RELATIONSHIP BETWEEN POLAROGRAPHIC OXYGEN MEASUREMENTS, METASTATIC ABILITY AND EF5 BINDING IN MURINE TUMOUR MODELS

by

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Relationship between polarographic oxygen measurements, metastatic ability and EF5 binding in murine tumour models

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Thesis Abstract

Hypoxia exists in most solid tumours. There is data to support that it is implicated in resistance to radiotherapy and chemotherapy and might contribute to tumour aggressiveness and metastasis. Therefore, methods that measure hypoxia are of significant interest. In this thesis the relationship between tumour oxygenation, as measured with the Eppendorf pO_2 Histograph and metastatic ability was examined in two rodent tumour models, KHT-C and SCC-VII. A significant increase in early pulmonary metastasis formation was observed in hypoxic KHT-C tumours. A similar trend was observed in SCC-VII tumours but it was not statistically significant. In addition, a comparison was made between the Eppendorf technique for measuring hypoxia and labelling of hypoxic cells using the marker EF5 in two human cervix cancer xenograft models, Me180 and HeLa. In Me180 tumours, a significant correlation was found between the two techniques but no correlation was found in HeLa tumours. It was hypothesised that histopathological characteristics such as extensive necrosis might have contributed to the disparate results.

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CHAPTER 1

INTRODUCTION

1.1 General concepts

1.1.1 Development of hypoxia in tumours

Hypoxia (low oxygenation) is an aberrant environmental condition that is present in a variety of rodent and human tumours. It is thought to arise from an imbalance between oxygen supply and consumption (Gulledge and Dewhirst, 1996). Firstly, tumours have a high demand for oxygen and nutrients due to their high proliferation rate and abnormal metabolism. Secondly, when tumours grow beyond a limited volume of 1 to 2 mm^3, passive diffusion of oxygen and nutrients is no longer adequate and tumours must stimulate the growth of new blood vessels (Weidner and Folkman, 1996). Rapid tumour growth does, however, not allow for vascular differentiation and often leads to the formation of an abnormal network of capillaries with chaotic branching and tortuous, leaky vessels. Increased vessel permeability facilitates accumulation of plasma in the interstitium, which in turn shuts down blood vessels (Jain, 1987). The result of these vascular malformations is an erratic oxygen and nutrient supply.

1.1.2 Chronic and acute hypoxia

Tumour hypoxia can occur in two forms. The first is termed chronic or diffusion-limited hypoxia and is believed to develop as a result of limitations in the diffusion distance of oxygen from a vessel due to cellular respiration. Beyond this diffusion distance, which is typically around 150 μm, the cells become starved, die off and necrosis develops (Thomlinson and Gray, 1955). The second way in which hypoxia can occur is transient in nature and is referred to as acute or perfusion-limited hypoxia. It results from intermittent,

partial or complete reductions in blood flow (Brown, 1979; Dewhirst, 1998). A complete shutdown would temporarily starve cells of both, nutrients and oxygen, while a partial occlusion would allow flow of plasma but not of blood cells and thus primarily compromise oxygen supply. Both types of hypoxia are present to some extent in solid tumours.

1.2 Methods of oxygen measurement in tumours

A large number of techniques to measure hypoxia in tumours has been developed and is undergoing extensive testing in experimental and clinical studies. Comprehensive surveys on currently available techniques have been published (Stone *et al*, 1993; Raleigh *et al*, 1996; Horsman *et al*, 1998). Most widely used techniques include the Eppendorf polarographic oxygen sensors, the comet assay, detection of nitroimidazole binding and magnetic resonance imaging techniques. The methods we have focussed on and which are currently applicable in the context of clinical studies in patients are the polarographic oxygen sensors and the quantification of binding of specific drugs (2-nitroimidazoles) to hypoxic cells. These techniques will be discussed in detail as they are most relevant to the work presented in this thesis (chapter 2 and 3).

1.2.1 Polarographic oxygen sensors

Polarographic histography relies on the chemical reduction of oxygen at an electrical conducting surface under the influence of a fixed negative polarising voltage applied between an anode (ground) and a cathode (fine needle electrode), which is inserted into the tissue of interest. The current, resulting from the oxidation-reduction reaction, flows in the measurement circuit and is proportional to the oxygen concentration adjacent to the

cathode. The applied voltage between anode and cathode is a critical parameter in polarographic oxygen tension measurements. Plotting the electrode current against the applied polarisation voltage at different constant oxygen concentrations results in a set of polarograms (figure 1.1). A characteristic plateau due to the diffusion limitation of oxygen from the tissue of interest to the cathode surface is observed. The polarisation voltage at which the oxygen sensors operate optimally lies in the middle of this plateau. Here, one may expect a stable output from the system because the current is, at that point, almost unaffected by minor fluctuations in polarisation voltage. The use of this plateau value for polarisation voltage results in a linear relationship between pO_2 and electrode current. At lower and higher voltages, incomplete or other reactions (hydrogen wave) take place respectively.

