues. Radiat Res 109:479-89.

Horsman M R, Chaplin D J, Brown J M (1989) Tumor radiosensitization by nicotinamide: a result of improved perfusion and oxygenation. Radiat Res 118:139-50.

Horsman M R, Hoyer M, Honess D J, Dennis I F, Overgaard J (1993) Nicotinamide pharmacokinetics in humans and mice: a comparative assessment and the implications for radiotherapy. Radiother Oncol 27:131-9.

Hoskin P J, Saunders M I, Phillips H, Cladd H, Powell M E B, Goodchild K, Stratford M I, Rojas A (1997) Carbogen and nicotinamide in the treatment of bladder cancer with radical radiotherapy. Br J Cancer 76:260-3.

Hoskin P J, Stratford M R, Saunders M I, Hall D W, Dennis M F, Rojas A (1995) Administration of nicotinamide during chart: pharmacokinetics, dose escalation, and clinical toxicity. Int J Radiat Oncol Biol Phys 32:1111-9.

Inch W R, McCredie J A, Kruuv J (1966) Effect of breathing 5 per cent carbon dioxide and 95 per cent oxygen at atmospheric pressure on tumour radiocurability. Acta Radiol Ther Phys Biol 4:17-25.

Ishizaka S, Kimoto M, Tsujii T (1992) Defect in generation of LAK cell activity under oxygen limited conditions. Immun Lett 32:209-14.

Jirtle R L (1988) Chemical modification of tumour blood flow. In J Hyperthermia 4:355-71.

Jonsson G G, Kjellen E, Pero R W, Cameron R (1985) Radiosensitization effects of nicotinamide on malignant and normal mouse tissues. Cancer Res 45:3609-14.

Kaanders J H A M, Pop L A M, Marres H A M, van der Maazen R W M, van der Kogel A J, van Daal W A J (1995) Radiotherapy with carbogen breathing in head and neck cancer: feasibility and toxicity. Radiother Oncol 37:190-8.

Kallman R F (1972) The phenomenon of reoxygenation and its implications for fractionated radiotherapy. Radiology 105:135-42.

Keresteci A G, Rider W D (1978) Use of orthobaric oxygen in the radiotherapy of bladder tumours. Canad J Surg 16:127-9.

Kjellen E, Joiner M C, Collier J M, Johns H, Rojas A (1991) A therapeutic benefit from combining normobaric carbogen or oxygen with nicotinamide in fractionated X-ray treatments. Radiother Oncol 22:81-91.

Kruuv J A, Inch W R, McCredie J A (1967) Blood flow and tumour oxygenation of tumours in mice. Cancer 20:51-8.

Lagendijk J, Hofmann P, Schipper J (1988) Perfusion analysis in advanced breast carcinoma. Int J Hyperthermia 5:479-95.

Laurence V, Ward R, Honess D, Bleehen N (1996). A study of carbogen breathing and pentoxifylline compared with carbogen and nicotinamide. Third International ARCON Workshop, Umea, Sweden.

Lee I, Levitt S H, Song C W (1993) Improved tumour oxygenation and radiosensitization by combination with nicotinamide and pentoxifylline. Int J Radiat Biol 64:237-44.

Lustig N, Lowe N, Prosnitz L, Spaulding M, Cohen M, Stitt S, Brannon R (1990) Fluosol and oxygen breathing as an adjuvant to radiation therapy in the treatment of locally advanced non-small cell carcinoma of the lung: results of a phase I/II study. Int J Radiat Oncol Biol Phys 19:97-102.

Lynch E M, Sampson L E, Khalil A A, Horsman M R, Chaplin D J (1995) Cytotoxic effect of tumour necrosis factor-alpha on sarcoma F cells at tumour relevant oxygen tensions. Acta Oncol 34:423-7.

Mantalya M, Heikkonen J, Licphil, Perkkio J (1988) Regional blood flow in human tumours measured with argon, krypton and xenon. Br J Radiol 61:379-82.

Mattsson J, Appelgren L, Hamberger B, Peterson H-I (1979). Tumour vessel innervation and influence of vasoactive drugs on tumour blood flow. In: Peterson H-I (ed) Tumour blood circulation. CRC Press, Boca Raton, FL p.129-35.

Minchinton A I, Durand R E, J C D (1990) Intermittent blood flow in the KHT sarcoma - flow cytometric studies using Hoechst 33342. Br J Cancer 62:195-200.

Mottram J C (1936) A factor of importance in the radio sensitivity of tumours. Br J Radiol 9:606-14.

Nishizawa K, Okunieff P, Elmaleh D, McKusick K A, Strauss H W, Suit H D (1991) Blood flow of human soft tissue sarcomas measured by thallium-201 scanning: prediction of tumor response to radiation. Int J Radiat Oncol Biol Phys 20:593-7.

Oberg P A (1990) Laser Doppler flowmetry. Crit Rev Biomed Eng 18:125-63.

Overgaard J (1995). Modification of hypoxia from Gottwals Schwarz to nicotinamide: Have we learned the lesson? In: Kogelnik HD (ed) Progress in radio-oncology V. Monduzzi Editore, Bologna p.469-75.

Overgaard J, Horsman M R (1996) Modification of hypoxia-induced radioresistance in tumours by the use of oxygen and sensitisers. Semin Radiat Oncol 6:10-21.

Overgaard J, Horsman M R (1997). Overcoming hypoxic cell radioresistance. In: Steel GG (ed) Basic Clinical Radiobiology. Arnold, p.141-51.

Overgaard J, Sand Hansen H, Overgaard M, Bastholt L, Berthelsen A, Specht L, Lindelov B, Jorgenson K (1998) A randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish Head and Neck Cancer Study (DAHANCA) Protocol 5-85. Radiother Oncol 46:135-46.

Pass H I (1993) Photodynamic therapy in oncology: Mechanisms and clinical use. J Natl Cancer Inst 85:443-56.

Pellat-Deceunynck C, Wietzerbin J, Drapier J-C (1994) Nicotinamide inhibits nitric oxide synthase mRNA induction in activated macrophages. Biochem 297:53-8.

Pigott K H, Hill S A, Chaplin D J, Saunders M I (1996) Microregional fluctuations in perfusion within human tumours detected using laser Doppler flowmetry. Radiother Oncol 40:45-50.

Reinhold H S, Blachiwiecz B, Blok A (1977) Oxygenation and reoxygenation in 'sandwich' tumours. Bibl Anat 15:270-2.

Robinson S P, Howe F A, Griffiths J R (1995) Non-invasive monitoring of carbogen-induced changes in tumour blood flow and oxygenation by functional magnetic resonance imaging. Int J Radiat Oncol Biol Phys 33:855-9.

Robinson S P, Howe F A, Rodrigues L M, Stubbs M, Griffiths J R (1998) Magnetic resonance imaging techniques for monitoring changes in tumour oxygenation and blood flow. Semin Radiat Oncol 8:197-207.

Rodrigues L M, Maxwell R J, McSheehy P M J, Pinkerton C R, Robinson S P, Stubbs M, Griffiths J R (1997) In vivo detection of ifosfamide by ^{31}P-MRS in rat tumours: increased uptake and cytotoxicity induced by carbogen breathing in GH3 prolactinomas. Br J Cancer 75:62-8.

Rojas A (1991) Radiosensitization with normobaric oxygen and carbogen. Radiother Oncol 20 Suppl 1:65-70.

Rojas A (1992) ARCON: accelerated radiotherapy with carbogen and nicotinamide. B J Radiol 24 (suppl):174-8.

Rojas A M, Johns H, Fiat P R (1993) Should carbogen and nicotinamide be given throughout the full course of fractionated radiotherapy regimens? Int J Radiat Oncol Biol Phys 27:1101-5.

Rose C M, Lustig R, Mcintosh N, Teicher B A (1986) A clinical trial of Fluosol-DA® 20% in advanced squamous cell carcinoma of the head and neck. Int J Radiat Oncol Biol Phys 12:1325-7.

Rowell N, Flower M, Cronin B, McCready V R (1993) Quantitative single-photon emission tomography for tumour blood flow measurement in bronchial carcinoma. Eur J Nucl Med 20:591-9.

Rubin P, Hanley J, Keys H M, Marcial V, Brady L (1979) Carbogen breathing during radiation therapy-the Radiation Therapy Oncology Group Study. Int J Radiat Oncol Biol Phys 5:1963-70.

Sampson L, Chaplin D J (1994) The influence of microenvironment on the cytotoxicity of TNF alpha *in vitro*. Int J Radiat Oncol Biol Phys 29:467-71.

Samulski T, Fessenden P, Valdagni R, Kapp D (1987) Correlations of thermal washout rate, steady state temperatures, and tissue type in deep seated recurrent or metastatic tumours. Int J Radiat Oncol Biol Phys 13:907-16.

Saunders M I, Hoskin P J, Pigott K, Powell M E B, Goodchild K, Dische S, Denekamp J, Stratford M, Dennis M F, Rojas A (1997) Accelerated radiotherapy, carbogen and nicotinamide (ARCON) in locally advanced head and neck cancer: a feasibility study. Radiother Oncol 45:159-66.

Schwarz G (1909) Ueber Desensibilisierung gegen Rontgen-und Radiumstrahlen. Munchener Medezinische Wochenschrift 24:1-2.

Siemann D W, Hill R P, Bush R S (1977) The importance of the pre-irradiation breathing times of oxygen and carbogen (5% CO2: 95% O2) on the in vivo radiation response of a murine sarcoma. Int J Radiat Oncol Biol Phys 2:903-11.

Siemann D W, Horsman M R, Chaplin D J (1994) The radiation response of KHT sarcomas following nicotinamide treatment and carbogen breathing. Radiother Oncol 31:117-22.

Song C W (1998). Modification of blood flow. In: Molls M and Vaupel P (eds) Blood perfusion and microenvironment of human tumours. Implications for clinical radiooncology. Springer, p.193-207.

Song C W, Makepeace C M, Griffin R J, Hasegawa T, Osborn J L, Choi I B, Nah B S (1994) Increase in tumor blood flow by pentoxifylline. Int J Radiat Oncol Biol Phys 29:433-7.

Stratford M, Dennis M (1992) High-performance liquid chromatographic determination of nicotinamide and its metabolites in human and murine plasma and urine. J Chromatogr 582:145-51.

Stratford M R, Rojas A, Hall D W, Dennis M F, Dische S, Joiner M C, Hodgkiss R J (1992) Pharmacokinetics of nicotinamide and its effect on blood pressure, pulse and body temperature in normal human volunteers. Radiother Oncol 25:37-42.

Suzuki M, Hori K, Abe I, Saito S, Sato H (1981) A new approach to cancer chemotherapy: selective enhancement of tumour blood flow with angiotensin II. J Natl Cancer Inst 67:663-9.

Teicher B A (1994) Hypoxia and drug resistance. Cancer met rev 13:139-68.

Thomlinson R H, Gray L H (1955) The histological structure of some human lung cancers and the possible implications for radiotherapy. Br J Cancer 9:539-49.

Tozer G M, Shaffi K M (1993) Modification of tumour blood flow using the hypertensive agent angiotensin II. Br J Cancer 67:981-8.

Tozer G M, Shaffi K M, Prise V E, Bell K M (1996) Spatial heterogeneity of tumour blood flow modification induced by angiotensin II: Relationship to receptor distribution. Int J Cancer 65:658-63.