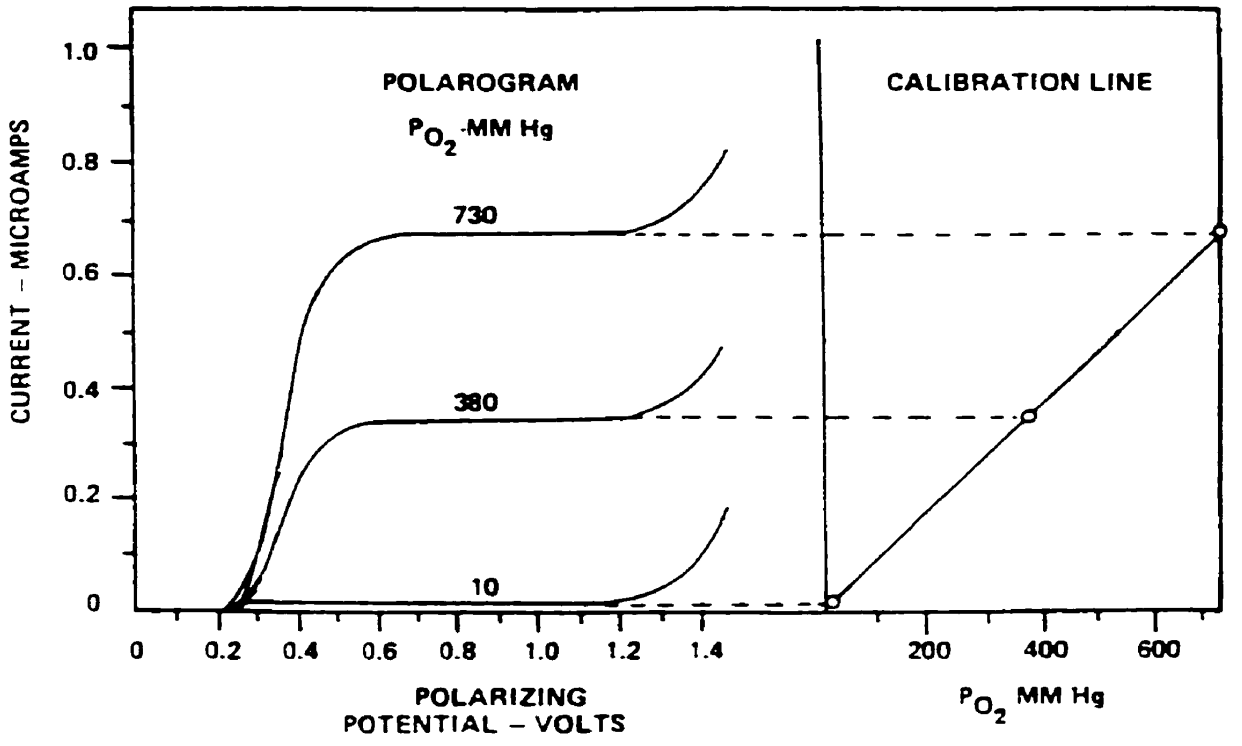

Figure 1.1: Idealised representation of the cathodic current as a function of applied polarising voltage in solutions of different oxygen tension (adapted from Fatt (1976))

1.2.1.1 The Eppendorf pO_2 Histograph

Polarographic electrodes for measuring local oxygen tension have been used in patients since the early 60s (Cater and Silver, 1960; Evans and Naylor, 1963). The first generation of polarographic techniques suffered from unsatisfactory design and unreliable performance. The wide use of polarographic oxygen measurements in patients started only recently following the development of the Eppendorf Histograph, Kimoc 6650, a technically improved and commercially available polarographic system. The introduction of the Eppendorf Histograph has led to extensive clinical testing in a number of easily accessible tumour sites such as head and neck, breast and cervix carcinoma, and sarcoma of the limbs (Gatenby *et al*, 1988; Vaupel *et al*, 1991; Lartigau *et al*, 1992a; Lartigau *et al*, 1993; Nordsmark *et al*, 1995; Brizel *et al*, 1996; Höckel *et al*, 1996; Nordsmark *et al*, 1996; Brizel *et al*, 1997; Sundfør *et al*, 1997; Fyles *et al*, 1998a). Several research groups have independently evaluated its potential value as a prognostic indicator so that the Eppendorf Histograph is now designated the 'gold standard' for measuring tumour oxygenation in patients (Stone *et al*, 1993).

The Eppendorf polarographic oxygen sensor uses a needle electrode which contains a gold micro-cathode (diameter 12 µm) recessed into the tip of an electrode shaft (diameter 300 µm). The glass-insulated cathode, which is covered with a Teflon membrane to prevent the measurements from being contaminated by proteins or other tissue constituents, is biased with a voltage of -700 mV against a silver-silver chloride anode, which is attached to the skin surface. The oxidation-reduction reaction which occurs at the level of anode and cathode is represented in figure 1.2. Before and after pO_2 measurements an oxygen electrode is calibrated using a saline solution equilibrated with 100% nitrogen or air (pO_2 of 145 mm Hg). The pO_2 Histograph is equipped with a microprocessor-controlled manipulator which allows stepwise advancement of the needle

probe through the tissue. A typical movement consists of a 1-mm forward step with subsequent rapid backward motion of 0.3 mm. This stepping procedure was designed to minimise tissue compression artefacts when the needle probe progresses in the tissue. After a response time of 1.4 seconds, which enables the sensor to adapt to the oxygen tension, the measured value at that needle position is recorded. This process is repeated automatically along the track of the electrode. The electrode signals are processed by computer and histograms of the resultant pO_2 values are used to extract parameters such as the percentage of measurements < 2.5 and 5 mm Hg and median pO_2 value.

(a) $4\,Ag + 4\,Cl^- \rightarrow 4\,AgCl + 4\,e^-$

(b) $O_2 + 2\,H_2O + 4\,e^- \rightarrow 4\,OH^-$

Figure 1.2: Oxidation (a) and reduction (b) reaction occurring at the level of the silver/silver-chloride anode and gold microcathode respectively.

Despite considerable improvements, there are still serious drawbacks/limitations in using the Eppendorf technique in patients. Good access to tumours is required, limiting the applicability to superficial tumours. A stringent and time consuming calibration procedure before and after each measurement is mandatory to assure stable output of the electrodes. The system also has a low signal-to-noise ratio at oxygen partial pressures between 0-10 mm Hg (which corresponds to the oxygen concentration range characteristic of radiobiological hypoxia), leading to increased uncertainty in the measurements at these low levels of oxygen. Furthermore, it is not always possible to distinguish measurements made in necrotic regions or normal tissue from those made in viable tumour tissue resulting in a potential over- or underestimation of

the degree of hypoxia. Finally, oxygen electrode data represent an average pO_2 value in tissue adjacent to the probe (estimated to be a volume equivalent to 50-100 cells), and thus do not provide information on oxygenation of individual clonogenic cells.

1.2.2 Nitroimidazole binding

Nitroimidazole-binding techniques are based on the observation that compounds such as 2-nitroimidazoles undergo hypoxia-dependent bioreduction by cellular nitroreductases (Workman, 1992) to produce reactive intermediates (Rauth *et al*, 1998). These reactive products can bind covalently to cellular macromolecules. In the presence of oxygen, the reduction is reversed at the first step and products are back oxidised (futile redox cycling). Hence, the proportion of hypoxic cells can be assessed from the amount of drug adducts retained in the tissue. A variety of detection methods has been developed to quantitate 2-nitroimidazole adduct binding.

Early studies utilised the ^{14}C and ^{3}H-labelled nitroimidazole, misonidazole, and have revealed heterogeneous distributions of radioactivity on autoradiographs of rodent and human tumour sections (Urtasun *et al*, 1986; Olive and Durand, 1989). More recently, 2-nitroimidazoles containing labels such as ^{123}I (Parliament *et al*, 1992; Urtasun *et al*, 1996), ^{18}F (Koh *et al*, 1992; Rasey *et al*, 1996; Hustinx *et al*, 1999; Evans *et al*, 1999) and ^{19}F (Raleigh *et al*, 1986; Maxwell *et al*, 1989; Raleigh *et al*, 1991; Aboagye *et al*, 1998) have been developed allowing non-invasive identification of drug binding by single photon emission tomography (SPECT), positron emission tomography (PET) and magnetic resonance spectroscopy (MRS). In addition, several groups started investigating detection techniques based on the recognition of nitroimidazole adducts by specific antibodies. Examples are CCI-103F (Raleigh *et al*, 1987; Cline *et al*, 1994), NITP (Hodgkiss and

Wardman, 1992), pimonidazole (Varia *et al*, 1998) and EF5 (Lord *et al*, 1993; Evans *et al*, 1995). Evaluation of binding has included analysis of sections stained with antibodies conjugated to fluorescent molecules (Hodgkiss *et al*, 1991; Raleigh *et al*, 1995) or biotin allowing immunoperoxidase-based immunohistochemical techniques (Cline *et al*, 1994; Kennedy *et al*, 1997). Also, flow cytometry and enzyme-linked immunosorbent assay (ELISA) analysis have been explored to measure binding to individual cells (Olive and Durand, 1983; Raleigh *et al*, 1987; Hodgkiss *et al*, 1991; Raleigh *et al*, 1992; Lord *et al*, 1993; Raleigh *et al*, 1994; Lee *et al*, 1996).