Trotter M J, Chaplin D J, Durand R E, L O P (1989) The use of fluorescent probes to identify regions of transient perfusion in murine tumours. Int J Radiat Biol Phys 82:672-6.

Trotter M J, Chaplin D J, Olive P L (1991) The effect of angiotensin II on intermittent tumour blood flow and hypoxia in the murine SCVII carcinoma. Eur J Cancer 27:887-93.

van der Maazen R W, Thijssen H O, Kaanders J H, de Koster A, Keyser A, Prick M J, Grotenhuis J A, Wesseling P, van der Kogel A J (1995) Conventional radiotherapy combined with carbogen breathing and nicotinamide for malignant gliomas. Radiother Oncol 35:118-22.

Vaupel P, Kallinowski F, Okunieff P (1989) Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumours: A review. Cancer Res 49:6449-65.

Vaupel P, Menke H (1989) Effect of various calcium antagonists on blood flow and red blood cell flux in malignant tumours. Prog Appl Microcirc 14:88-103.

Vaupel P, Schlenger K, Knoop C, Hoeckel M (1991) Oxygenation of human tumours: evaluation of tissue oxygen distribution in breast cancers by computerised O_2 tension measurements. Cancer Res 51.

Wahlberg E, Olofsson P, Swedenborg J, Fagrell B (1991) Effects of local hyperemia and edema on the biological zero in laser Doppler fluxmetry (LD). Int J Microcirc Clin Exp 11:157-65.

Warburg O (1930). The metabolism of tumours. Constable, London.

Wilson C B H, Lammertsma A A, McKenzie C G, Sikora K, Jones T (1992) Measurements of blood flow and exchanging water space in breast tumours using positron emission tomography: A rapid and noninvasive dynamic method. Cancer Res 52:1592-7.

Wood P, Hirst D (1989) Modification of tumour response to calcium antagonists in the SCCVII/st tumour implanted at two different sites. Int J Radiat Biol 56:355-67.

Wood P J, Hirst D G (1989) Cinnarizine and flunarizine as radiation sensitizers in two murine tumours. Br J Cancer 58:742-5.

Yoshimoto S, Ishizaki Y, Sasaki T, Murota S (1991) Effect of carbon dioxide and oxygen on endothelin production by cultured porcine cerebral endothelial cells. Stroke 22:378-83.

Zackheim H S, Vasily D B, Westphal M L, Hastings C W (1981) Reactions to niacinamide. J Am Acad Dermatol 4:736-7.

Zackrisson B, Franzen L, Henriksson R, Littbrand B, Stratford M, Dennis M, Rojas A M, Denekamp J (1994) Acute effects of accelerated radiotherapy in combination with carbogen breathing and nicotinamide (ARCON). Acta Oncol 33:377-81.

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Microregional blood flow in murine and human tumours assessed using laser Doppler microprobes

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Summary A multichannel laser Doppler system has been used to measure microregional fluctuations in perfusion in the HT29 human tumour xenograft and in patients with advanced malignant disease. A comparison is made with previously obtained data for the SaF, a transplantable murine tumour. The 300 μm diameter probes recorded fluctuations in erythrocyte flux in tumour microregions with an estimated volume of 10^{-2} mm^3. Of the 66 human tumour microregions sampled, 26% showed a change in erythrocyte flux by a factor of 2 or more over the 60 min measurement period, compared with 37% of HT29 and 48% of SaF microregions. In each of the studies more than 50% of changes were completed within 20 min, although slower changes were more common in the human tumours than in the experimental systems. Within the 1 h monitoring period at least 30% of the changes were reversed (human tumours 30%, HT29 45%, SaF 31%). These findings demonstrate that microregional changes in erythrocyte flux, consistent with transient, perfusion-driven changes in oxygenation, are a feature of human malignancies as well as experimental transplanted tumours.

Keywords: hypoxia; erythrocyte flux; SaF murine tumour; HT29 tumour

The presence of hypoxic cells within tumours is considered to be an important factor in determining treatment outcome following radiotherapy (Dische, 1985, Overgaard, 1992, Höckel *et al.*, 1993). Since Thomlinson and Gray (1955) first proposed the existence of hypoxic cells beyond the diffusion distance of oxygen, efforts have continued to devise strategies to increase tumour oxygenation effectively. Hyperbaric or normobaric oxygen or carbogen breathing and the use of oxygen-mimetic radiosensitisers, while producing some successes, have proved generally disappointing in the clinic (Henk, 1981; Rubin *et al.*, 1979; Dische, 1985; Overgaard, 1992). Part of this failure may be because not all hypoxic cells arise as a result of diffusion limitations. More recently, attention has focused on overcoming both acute and chronic hypoxia. To this aim, based on the success of studies using murine tumour models (Kjellen *et al.*, 1991), clinical studies have been initiated to evaluate the combination of nicotinamide and carbogen breathing in accelerated radiotherapy regimes (ARCON) (Zackrisson *et al.*, 1994). While the use of carbogen is designed to overcome chronic, diffusion-limited hypoxia, the vitamin B derivative, nicotinamide, is believed to overcome acute hypoxia resulting from transient fluctuations in tumour blood flow (Horsman *et al.*, 1988; Chaplin *et al.*, 1990). Acute or perfusion-limited hypoxia was postulated to result from blood flow irregularities at the microvascular level by Brown (1979). More direct evidence was provided by observations of temporary non-perfusion of vessels in tumours grown as 'sandwich' preparations, between transparent plates (Reinhold *et al.*, 1977). Further studies using fluorescent perfusion markers combined with flow cytometric and histological techniques have confirmed that intermittent perfusion is a common feature of transplantable murine tumours and some human tumour xenografts (Chaplin *et al.*, 1987; Trotter *et al.*, 1989; Chaplin and Trotter, 1991). The techniques employed in experimental tumour systems are not applicable to the clinic however and to date, no evidence exists to indicate that transient perfusion and hence acute hypoxia, occurs in human tumours. Such information is clearly essential for the design of effective treatment strategies. The recent development of a multichannel laser Doppler system has made possible the real time monitoring of erythrocyte flux in tumour microregions, since each 300 μm probe has an estimated sampling volume of approximately 10^{-2} mm^3. We have recently reported the successful use of this system in measuring apparently spontaneous fluctuations in perfusion in transplantable murine tumours (Chaplin and Hill, 1995). We have also reported that nicotinamide can reduce the number of flow reductions measured in such a system (Hill and Chaplin, 1995). An initial study has already established the feasibility of using laser Doppler microprobes to detect changes in microregional erythrocyte flux in human tumours (Pigott *et al.*, 1996). The purpose of the current study was to use laser Doppler flowmetry to investigate the incidence of perfusion fluctuations in the xenografted human colonic carcinoma HT29 as well as a range of human malignancies, for comparison with the murine tumour studies.

Materials and methods

Experimental tumour models

Two experimental tumour systems were used in this study: the undifferentiated murine sarcoma SaF and the human colonic adenocarcinoma HT29. Subcutaneous tumours were produced by injecting 0.05 ml of a crude SaF cell suspension or 5×10^5 tissue culture maintained HT29 cells dorsally into 12-16 week old CBA/Gy f TO (SaF) or SCID (HT29) mice. Animals were selected when their tumours reached 5-6.5 mm geometric mean diameter (150-300 mg), and were restrained, unanaesthetised, in Perspex jigs for the duration of the measurements, at the end of which a lethal dose of sodium pentobarbitone was injected via a previously inserted tail vein catheter.

Patients

Following written informed consent, 13 superficial tumours were entered into the study. The lesions, chosen for their accessibility, comprised primary, recurrent and metastatic deposits in different sites and of varying histological type. Patients lay comfortably on a couch and were requested to remain as still as possible during recording.

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Laser Doppler flowmetry

Erythrocyte flux was monitored using the Oxford array multiple channel laser Doppler system (Oxford Optronix, Oxford, UK). Up to six custom-designed microprobes (300 μm diameter), each monitoring a nominal sampling volume of 10^{-2} mm^3, were inserted into each tumour, allowing simultaneous measurements of perfusion in several discrete locations. Once stable readings were obtained from each probe, erythrocyte flux was monitored for 60 min.

Data analysis

Each of the Doppler channels recorded 20 readings per second. From these values a single average was calculated for each 2 min interval, for each channel. In the animal studies, a final 2 min average was calculated for the readings recorded after the lethal injection. This value was then subtracted from all the preceding calculated averages: it is most likely due to the Brownian motion of free red blood cells in front of the probe. In order to allow for this 'background' component of the signal in the clinical studies, data from several murine experiments were examined. When the final, post mortem, average was expressed as a percentage of the last 2 min average calculated while the animal was alive, a mean value of 30% was calculated (Chaplin and Hill, 1995; Pigott *et al*., 1996). Thus, for the clinical studies, 30% of the last average calculated for each channel was subtracted from the data. The final plots of erythrocyte flux against time were compared with the original recorded data in order to eliminate any changes associated either with animal/patient movement or probe movement (detected as an abrupt change in the backscatter signal).

Results

For comparison with the 36 SaF tumours previously studied (Hill and Chaplin, 1995), blood flow measurements were performed on 18 individual HT29 tumours and 13 human tumours. The latter included both primary and recurrent breast carcinomas as well as metastases to regional lymph nodes and skin from a variety of tumours of different histologies. Patients ranged in age from 47 to 80 years old and their tumours ranged in size from approximately 3×3 cm (see Figure 1) to 10×10 cm. Figure 1 shows five needle probes inserted in a metastatic colon adenocarcinoma skin nodule via 20 gauge cannulas. Depending on the size and morphology of the tumour, the probes were inserted such that their tips might be separated by anything from a few millimetres to 1 or 2 cm.

Four individual traces from different probes inserted into a primary breast carcinoma are shown in Figure 2 and four traces from a recurrent non-Hodgkin's lymphoma neck node are shown in Figure 3. It can be seen that within the same tumour, fluctuations in erythrocyte flux occurred independently in two of the microregions sampled. Individual traces showed great variation, some showed no change over the whole 60 min measurement period, while others showed increases or decreases in erythrocyte flux. Some traces showed more than one change. The apparent rapid decrease in perfusion indicated in one of the traces shown in Figure 2

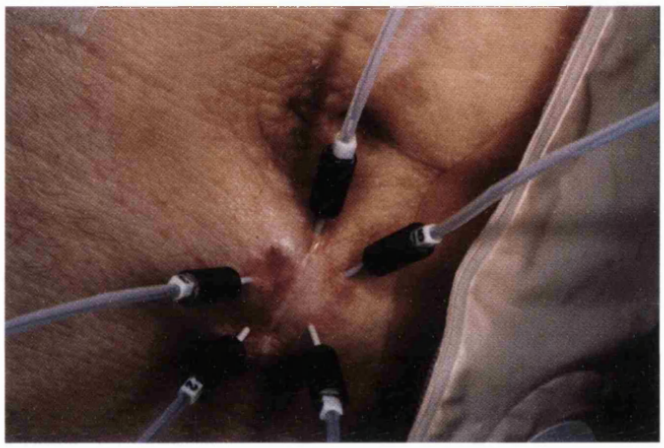

Figure 1 Five needles probes positioned within a colon carcinoma metastatic skin nodule. The 20 gauge cannulas through which the probes were introduced are also visible.

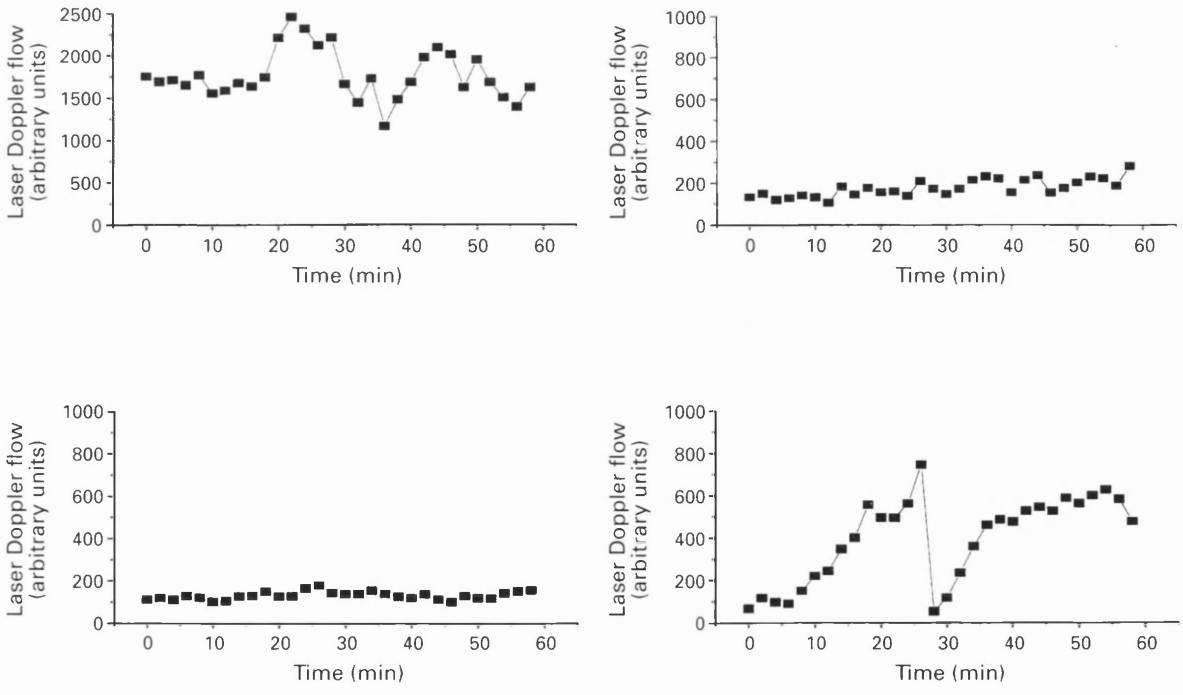

Figure 2 An example of four recorded traces from separate probes positioned in different microregions of a primary breast carcinoma. Each point represents the average of 2400 readings taken over a 2 min sampling period.

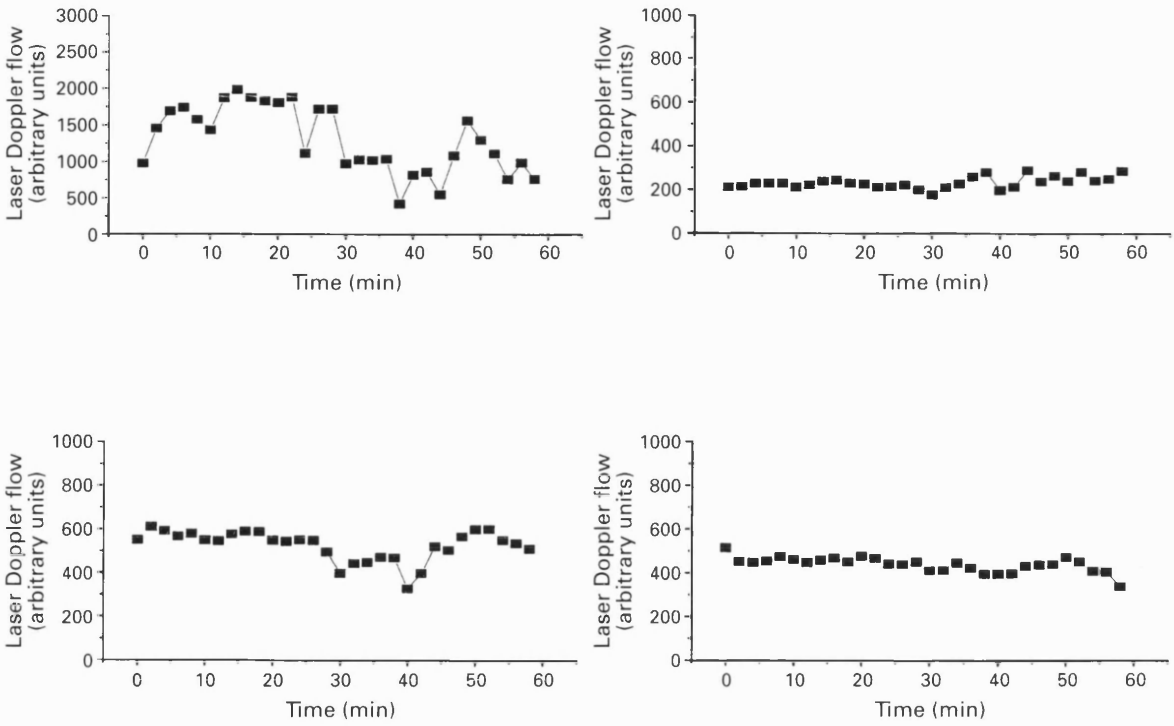

Figure 3 An example of four individual traces from a non-Hodgkin's lymphoma neck node. Each point represents the average of 2400 readings taken over a 2 min sampling period.

Table I Changes in microregional erythrocyte flux during 60 min observation periods

	Murine SaF			*Xenograft HT29*			*Human tumours* in situ *Various histologies*		
Factor of change	*Percentage of traces showing change (n-116)*	*Direction of change*		*Percentage of traces showing change (n=63)*	*Direction of change*		*Percentage of traces showing change (n=66)*	*Direction of change*	
⩾1.5	–			–			58	38↑	22↓
⩾2	48	39↑	49↓	37	20↑	22↓	26	15↑	9↓
⩾5	16	10↑	14↓	10	4↑	6↓	3	3↑	0↓

was accompanied by an abrupt change in the recorded backscatter signal, indicating a change in the position of the probe and was therefore excluded from the analysis.

Data from each probe were analysed separately and a summary of the changes in erythrocyte flux measured in all of the individual traces obtained is shown in Table I for both the HT29 and the human tumours, together with previously published data for the murine SaF (Hill and Chaplin, 1995). In the human tumours, 26%, of the sampled microregions showed a change in erythrocyte flux by a factor of 2 or more over the 1 h measurement period, compared with 37% of HT29 microregions and 48% of SaF microregions. The incidence of changes by at least a factor of 5 is also indicated, as is a smaller change, by a factor of 1.5 or more for the human tumours. Approximately equal numbers of increases and decreases in flow were measured in the SaF and the HT29 compared with the human tumour measurements which indicated a greater number of flow increases. We believe this may reflect haemorrhage in front of the probe, since one patient with a very low platelet count showed increases in erythrocyte flux in five of his six tumour microregions monitored.

We are now able to monitor erythrocyte flux in skin, simultaneously with the tumour measurements. Limited data are available at this point, since the skin probes have so far been used for only three patients. However, only one of the nine evaluable traces shows a change in erythrocyte flux by a factor of 1.5.

Table II Kinetics of change in microregional erythrocyte flux

Time from max to min or min to max	*Murine SaF*	*Xenograft HT29*	*Human tumours*
Rate of change (%)			
Within 10 min	49	42	25
Within 20 min	75	69	56
Changes reversed within observation period (%)	31	45	30

As well as the incidence of perfusion fluctuations, the laser Doppler system provides kinetic information on the speed and duration of the changes measured. These data are summarised in Table II. Although some traces show a very slow rate of change, all three studies indicate that more than 50% of changes occur over a period of 20 min or less. The human tumours show a greater number of slow changes than the HT29 or the SaF. In each study, a significant proportion of the changes was reversed within the observation period.

Discussion

With the advent of the Eppendorf polarographic electrode system direct measurements of tumour tissue oxygenation are now possible in patients, and the existence of regions of low oxygen partial pressure has been demonstrated in a number

of studies (Kallinowski *et al.*, 1990; Vaupel *et al.*, 1991; Höckel *et al.*, 1993). However the global picture of pO_2 distribution throughout a tumour will not reflect changes in oxygen tension which may occur transiently in different regions of the tumour due to periods of impaired perfusion or vessel closure. At present no system is available to measure temporal changes in oxygenation directly at single or multiple fixed points within a tissue. However, since it has been demonstrated that clonogenically hypoxic cells can result from dynamic perfusion changes (Chaplin *et al.*, 1987) studies of acute hypoxia and its elimination have focused on the measurement of microregional fluctuations in tumour perfusion. Studies involving many rodent tumour models have indicated the widespread incidence of temporary flow reductions and the development of strategies designed to eliminate them remains a focus of experimental research. It is therefore important that the presence or absence of acute hypoxia, or perfusion changes leading to it, is established in human tumours.

This study demonstrates that microregional fluctuations in erythrocyte flux can be detected in human tumours using laser Doppler flowmetry. The temporal changes in flow measured in the HT29, a human tumour growing as a xenograft, and in primary and metastatic tumours in patients are similar to those reported previously for two transplantable murine tumours (Chaplin and Hill, 1995). Over the 1 h monitoring period, 37% of HT29 microregions, and 26% of the human tumour microregions sampled showed a change in erythrocyte flux by a factor of 2 or more. This compares with 48% in the SaF. The figure for human tumours could be an exaggeration, if some of the flow increases actually reflect haemorrhage in front of the probe. If the true number of increases was equal to the number of decreases measured, the percentage of traces showing a 2-fold change would still be approximately 20% however. A factor of 2 reduction in perfusion could correspond at one extreme to the complete closure of 50% of the vessels contained in the sampling volume or at the other to a 50% reduction in flow in all of the vessels in the region. Likewise, a factor of 1.5 decrease would result if flow ceased in 30% of the sampled vessels. In each case, an increased level of hypoxia would result, either by the creation of new foci of hypoxic cells or by decreasing the supply of oxygen and thereby causing an expansion of the rim of hypoxic cells. In the human tumours, as in the experimental models, a high proportion of the changes measured occurred within 20 min and in at least 30% of cases the change was reversed within the 60 min of recording.

In summary, the current study establishes that temporal changes in erythrocyte flux consistent with the occurrence of perfusion-limited hypoxia are a common feature of both human and murine tumours. This finding has important implications for tumour biology and therapy.

References

BROWN JM. (1979). Evidence for acutely hypoxic cells in mouse tumours and a possible mechanism for reoxygenation. *Br. J. Radiol.*, **52**, 650–656.

CHAPLIN DJ AND HILL SA. (1995). Temporal heterogeneity in microregional erythrocyte flux in experimental solid tumours. *Br. J. Cancer*, **71**, 1210–1213.

CHAPLIN DJ AND TROTTER MJ. (1990). Chemical modifiers of tumour blood flow. In *Tumour Blood Supply and Metabolic Microenvironment*, Vaupel P and Jain R. (eds), pp. 65–85. Gustav Fischer: Stuttgart.

CHAPLIN DJ, OLIVE PL AND DURAND RE. (1987). Intermittent blood flow in a murine tumour: radiobiological effects. *Cancer Res.*, **47**, 597–601.

CHAPLIN DJ, HORSMAN MR AND TROTTER MJ. (1990). Effect of nicotinamide on the microregional heterogeneity of oxygen delivery within a murine tumour. *J. Natl Cancer Inst.*, **82**, 672–676.