1.2.2.1 EF5 binding

The compound EF5[1] (figure 1.3) is a pentafluorinated derivative of etanidazole (a 2-nitroimidazole). It is a relatively new member of this class of compounds and holds great promise for clinical application. At present, the use of EF5 requires a biopsy of the tissue of interest. The unusual nature of the -CF2CF3 side chain terminus has contributed to the successful production of highly specific monoclonal antibodies ('ELK3-51') (Lord *et al*, 1993). The monoclonal antibodies can be tagged either with detector moieties such as biotin or with the fluorochromes 'Cy3' or 'Cy5' allowing immunohistochemical and flow cytometric quantitation. The presence of fluorine atoms provides opportunities for detection by non-invasive assays such as nuclear magnetic resonance. Recently, synthesis of ^{18}F-EF5 and its analogue ^{18}F-EF1 (Hustinx *et al*, 1999) has been reported, allowing imaging of drug-adduct distribution by positron emission tomography. EF5 binding has been demonstrated to be specific, oxygen dependent and sensitive at very low oxygen levels (Lord *et al*, 1993; Evans *et al*, 1995). In addition, the EF5 binding technique, like other nitro-

[1] EF5 = [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide]

imidazole techniques, is capable to discern variations in oxygenation at the cell-cell level. It also has the advantage of providing a positive signal in the absence of oxygen as opposed to other methods such as the Eppendorf technique, which provide a positive signal in aerobic cells.

At the time of writing, pimonidazole and EF5 are the only 2-nitroimidazole compounds that have been approved for testing in clinical trials (Evans *et al*, 1999). For the experiments described in chapter 3 we used the 2-nitroimidazole EF5, as this drug was kindly provided to us by Dr. C. Koch, University of Pennsylvania, within the scope of a research collaboration.