DISCHE S. (1985). Chemical sensitizers for hypoxic cells: a decade of experience in clinical radiotherapy. *Radiother. Oncol.*, **3**, 95–115.

HENK JM. (1981). Does hyperbaric oxygen have a future in radiation therapy? *Int. J. Radiat. Oncol. Biol. Phys.*, **7**, 1125–1128.

HILL SA AND CHAPLIN DJ. (1995). The effect of nicotinamide on microregional blood flow within tumours assessed using laser Doppler probes. *Acta Oncol.*, **34**, 401–404.

HÖCKEL H, KNOOP C, SCHLENGER K, VORNDRAN B, BAUMANN E, MITZE M, KNAPSTEIN PG AND VAUPEL P. (1993). Intertumoral pO_2 predicts survival in advanced cancer of the uterine cervix. *Radiother. Oncol.*, **26**, 45–50.

HORSMAN MR, BROWN JM, HIRST VK, LEMMON MJ, WOOD PJ, DUNPHY EP AND OVERGAARD J. (1988). Mechanism of action of the selective tumour radiosensitizer nicotinamide. *Int. J. Radiat. Oncol. Biol. Phys.*, **15**, 685–690.

KALLINOWSKI F, ZANDER R, HÖCKEL H AND VAUPEL P. (1990). Tumour tissue oxygenation as evaluated by computerised pO_2 histography. *Int. J. Radiat. Biol. Oncol. Phys.*, **19**, 953–961.

KJELLEN E, JOINER MC, COLLIER JM, JOHNS H AND ROJAS A. (1991). A therapeutic benefit from combining normobaric carbogen or oxygen with nicotinamide in fractionated X-ray treatments. *Radiother. Oncol.*, **22**, 81–91.

OVERGAARD J. (1992). Importance of tissue hypoxia in radiotherapy. A meta-analysis of controlled clinical trials. *Radiother. Oncol.*, (suppl.), **24**, 564.

PIGGOTT KH, HILL SA, CHAPLIN DJ AND SAUNDERS MI. (1996). Microregional fluctuations in perfusion within human tumours detected using laser Doppler flowmetry. *Radiother. Oncol.* (in press).

REINHOLD HS, BLACHIWIECZ B AND BLOK A.(1977). Oxygenation and reoxygenation in 'sandwich' tumours. *Bibl. Anat.*, **15**, 270–272.

RUBIN P, HANLEY J AND KEYS HM. (1979). Carbogen breathing during radiation therapy. *Int. J. Radiat. Oncol. Biol. Phys.*, **5**, 1963–1970.

THOMLINSON RH AND GRAY LH. (1955). The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br. J. Cancer*, **9**, 539–549.

TROTTER MJ, CHAPLIN DJ, DURAND RE AND OLIVE PL. (1989). The use of fluorescent probes to identify regions of transient perfusion in murine tumours. *Int. J. Radiat. Oncol. Biol. Phys.*, **16**, 931–935.

VAUPEL P, SCHLENGER K, KNOOP C AND HÖCKEL H. (1991). Oxygenation of human tumours: evaluation of tissue oxygen distribution in breast cancers by computerised O_2 tension measurements. *Cancer Res.*, **51**, 3316–3322.

ZACKRISSON B, FRANZEN L, HENRIKSSON R, LITTBRAND B, STRATFORD M, DENNIS M, ROJAS A AND DENEKAMP J. (1994). Acute effects of accelerated radiotherapy in combination with carbogen breathing and nicotinamide (ARCON). *Acta Oncol.*, **33**, 377–381.

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Effect of carbogen breathing on tumour microregional blood flow in humans

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Abstract

Background and purpose: Carbogen is currently being re-evaluated as a radiosensitiser. It acts primarily by increasing tissue pO_2, although there is evidence to suggest that enhanced tumour blood flow may also be a component of its action.

Materials and methods: Ten tumours in eight patients with advanced malignant disease were studied. Up to six microprobes, each with an estimated sampling volume of 10^{-2} mm^3, were inserted into the tumours. Ten min of baseline readings were taken prior to a 10 min carbogen (95% O_2/5% CO_2) breathing period, measurements were continued for a further 10 min.

Results: The results show that in 34 microregions analysed no overall change in tumour perfusion was seen with carbogen breathing. Individual tumour analysis demonstrated variation in response between patients to carbogen – after 6 min of carbogen four tumours showed an increase in blood flow by more than 10% of the pre-breathing value, two a decrease and four no change. The magnitude of change was small, with only two tumours fluctuating by more than 25%.

Conclusions: These findings confirm the presence of transient fluctuations in microregional blood flow in human tumours but suggest that the radiosensitising action of carbogen lies primarily in its effect on increasing the oxygen capacity of blood. This supports the addition of agents such as nicotinamide with carbogen in order to overcome both diffusion and perfusion limited hypoxia.

Keywords: Carbogen; Microregional tumour perfusion; Laser Doppler flowmetry (LDF)

1. Introduction

The presence of hypoxic cells within tumours is regarded as an important biological factor in the failure of radical radiotherapy to secure local control. The use of hyperbaric oxygen, normobaric oxygen, carbogen, and hypoxic cell radiosensitisers have met with limited success in individual trials, although an overview suggests a small benefit for this approach [15]. This may in part be because these manoeuvres were based on a diffusion model of hypoxia as proposed by Thomlinson and Gray [24].

It is now well established that this is not the only form of hypoxia that exists in tumours, with convincing evidence that perfusion driven changes in tumour oxygenation, as a result of fluctuations in microregional blood flow, are a major contribution to radiobiological hypoxia in experimental tumour systems [4,11,25]. Recent studies from our own Institution have indicated that such perfusion changes are a frequent occurrence in human tumours [16]. The radiosensitising effect of carbogen (95% O_2/5% CO_2) can vary in different animal tumour models with some tumours showing enhancement ratios of up to 1.6 [19]. Results from randomised controlled clinical trials, however, have been disappointing [17,20]. The failure of these early studies to show a benefit may at least in part be explained by the use of pre-breathing times of up to 90 min. Animal studies have shown that the pre-irradiation breathing time can be a critical factor in determining the degree of tumour sensitisation by carbogen [3,21]. Direct measurements of tumour oxygenation using polargraphic electrodes have confirmed the existence of time-dependent changes in pO_2 during carbogen breathing. Falk et al. demonstrated that carbogen induces only a transitory rise in pO_2 in human tumours reaching a maximum within 8–12 min and falling after 18 min [9]. As a result of this and the animal data, carbogen is currently being re-evaluated in clinical studies.

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Carbogen sensitisation is thought to act primarily by increasing the amount of dissolved O_2 in the blood, with the CO_2 component causing tachycardia, respiratory stimulation, vasodilatation and a shift of the oxygen dissociation curve to the right thereby favouring unloading of O_2 in the tissues. Recent animal work, however, using ^{86}Rb in RIF-1 tumours has suggested that carbogen-induced increases in tumour blood flow may be a more important component of enhanced tumour oxygenation than was previously thought [12]. If this is the case for human tumours, then carbogen breathing may be a useful adjunct to not only radiotherapy but also drug therapies, by improving the tumour delivery of systemically administered agents. However, investigation of flow changes at the microregional level are needed to determine if carbogen has a role in modulating not only diffusion limited hypoxia but also perfusion-driven changes in oxygenation.

The aim of this study was to evaluate the influence of carbogen breathing on microvascular perfusion in human tumours using a newly developed multi-channel laser Doppler system. These probes have been used to demonstrate microregional fluctuations in perfusion over time in both human and murine tumours [2,11,16].

2. Materials and methods

2.1. Patients

Eight patients were studied, six male, two female, median age 63 years (range 46 to 86), all with histologically proven advanced malignancy and all undergoing treatment at Mount Vemon Centre for Cancer Treatment. Tumour characteristics are shown in Table 1. Two patients (number 6 and 7) with multiple sites of disease were studied on two separate occasions. Because of the length of the laser Doppler probes (25 mm) only superficial lesions could be studied. Written informed consent was obtained from all patients prior to measurements being taken, and approval for the study was given by Local Ethics Committee.

2.2. Laser Doppler flowmetry

Relative changes in microvascular perfusion were measured using the Oxford Array multi-channel Laser Doppler system (Oxford Optronix, Oxford UK). The system incorporates up to 12 cylindrical probes each measuring 25 mm in length and 300 μm in diameter with an estimated sampling volume of 10^{-2} mm^3. A laser diode of wavelength 780 nm is coupled to the probes which contain optical fibres (130 μm in diameter) delivering and collecting light to the tissue. Movement of red blood cells causes interaction with the photons of light which are then Doppler shifted, this change in frequency is a measure of blood velocity. The number of Doppler shifts is proportional to erythrocyte concentration (or blood volume). The product of blood velocity and volume is directly proportional to red blood cell (RBC) flux (or blood flow) and is generated as a signal [14].

An additional signal, the backscatter, is also recorded which is proportional to the total amount of light detected by the probe. This is used to monitor movement of a probe which will result in a change in the probe/tissue interface and an abrupt alteration in the backscatter signal. Apparent changes in red blood flux accompanied by a precipitous change in the backscatter reading were excluded from the analysis.

2.3. Experimental set-up

Patients lay on a bed and up to six microprobes were inserted into the tumour. The probes are blunt ended and to facilitate entry into the lesion a 20-gauge cannula was inserted into the skin overlying the tumour. The probe was then advanced down the plastic sleeve, and lodged within the tumour (Fig. 1). Subcutaneous lignocaine (1%) was used to anaesthetise the skin prior to insertion of the cannula. Since measurements were taken some distance below the skin it was felt lignocaine would be unlikely to affect the readings. After a period of equilibration baseline measurements were taken for 10 min. In all patients the probes were well tolerated for the observation period.

Table 1
Tumour characteristics and relative blood flow changes seen 6 min after commencing carbogen breathing

Patient no.	Tumour details	Histology	Relative blood flow 6 min into carbogen (± S.E.M.)
1*	Abdominal skin deposit 4 cm	Adenocarcinoma pancreas	1.13 (±0.05)
2	Breast 10 cm	Adenocarcinoma	1.01 (±0.11)
3	Neck node 3.5 cm	High grade non-Hodgkin lymphoma	0.85 (±0.07)
4**	Neck node 3 cm	High grade non-Hodgkin lymphoma	2.24 (±0.13)
5	Neck node 6 cm	Squamous cell cancer supraglottis	1.07 (±0.19)
6	Skin deposit abdomen 3 cm	Adenocarcinoma colon	1.14 (±0.18)
	Skin deposit abdomen 3.5 cm		1.06 (±0.05)
7	Groin node (R) 4 cm	High grade non-Hodgkin lymphoma	0.81 (±0.06)
	Groin node (L) 3 cm		0.96 (±0.06)
8	Axillary lymph node 3 cm	Melanoma	1.25 (±0.28)

Analysis of 10 min carbogen breathing period only because: *only 4 min post-carbogen observation period; **all probes dislodged 3 min into post-carbogen observation period.

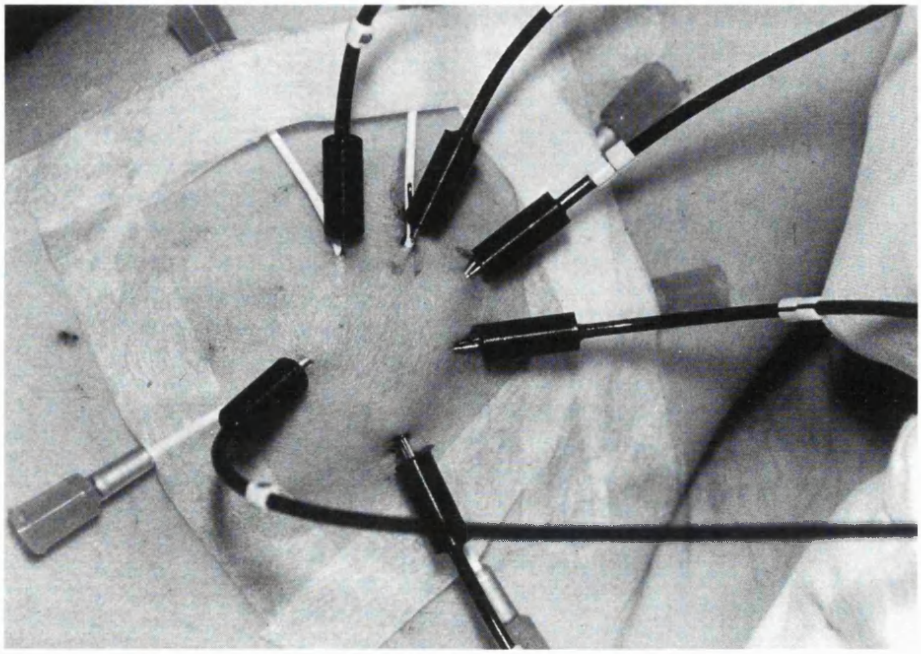

Fig. 1. Photograph showing six laser Doppler probes in situ within a skin nodule.

2.4. Carbogen breathing

Following acquisition of baseline readings, a 10-min period of carbogen breathing was commenced using a close-fitting face mask (Intersurgical, UK.) covering both the nose and mouth. The carbogen (95% oxygen and 5% carbon dioxide) was delivered at a flow rate of 15 l min^{-1} through a closed system using a 1-way valve and a 3-l breathing bag.

Measurements were continued for a further 10 min after the cessation of carbogen breathing with the patient breathing room air.

2.5. Data analysis

Individual probes generate 20 readings per second. From this, an average flow reading is calculated for each 2-min interval in all channels and plotted against time.

A proportion of the signal is due to a biological zero [1,6] which is partly due to Brownian motion of the erythrocytes in front of the probe. Animal data gives values for this background 'noise' of 30% and this correction factor was applied to all of the readings [2,11,16]. The final plots of red blood cell flux together with the original recorded data were examined and any traces showing evidence of patient movement or probe movement were excluded from analysis.

Flow during carbogen breathing was related to a baseline value which was a single mean calculated from the measurements recorded over the 10-min period prior to breathing carbogen, and was designated as the flow at time zero. Blood flow averages at 6, 10, 14 and 20 min after starting carbogen breathing were compared with the time zero value using a paired two-tailed t-test where $P<0.05$ was considered to be significant.

3. Results

Blood flow measurements were analysed in a total of 50 leads in ten tumours on eight patients. Patient number 6 with multiple skin metastasis and patient number 7 with bilateral inguinal lymphadenopathy were assessed on two separate occasions.

Carbogen breathing was tolerated without difficulty in all patients and thus the 10-min carbogen breathing period could be analysed in all 50 traces (seven were excluded due to movement artefact). However, two patients were not evaluable for the entire 10-min post-carbogen observation period; patient number 1 had only a 4-min post-carbogen observation period, and patient number 4 dislodged all of the probes on movement 3 min after cessation of carbogen. This allowed analysis of the whole carbogen and post-carbogen breathing time in 39 traces from eight tumours (five traces were excluded due to movement).

Fig. 2 shows complete data from these eight tumours (34 traces). The values are expressed as a mean±standard error of the mean. The data indicates that breathing carbogen does not influence RBC flux. As the patients switch to air breathing there is a marginal increase in flux which then falls. Statistical analysis of relative blood flow at 6, 10 and 14 min did not show a significant variation from the pre-carbogen value ($P=0.94$, $P=0.90$, $P=0.33$, respectively). The relative decrease in erythrocyte flux after cessation of carbogen breathing, however, did give a

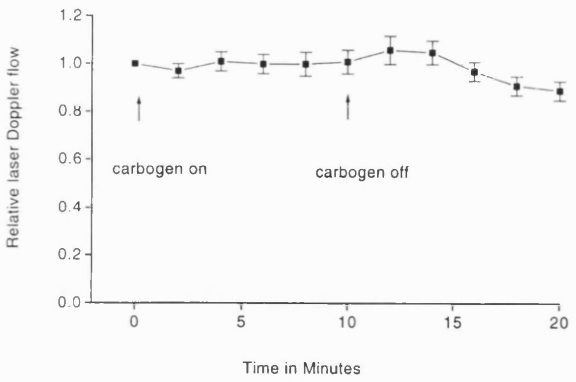

Fig. 2. Relative change in mean blood flow (±S.E.M.) from eight tumours (34 leads) during and after 10 min of carbogen breathing.

statistically significant value at 20 min ($P=0.005$) from time zero.

Data recorded from each of the ten tumours was analysed separately. The aim was first to acquire an overall view of the carbogen effect on blood flow in a particular tumour and then, to study variation of flux in individual rnicroregions. Fig. 3, which shows complete data from four patients, demonstrates the variation in response between patients to breathing carbogen.

Table 1 shows the relative blood flow changes of the ten tumours at 6 min. This time was selected since, as an average of the readings between 4 and 6 min after the start of carbogen, it best reflects the use of carbogen in clinical practice [8] and would indicate the effect of carbogen on blood flow at the start of radiation. Four out of ten tumours showed an increase in flow after 6 min of carbogen breathing greater than 10% of the pre-breathing value, whilst two tumours showed a decrease and in two the flow was unchanged.

Variation of erythrocyte flux and response to carbogen in different microregions of a single tumour are illustrated in Fig. 4, where the traces of four probes from patient 5 are shown (one trace was excluded due to excessive probe movement). There is marked heterogeneity between the four microregions not only in terms of magnitude but also the direction of change in blood flow. A summary of the effect of carbogen on RBC flux in all of the individual traces is shown in Table 2. At 6 min, 60% of traces demonstrated a change in blood flow (37% increased, 23% decreased) and at 10 min 68% had altered (42% increased, 26% decreased). The magnitude of change was modest, however, with only 33% at 6 min and 42% at 10 min varying by 25% or more.

4. Discussion

Tumour oxygenation depends upon blood flow and oxygen diffusion. The addition of 5% CO_2 to 95% oxygen aimed to overcome the vasoconstrictive effect of oxygen. There is evidence from normal tissue studies that carbon dioxide acts on blood vessels causing enhanced blood flow by vasodilatation, capillary recruitment and increased blood flow velocity [5]. Improved perfusion has been reported in murine tumours during carbogen breathing,

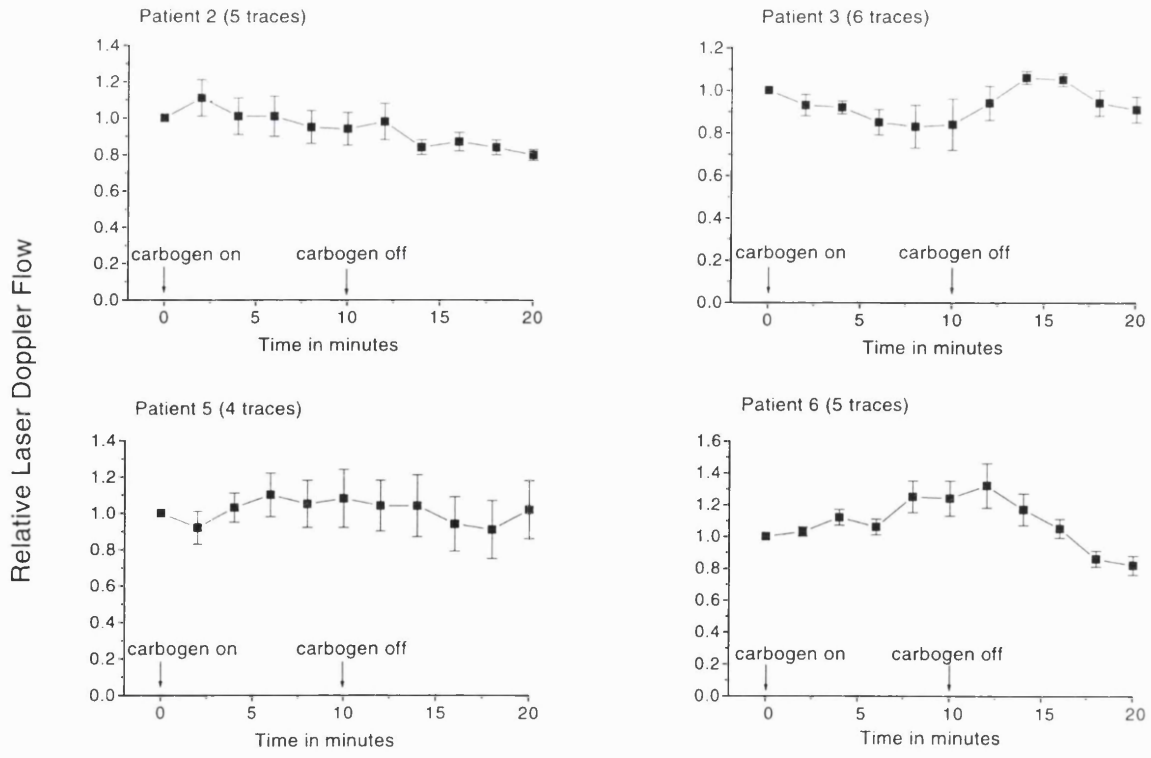

Fig. 3. Relative change in mean blood flow (±S.E.M.) during and after 10 min of carbogen breathing in four different tumors.

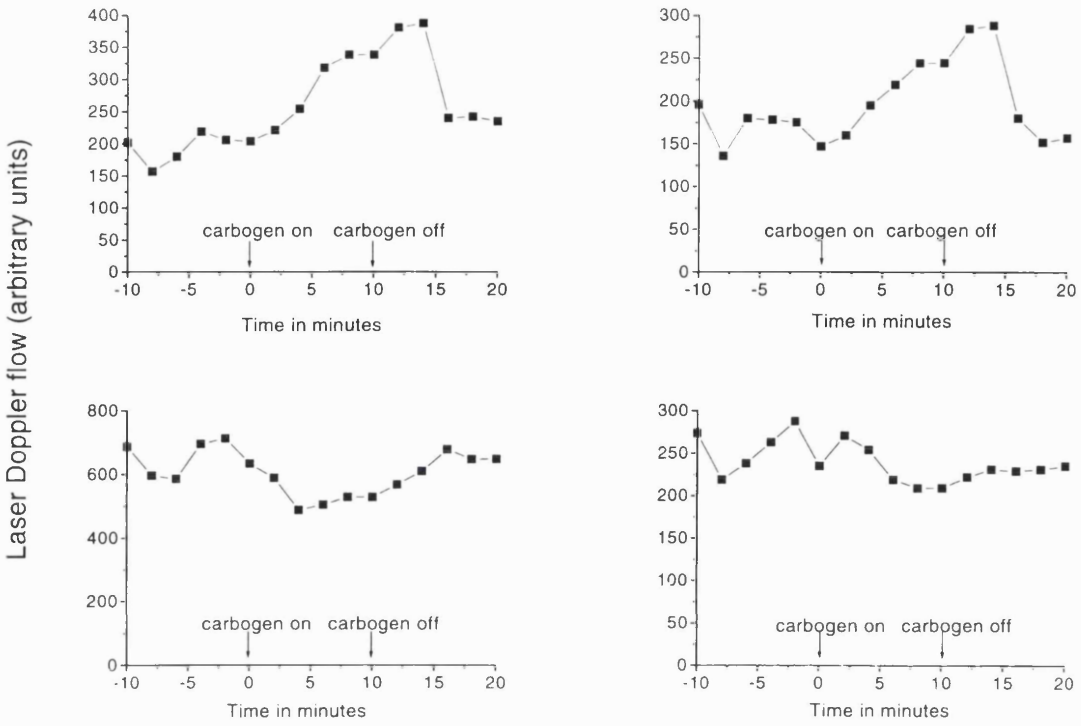

Fig. 4. Individual traces showing variation in blood flow in four separate microregions in patient 6.

[12,13] although this has not been substantiated by other groups [7,10]. In addition, using functional MRI to assess the effect of carbogen in both experimental rat tumours and human tumours changes in signal have been reported [18,23]. These MR signal changes, are believed to reflect, however, a complex measure of both blood flow and oxygenation.

This study has demonstrated that laser Doppler microprobes are well tolerated by patients and that small changes in erythrocyte flux can be detected. By taking an average of the readings from every microregion sampled an overall picture of tumour blood flow can be obtained. It has been shown that if tumour blood flow readings from all areas sampled are analysed, whilst individual fluctuation occurs, overall perfusion of human tumours remains essentially unchanged during carbogen breathing.

This data confirms previous animal and human studies [3,22] showing that the response of individual tumours to carbogen can vary and even where increases in flow are seen during carbogen breathing these are relatively small, with only two tumours in this series showing fluctuation of more than 25%. Overall change in blood flow in a particular tumour was obtained by averaging readings from up to 6 probes inserted into that tumour. Each probe has a sampling volume of 10^{-2} mm^3 containing only a few capillaries, thus information is only available about blood flow changes in selected microregions of the tumour. However, since fluctuations in flow in discrete microregions have been implicated in radiation resistance, through the existence of perfusion limited hypoxia [4], investigation of flow changes on this level are potentially important. If we assume that on average eight capillaries are sampled in each microregion, then an alteration in flux of 25% could reflect an equal flow change in each vessel or perhaps two vessels opening or closing.

At 6 min into carbogen breathing one-third of all microregions show a variation in flow of at least 25% with an approximately equal number of increases and decreases

Table 2
Changes in microregional flux at 6 and 10 min after the commencement of carbogen breathing

Factor of change	No. of traces showing change (%) at 6 min		No. of traces showing change (%) at 10 min	
	↑	↓	↑	↓
≥1.1	16 (37%)	10 (23%)	18 (42%)	11 (26%)
≥1.25	8 (19%)	6 (14%)	11 (26%)	7 (16%)
≥1.5	5 (12%)	1 (2%)	6 (14%)	2 (5%)
≥2	3 (7%)	0	2 (5%)	0
Total number of traces showing change ≥1.1	26 (60%)		29 (68%)	

and in the small percentage of regions which display a variation of greater than 50%, increases outnumber decreases by a factor of 5 to 1. This may indicate that carbogen has, at least in some microregions, a limited role in modulating perfusion limited hypoxia, and highlights the need for further investigation in a larger number of tumours if this concept is to be used to select patients in whom carbogen may have therapeutic value. It should be noted, however, that random fluctuations of more than 50% over a 60-min period have been reported [11,16] suggesting that quite large random variations in blood flow can occur without the introduction of any other agents in such a system.

The flow rate of inhaled carbogen is considered to be important in enhancing tumour blood flow, and above 330 ml/min perfusion changes are abolished [12]. The relatively high flow rate of 15 l/min used in this series might explain why no overall perfusion changes were evident. In practice, however, lower flow rates are tolerated poorly due to the increased respiratory drive provoked by the CO_2 content of carbogen.

Transient fluctuations in microregional blood flow consistent with perfusion limited hypoxia have been shown to occur in both experimental and human tumours. Whilst these have been confirmed in this study, no consistent effect of carbogen on blood flow has been demonstrated. Although these findings may offer some credence to the hypothesis that the carbon dioxide content abolishes vasoconstriction provoked by the high levels of oxygen, they suggest that the radiosensitising action of carbogen lies primarily in its ability to increase the oxygen carrying capacity of blood. This supports the introduction of agents such as nicotinamide with carbogen breathing to allow eradication of both diffusion and perfusion-limited hypoxia.

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References

[1] Abbot, N.C. and Beck, J.S. Biological zero in laser Doppler measurements in normal ischaemic and inflamed human skin. Int. J. Microcirc. Clin. Exp. 12: 89–98, 1993.

[2] Chaplin, D.J. and Hill, S.A. Temporal heterogeneity in microregional erythrocyte flux in experimental solid tumours. Br. J. Cancer 71: 1210–1213, 1995.

[3] Chaplin, D.J., Horsman, M.R. and Sieman, D.W. Further evaluation of nicotinamide and carbogen as a strategy to re-oxygenate hypoxic cells in vivo importance of nicotinamide dose and pre-irradiation breathing time. Br. J. Cancer 68: 269–273, 1993.

[4] Chaplin, D.J., Olive, P.L. and Durand, R.E. Intermittent blood flow in the murine tumour: radiobiological effects. Cancer. Res. 47: 597–601, 1987.

[5] Chen, J.L., Wei, L., Acuff, V., Bereczki, D., Hans, F.J., Otsukat, T., Finnegan, W., Patrak, C. and Feinstermacher, J. Slightly altered permeability-surface area products imply some cerebral capillary recruitment during hypercapnia. Microvasc. Res. 48: 190–211, 1994.

[6] Colantuoni, A., Bertuglia, S. and Intaglietta, M. A biological zero of laser Doppler fluxmetry — microcirculatory correlates in the hamster cheek pouch during flow and no flow conditions. Int. J. Microcirc. Clin. Exp. 13: 125–136, 1993.

[7] Dewhirst, M.W., Ong, E.T., Rosner G.L., Rehmus S.W., Shan, S., Braun, R.D., Brizel, D.M. and Secomb, T.W. Arteriolar oxygenation in tumour and subcutaneous arterioles: effects of inspired air oxygen content.. Br. J. Cancer 74: 241–246, 1996.

[8] Dische, S., Rojas, A., Rugg, T., Hong, A. and Michael, B.D. Carbogen breathing: a system for use in man. Br. J. Radiol. 65: 87–90, 1992.

[9] Falk, S.J., Ward, R. and Bleehan, N.M. The influence of carbogen breathing on tumour tissue oxygenation in man evaluated by computerised pO_2 histography. Br. J. Cancer 56: 919–924, 1992.

[10] Grau, R.S., Horsman, M.R. and Overgaard, J. Improving the radiation response in a C3H mouse mammary carcinoma by normobaric oxygen or carbogen breathing. Int. J. Radiat. Oncol. Biol. Phys. 22: 415–419, 1995.

[11] Hill, S.A., Pigott, K.H., Saunders, M.I., Powell, M.E.B., Arnold, S., Obeid, A., Ward, G., Leehey, M., Hoskin, P.J. and Chaplin, D.J. Microregional blood flow fluctuations in murine and human tumours assessed using laser Doppler microprobes. Br. J. Cancer 74: 260–263, 1996.

[12] Honess, D.J. and Bleehan, N.M. Perfusion changes in the RIF-1 tumour and normal tissues after carbogen and nicotinamide, individually and combined. Br. J. Cancer 71: 1175–1180, 1995.

[13] Kruuv, J.A., Inch, W.R. and McCredie, J.A. Blood flow and oxygenation of tumors in mice. Cancer 20: 51–58, 1967.

[14] Oberg, P.A. Laser-Doppler flowmetry. Crit. Rev. Biomed. Eng. 18: 125–163, 1990.

[15] Overgaard, J. Modification of hypoxia from Gottwals Schwarz to nicotinamide: have we learned the lesson? In: Danish Cancer Society. Progress in Radio-Oncology V, pp. 469–475. Editors: H.D. Kogelnik. Monduzzi Editore, Bologna, 1995.

[16] Pigott, K.H., Hill, S.A., Chaplin, D.J. and Saunders, M.I. Microregional fluctuations in perfusion within human tumours detected using laser Doppler flowmetry. Radiother. Oncol. 40: 45–50, 1996.

[17] Rider, W.D., Kersesteci, A.G., Hawkins, N.V. and Bush, R.S. A trial of 'orthobaric oxygen' as an adjunct of radiotherapy of bladder cancer. In: Proceedings of Radiological Society of North America, 5th Scientific Assembly, Chicago, 1969.

[18] Robinson, S.P., Howe, F.A., Griffiths, J.R. Non-invasive monitoring of carbogen-induced changes in tumor, blood flow and oxygenation by functional magnetic resonance imaging. Int. J. Radiat. Oncol. Biol. Phys. 33: 855–859, 1995.

[19] Rojas, A. Radiosensitisation with normobaric oxygen and carbogen. Radiother. Oncol. 20: 65–70, 1991.

[20] Rubin, P., Hanley, J., Keys, H.M., Marcial, V. and Brady, L. Carbogen breathing during radiation therapy. Int. J. Radiat. Oncol. Biol. Phys. 5: 1963–1970, 1979.

[21] Siemann, D.W., Hill, R.P. and Bush, R.S. The importance of the pre-irradiation breathing times, oxygen and carbogen (5% CO_2) on the in vivo radiation response of a murine sarcoma. Int. J. Radiat. Oncol. Biol. Phys. 2: 903–911, 1977.

[22] Siemann, D.W., Horsman, M.R. and Chaplin, D.J. The radiation response of KHT sarcomas following nicotinamide treatment and carbogen breathing. Radiother. Oncol. 31: 117–122, 1994.

23] Taylor, N.J., Griffiths, J.R. Howe, F.A., Saunders, M.I., Robinson, S.P., Thoumine, M., Caine, L.A., Hoskin, P.J., Powell, M., Chaplin, D.J. and Baddeley, H. Carbogen-induced oxygenation and blood flow changes within human tumours, monitored by Gradient Echo Magnetic Resonance Imaging. In: SMRM 4th Fourth Annual Meeting, p. 1313, 1996 (Abstract).

24] Thomlinson, R.H. and Gray, L.H. The histological structure of some human lung cancers and the possible implications for radiotherapy. Br. J. Cancer 9: 539–549, 1955.

[25] Trotter, M.J., Chaplin, D.J., Durand, R.E. and Olive, P.L. The use of fluorescent probes to identify regions of transient perfusion in murine tumours. Int. J. Radiat. Oncol. Biol. Phys. 16: 931–935, 1989.

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Advances in Brief

Human Tumor Blood Flow Is Enhanced by Nicotinamide and Carbogen Breathing[1]

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Abstract

Perfusion insufficiency and the resultant hypoxia are recognized as important mechanisms of resistance to anticancer therapy. Modification of the tumor microenvironment to increase perfusion and oxygenation of tumors may improve on the efficacy of these treatments. Using laser Doppler probes to measure microregional RBC flux, this study examines the influence of nicotinamide and carbogen on human tumor perfusion. Ten patients with advanced cancers were studied. Nicotinamide (80 mg/kg) was given p.o., and 60 min later, up to six probes were inserted into the tumor. Readings were taken for 1 h, followed by 10 min of carbogen breathing and 10 additional min of breathing room air. Results were compared with those from a similar group of eight control patients who were not given nicotinamide, but who breathed carbogen. In 44 microregions analyzed, 33 (73%) showed perfusion fluctuations of 50% or more, and 20 (44%) by 100% or more. This compared with the control group in whom 62% and 27% of microregions varied by 50% or more and 100% or more, respectively. Perfusion increases outweighed decreases by 30% with nicotinamide and 20% in the controls. On breathing carbogen, patients pretreated with nicotinamide showed an increase in tumor perfusion of 17% at 5 min and 22% at 10 min, compared with only 0% and 1% in the control group. Pretreatment with nicotinamide made little difference to the random blood flow fluctuations seen in controls. However, when carbogen was introduced, tumor perfusion increased compared with the control group. This may have important therapeutic implications by improving response to treatment and allowing better delivery of systemically administered agents.

Introduction

Modification of the tumor microenvironment may be a method of improving on the therapeutic success not only of conventional anticancer treatment, such as chemotherapy and radiotherapy, but also novel anticancer therapies, such as photodynamic therapy, gene therapy, and biological therapies. This, however, requires a greater understanding of tumor physiology and factors that may influence it. Tumor blood flow is clearly an important factor in the delivery and, hence, the cytotoxic effect of systemically administered agents. It also determines the degree of hypoxia within a tumor, which, in turn, is recognized to be a major cause of resistance to radiation, photodynamic therapy, certain chemotherapeutic drugs, and biological agents (1–4). Hypoxic areas, as defined by a pO_2 of less than 10 mm Hg, are recognized to be a common feature of both experimental and human cancers (2, 5, 6). It was originally believed that such regions of low oxygen tension were due solely to the limited diffusing capacity of oxygen, with cells distant from blood vessels being relatively starved of oxygen and nutrients for long periods (7). More recently, however, it has been shown that regions of hypoxia can also result from transient fluctuations in tumor blood flow (8).

Improving tumor oxygenation and perfusion is now under renewed scrutiny, with both animal and clinical studies under way evaluating the hypoxic cell sensitizers carbogen and nicotinamide (9–11). The principal mode of action of carbogen (95% oxygen, 5% carbon dioxide) is to increase the amount of dissolved oxygen within the blood, thereby enhancing oxygenation of diffusion-limited or chronically hypoxic cells. Nicotinamide, the amide derivative of vitamin B_3, is believed to overcome perfusion-limited or acute hypoxia by minimizing the frequency and magnitude of changes in microregional tumor erythrocyte flux (12). Animal studies have shown that, combined, carbogen and nicotinamide can achieve a 2-fold enhancement ratio for local tumor control (13).

We have recently developed a clinical technique to monitor real-time fluctuations in microregional erythrocyte flux in experimental and human tumors (14, 15) using commercially available multichannel laser Doppler microprobes.

The aim of this study was to use laser Doppler probes to evaluate the effect of carbogen with and without nicotinamide on microregional RBC flux.

Materials and Methods

Patients. Patients participating in this study all had histologically proven malignancy and gave informed consent to participate in this study. Approval for the study was obtained from the local ethics committee.

Ten patients given nicotinamide were compared to a control group of eight patients previously studied, who had not received nicotinamide. The control group included six patients, two of whom had multiple tumors and were studied on two separate occasions. Patient and tumor details are shown in Table 1.

Laser Doppler Flowmetry. RBC flux was measured using the Oxford Array multichannel laser Doppler system (Oxford Optronix, Oxford, UK), which allows simultaneous measurement of blood flow in up to 12 discrete microregions. The system used in this study comprised six custom-made, cylindrical probes, each measuring 25 mm in length and 300 μm in diameter and with an estimated sampling volume of 0.01 mm^3.

Nicotinamide Administration. Nicotinamide was given p.o. in a dose of 80 mg/kg. This dose, which is used in clinical practice, is well tolerated by patients and achieves effective plasma levels in most (10, 16). Salivary or plasma levels of nicotinamide were measured by high-performance liquid chromatography (17, 18) at 15–20-min intervals during the study period.

Carbogen Breathing. Carbogen (95% O_2, 5% CO_2) breathing was carried out using a technique pioneered for clinical use at our center (19). A close-fitting face mask (Intersurgical, Wokingham, UK) covers both the nose and mouth, and the gas is delivered at a flow rate of 15 liters/min through a closed system using a one-way valve and a 3-liter breathing bag.

Experimental Setup. Each patient lay on a bed, and up to six microprobes were inserted into the tumor. The skin adjacent to the lesion was anesthetized using 1% lignocaine, and each microprobe was introduced into the tumor through a 20-gauge cannula. Because LDF[3] measurements were taken some distance from the skin it was felt lignocaine would be unlikely to influence the readings. Laser Doppler readings began, and after 60 min of "baseline"

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[3] The abbreviation used is: LDF, laser Doppler flux.

Table 1 *Details of patients and tumors studied, including nicotinamide levels and change in LDF with carbogen breathing*[a]

Case	Site and size of tumor	Histology and site of primary tumor	Plasma nicotinamide levels (nmol/ml)		Relative change in LDF with carbogen after	
			60′	start of carbogen	5 min	10 min
1	Node (SCF), 3 cm	SCC lung	1112	1022	1.16 ± 0.11	1.18 ± 0.05
2	Skin (abdomen), 3 cm	AC colon	2024	1452	1.02 ± 0.04	1.10 ± 0.04
3	Node (neck), 6 cm	SCC pharynx	1595	1054	1.05 ± 0.07	0.92 ± 0.05
4	Skin (thigh), 5 cm	Melanoma	1832	834	1.09 ± 0.07	1.16 ± 0.09
5	Breast, 7 cm	AC breast	3217	1768	1.17 ± 0.12	1.26 ± 0.14
6[b]	Skin (abdomen), 3 cm	AC colon	378	680	1.18 ± 0.06	125 ± 0.07
7[c]	Skin (arm), 3 cm	SCC lung	1364	1246		
8	Skin (flank), 3 cm	TCC kidney	1849	1546	1.11 ± 0.03	1.07 ± 0.03
9	Node (axilla), 5 cm	NHL	721	1676	0.97 ± 0.19	0.96 ± 0.19
10	Node (groin), 4 cm	NHL	1078	913	1.77 ± 0.39	1.77 ± 0.39
1[d]	Breast, 10 cm	AC			1.01 ± 0.11	0.94 ± 0.11
2[d]	Node (neck), 3.5 cm	NHL			0.85 ± 0.07	0.84 ± 0.11
3[d]	Node (neck), 6 cm	SCC larynx			1.10 ± 0.19	1.08 ± 0.12
4[d]	Skin (abdomen), 3 cm	AC colon			1.14 ± 0.18	1.06 ± 0.05
	Skin (abdomen), 3.5 cm				1.21 ± 0.23	1.24 ± 0.11
5[d]	Node (right groin), 4 cm	NHL			0.81 ± 0.06	0.96 ± 0.06
	Node (left groin), 3 cm				0.79 ± 0.20	1.00 ± 0.06
6[d]	Node (axilla), 3 cm	Melanoma			1.25 ± 0.28	1.00 ± 0.05

[a] SCC, squamous cell carcinoma; TCC, transitional cell carcinoma; NHL, non-Hodgkin's lymphoma; AC, adenocarcinoma; SCF, supraclavicular fossa.
[b] Excluded from 60-min observation.
[c] Unable to breathe carbogen.
[d] Control patients (no nicotinamide, breathed carbogen only).

measurements, a 10-min carbogen breathing period commenced. Finally, an additional 10 min of LDF readings were acquired, with the patient breathing room air.

Patients given nicotinamide waited 60 min postadministration before LDF measurements began. This timing was chosen to conform with current clinical practice, in which patients receive radiotherapy and concomitant carbogen 2 h afterward (10, 11). This is based on experimental evidence that suggests that radiosensitization is maximal when radiation is given at peak plasma nicotinamide levels occurring between 30 min and 3 h after administration (16).

Data Analysis. Individual probes generate 20 readings per second. From this, an average flow reading is calculated for each 2-min interval in all channels and plotted against time. The final plots of RBC flux, together with the original recorded data, were examined, and traces showing evidence of patient or probe movement were excluded from analysis.

Flow during carbogen breathing was related to a baseline value that was a single mean calculated from the measurements recorded over the 10-min period prior to breathing carbogen and was designated as the flow at time 0. Blood flow averages at 5 and 10 min after starting carbogen breathing were compared with the time 0 value using a paired two-tailed t test, where $P < 0.05$ was considered to be significant.

Results

Therapeutic levels of nicotinamide (>700 μmol) were achieved in all patients except patient 6, in whom levels were slow to rise and were just suboptimal at the time of carbogen breathing (Table 1). Her readings were excluded from the 60-min observation period but were included for analysis of carbogen breathing. Patient 7 was unable to tolerate the face mask and did not breathe carbogen.

Assessment of the 1-h observation period was made on 52 traces in nine patients. Eight traces were excluded due to probe or patient movement, allowing the RBC flux in 44 separate microregions to be analyzed. As noted in our previous studies, striking heterogeneity of perfusion changes was seen between individual tumors and the separate microregions studied in a tumor (14, 15). Temporal fluctuations in perfusion by a factor of 50% or more occurred in 33 traces (73%), and 20 traces (44%) showed changes of 100% or more (Table 2). In 12 (27%) of these traces, the initial changes in RBC flux were subsequently reversed; *i.e.*, an increase in blood flow was followed by a period of decreased perfusion or *vice versa*. Table 2 compares fluctuations in microregional RBC flux seen over 60 min in the nicotinamide group with changes observed in the control group. Seven patients whose tumors were included in the latter group did not breathe carbogen and formed part of a previous study (14). Although a slightly higher percentage of the nicotinamide-treated tumors showed fluctuations in RBC flux by a factor of 1.5 or more, the ratio of increases:decreases was lower than in the control group (1.2 in the nicotinamide tumors and 1.3 in the controls). However, taking the hour as a whole, blood flow remained constant (Fig. 1).

Fig. 2 shows the relative changes in RBC flux from nine nicotinamide and eight control patients as a result of carbogen breathing. Values are expressed as means ± SE. The data indicate that in patients pretreated with nicotinamide, erythrocyte flux increased with carbogen, appearing to plateau at 8 minutes. This compares with the control group in whom carbogen effected no overall change in blood flow. Analysis after 5 and 10 min of carbogen breathing showed blood flow in the nicotinamide group to have increased by 17% and 22% relative to the precarbogen value, which reaches statistical significance at $P < 0.004$ and $P < 0.001$, respectively. This compares with the control group, which showed fluctuations of 0 and 1% at 5 and 10 min ($P = 0.94$ and $P = 0.9$, respectively). Table 1 summarizes the effect of carbogen on RBC flux in individual patients.

Discussion

There are reports that in certain experimental tumors, carbogen can improve tumor blood flow and oxygenation (9, 20). If such increases in perfusion were a common feature of human tumors, the finding would be of importance not only to radiotherapy but also to systemically delivered treatments, such as chemotherapy and biological

Table 2 *Changes in microregional erythrocyte flux over a 60-min observation period in 15 control tumors and 10 treated with nicotinamide*[a]

Factor of change	Controls		Nicotinamide	
	No. of traces changing	No. and direction of changes	No. of traces changing	No. and direction of changes
≥1.5	45 (62%)	46 (+) 35 (−)	33 (73%)	28(+) 23 (−)
≥2	20 (27%)	17 (+) 13 (−)	20 (44%)	14(+) 10 (−)

[a] +, increase; −, decrease.

therapies. Our clinical data indicate that blood flow response to carbogen can be variable, with both increases and decreases occurring (15). This agrees with more recent detailed data from animal systems, which show that decreases as well as increases in tumor blood flow are observed in response to carbogen (21, 22). The current study shows that in a heterogeneous group of tumors, pretreatment with nicotinamide more reliably enhances tumor perfusion compared with carbogen alone, with only one tumor not showing increased perfusion.

Nicotinamide is believed to improve oxygenation by improving the homogeneity of microregional tumor blood flow. It might therefore be expected that nicotinamide would lead to a rise in blood flow, or at least an alteration in the ratio of blood flow fluctuations with fewer decreases occurring. These data, however, show a similar result to random blood flow fluctuations seen in a group of untreated patients in whom 62% of traces changed by 50% or more (73% in this series) and 27% changed by 100% (44% in this series), with increases in blood flow outnumbering decreases by 1:1.3 (Ref. 14; 1:1.2 in this study). Thus, nicotinamide, at the doses administered in the clinic, does not prevent transient fluctuations in human tumor microregional blood flow. This finding contrasts with studies in experimental tumor systems in which nicotinamide has been shown to reduce or eliminate such blood flow instability. It should be emphasized, however, that animal studies have used much higher doses of nicotinamide that cannot be achieved in humans without unacceptable toxicity (16).

Our study demonstrates that, together, carbogen and nicotinamide give rise to an increase in blood flow. The overall increase of 22% may seem modest, but such a global view ignores the spatial heterogeneity of both blood flow and oxygenation known to exist in tumors. If increases were occurring where capillary flow has either ceased or is severely reduced, *i.e.,* where microregional perfusion is most compromised, they could bring important benefits for drug delivery and the eradication of hypoxia.

The mechanism responsible for the combination of carbogen and nicotinamide increasing tumor blood flow is unclear but may involve a complex interaction between tumor cells and tumor vasculature. For example, it is known that tumor and incorporated host cells produce vasoactive compounds, such as the vasodilator nitric oxide and the vasoconstrictor peptide endothelin. Their production, which may vary quite markedly between different tumor types, is independently altered by both carbon dioxide levels and nicotinamide (23, 24). Thus, a selective alteration in local production of such vasoactive compounds that might, *e.g.,* prevent vasoconstriction of vessels within a tumor, may be a possible explanation for the effects on tumor perfusion seen in our study with the combination of carbogen and nicotinamide.

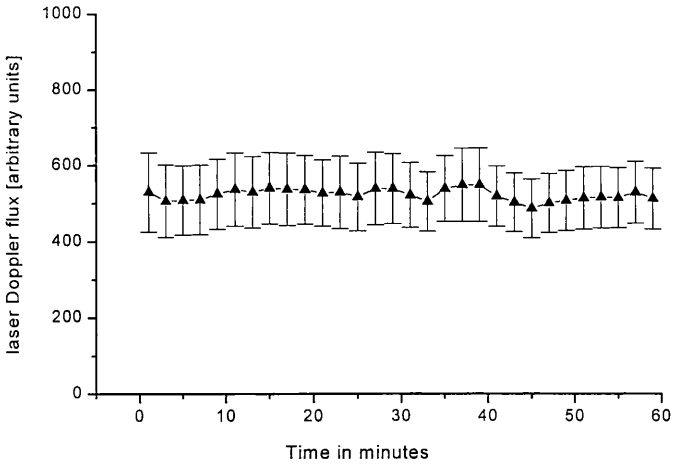

Fig. 1. Change in LDF over 1 h in patients pretreated with nicotinamide (44 traces). Data are means; *bars,* SE.

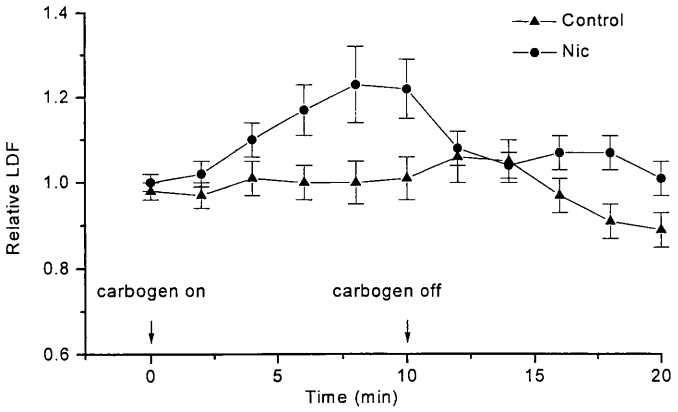

Fig. 2. The effect of carbogen breathing on relative LDF in control patients (34 traces) and patients pretreated with nicotinamide (45 traces). Data are means; *bars,* SE.

Our study confirms that tumor perfusion can be increased with the addition of carbogen and nicotinamide. This may, in turn, lead to higher tumor cure rates by enhanced radioresponsiveness and improved delivery of systemic treatments. Clearly, consistent increases are necessary if the use of nicotinamide and carbogen is to become routine. Our work, however, provides a basis for further investigation of a wider range of tumors and the evaluation of other agents that might offer larger and more dependable improvements in tumor perfusion.

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References

1. Overgaard, J., and Horsman, M. R. Modification of hypoxia-induced radioresistance in tumours by the use of oxygen and sensitisers. Semin. Radiat. Oncol., *6:* 10–21, 1996.
2. Hockel, M., Knoop, C., Schlenger, K., Vorndran, B., Baubmann, E., Mitze, M., Knapstein, P., and Vaupel, P. Intratumoural pO_2 predicts survival in advanced cancer of the uterine cervix. Radiother. Oncol., *26:* 45–50, 1993.
3. Ishizaka, S., Kimoto, M., and Tsujii, T. Defect in generation of LAK cell activity under oxygen limited conditions. Immunol. Lett., *32:* 209–214, 1992.
4. Sampson, L., and Chaplin, D. J. The influence of microenvironment on the cytotoxicity of TNF α *in vitro*. Int. J. Radiat. Oncol. Biol. Phys., *29:* 467–471, 1994.
5. Vaupel, P., Frinak, S., and Bicher, H. I. Heterogenous oxygen partial pressure and pH distribution in C3H mouse mammary adenocarcinoma. Cancer Res., *41:* 2008–2013, 1981.
6. Vaupel, P., Schlenger, K., Knoop, C., and Höckel, M. Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O_2 tension measurements. Cancer Res., *51:* 3316–3322, 1991.
7. Thomlinson, R. H., and Gray, L. H. The histological structure of some human lung cancers and the possible implications for radiotherapy. Br. J. Cancer, *9:* 539–549, 1955.
8. Chaplin, D. J., Olive, P. L., and Durand, R. E. Intermittent blood flow in a murine tumor: radiobiological effects. Cancer Res., *47:* 597–601, 1987.
9. Rodrigues, L. M., Maxwell, R. J., McSheehy, P. M. J., Pinkerton, C. R., Robinson, S. P., Stubbs, M., and Griffiths, J. R. *In vivo* detection of ifosfamide by ^{31}P-MRS in rat tumours: increased uptake and cytotoxicity induced by carbogen breathing in GH3 prolactinomas. Br. J. Cancer, *75:* 62–68, 1997.
10. Hoskin, P. J., Saunders, M. I., Phillips, H., Cladd, H., Powell, M. E. B., Goodchild, K., Stratford, M. I., and Rojas, A. Carbogen and nicotinamide in the treatment of bladder cancer with radical radiotherapy. Br. J. Cancer, *76:* 260–263, 1997.
11. Saunders, M., and Dische, S. Clinical results of hypoxic cell radiosensitisation from hyberbaric oxygen to accelerated radiotherapy, carbogen and nicotinamide. Br. J. Cancer, *74:* 271–278, 1996.
12. Chaplin, D. J., Horsman, M. R., and Trotter, M. J. Effect of nicotinamide on the microregional heterogeneity of oxygen delivery within a murine tumor. J. Natl. Cancer Inst., *82:* 672–676, 1990.
13. Rojas, A., Hirst, V. K., Calvert, A., and Johns, H. Carbogen and nicotinamide as radio-sensitisers in a murine mammary carcinoma using conventional and accelerated radiotherapy. Int. J. Radiat. Oncol. Biol. Phys., *34:* 357–365, 1996.
14. Hill, S. A., Pigott, K. H., Saunders, M. I., Powell, M. E. B., Arnold, S., Obeid, A., Ward, G., Leahy, M., Hoskin, P. J., and Chaplin, D. J. Microregional blood flow

fluctuations in murine and human tumours assessed using laser Doppler microprobes. Br. J. Cancer, *74:* 260–263, 1996.
15. Powell, M. E. B., Hill, S. A., Saunders, M. I., Hoskin, P. J., and Chaplin, D. J. Effect of carbogen breathing on tumour microregional blood flow in humans. Radiother. Oncol., *41:* 225–231, 1996.
16. Hoskin, P. J., Stratford, M. R., Saunders, M. I., Hall, D. W., Dennis, M. F., and Rojas, A. Administration of nicotinamide during chart: pharmacokinetics, dose escalation, and clinical toxicity. Int. J. Radiat. Oncol. Biol. Phys., *32:* 1111–1119, 1995.
17. Stratford, M., and Dennis, M. High-performance liquid chromatographic determination of nicotinamide and its metabolites in human and murine plasma and urine. J. Chromatogr., *582:* 145–151, 1992.
18. Stratford, M., Dennis, M., Hoskin, P., Phillips, H., Hodgkiss, R., and Rojas, A. Nicotinamide pharmacokinetics in humans: effect of gastric acid inhibition, comparison of rectal *versus* oral administration and the use of saliva for drug monitoring. Br. J. Cancer, *74:* 16–21, 1996.
19. Dische, S., Rojas, A., Rugg, T., Hong, A., and Michael, B. D. Carbogen breathing: a system for use in man. Br. J. Radiol., *65:* 87–90, 1992.
20. Honess, D. J., and Bleehen, N. M. Perfusion changes in the RIF-1 tumour and normal tissues after carbogen and nicotinamide, individually and combined. Br. J. Cancer, *71:* 1175–1180, 1995.
21. Dewhirst, M. W., Ong, E. T., Rosner, G. L., Rehmus, S. W., Shan, S., Braun, R. D., Brizel, D. M., and Secomb, T. W. Arteriolar oxygenation in tumour and subcutaneous arterioles: effects of inspired air oxygen content. Br. J. Cancer, *74:* S241–S246, 1996.
22. Hill, S. A., Collingridge, D. R., Vojnovic, B., and Chaplin, D. J. Tumour radiosensitization by high oxygen content gases: influence of the carbon dioxide content of the inspired on pO_2, microcirculatory function and radiosensitivity. Int. J. Radiat. Oncol. Biol. Phys., in press, 1998.
23. Yoshimoto, S., Ishizaki, Y., Sasaki, T., and Murota, S. Effect of carbon dioxide and oxygen on endothelin production by cultured porcine cerebral endothelial cells. Stroke, *22:* 378–383, 1991.
24. Pellat-Deceunynck, C., Wietzerbin, J., and Drapier, J-C. Nicotinamide inhibits nitric oxide synthase mRNA induction in activated macrophages. Biochemistry, *297:* 53–58, 1994.