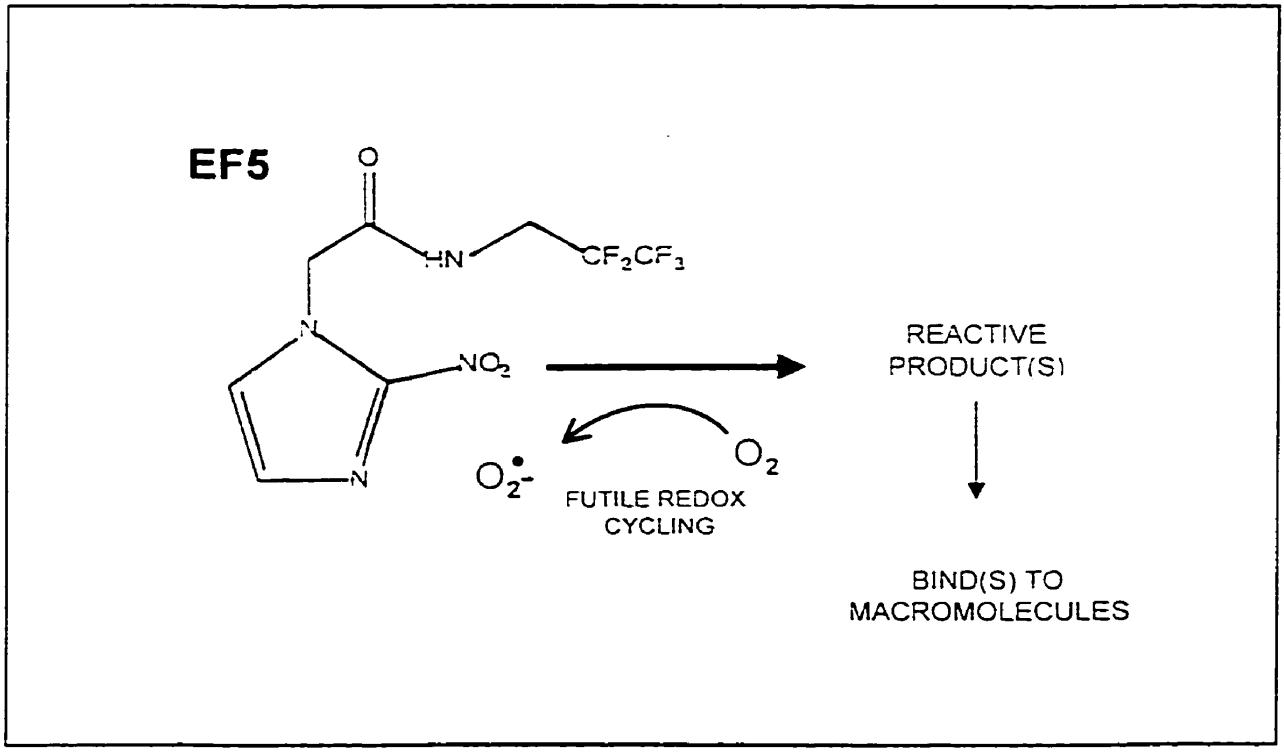

Figure 1.3: EF5 binding to macromolecules. Under anoxic conditions, intracellular reduction of the nitro (NO_2) group produces reactive intermediates that can covalently bind to macromolecules in the cell. In the presence of oxygen, futile redox cycling of the drug takes place preventing reactive intermediates from being formed.

1.3 Characteristics of oxygenation in tumours

1.3.1 Intra- and inter-tumour heterogeneity

Most experimental data on tumour oxygenation has been obtained in rodent tumours using indirect techniques, mainly radiobiological assays. A detailed literature review has been published by Rockwell *et al* (1984). Wide tumour-to-tumour variability in hypoxic proportions has been observed ranging from 0% to almost 100%. A similar variability has been reported in human tumours, xenografted into mice (Rockwell and Moulder, 1990). It has been postulated that possible sources of tumour-to-tumour variability could include differences in tumour size or cellularity, inherent clonogenicity of cells in individual tumours and host response to tumours (Moulder and Rockwell, 1984; Rockwell and Moulder, 1990). However, more recently, differences in oxygenation amongst tumours from identical cell line origin, grown in the same host and measured at almost identical size, have been observed by several investigators using radiobiological assays (Kavanagh *et al*, 1999a) or other techniques of oxygen measurement like the comet assay (Aquino-Parsons *et al*, 1999), 2-nitroimidazole binding and Eppendorf pO_2 Histography (Kavanagh *et al*, 1996; De Jaeger *et al*, 1998; Kavanagh *et al*, 1999a; Adam *et al*, 1999; Aquino-Parsons *et al*, 1999). In most of these studies, the variability in oxygenation within individual tumours was also studied and generally found to be less than the variability between tumours. It was postulated that this inter-tumour heterogeneity in oxygenation might reflect stochastic variations in the development of tumour vasculature during tumour growth (Rockwell *et al*, 1990; De Jaeger *et al*, 1998; Kavanagh *et al*, 1999a).

The Eppendorf pO_2 Histograph is the only technique allowing direct measurements of tumour oxygenation *in vivo*. Interestingly, Adam *et al* (1999) recently

reported comparisons of oxygenation, as measured with the Eppendorf technique, in a series of murine tumours, human xenografts transplanted into mice, and patient tumours. Their data indicate that the intra- and inter-individual variability in tumour oxygenation is far more pronounced in patients as compared to experimental tumours and that transplanted tumours are considerably more hypoxic than patient tumours. Our lab has observed a similar shift towards higher oxygen values in human cervix tumours (Fyles *et al*, 1998a) as compared to rodent tumours (De Jaeger *et al*, 1998; Kavanagh *et al*, 1999a). The reason for this difference between patient and rodent tumours is not clear. Possible explanations are differences in tumour growth rate and induction of angiogenesis, site of transplantation (all experimental tumours were transplanted s.c. or i.d. while patient tumours grow 'orthotopically'), or host factors.

The observation that there is considerable heterogeneity in tumour oxygenation has at least two important consequences:

1) The validity of methods of measuring tumour oxygenation as a predictive outcome assay will depend on their ability to demonstrate that intra-tumour heterogeneity is less than inter-tumour heterogeneity (Brizel *et al*, 1995).

2) The effect of tumour-to-tumour variability in studies performed on groups of animals whereby results of individual animals are pooled will be masked by the pooling process (Rockwell *et al*, 1984; Evans *et al*, 1997) underlining the importance of making measurements of tumour oxygenation in individual tumours.

1.4 Effects of hypoxia on tumour behaviour

Currently, the impact of hypoxia on tumour behaviour is thought to be three-fold. First, it is well documented that the presence of hypoxia increases the resistance to ionising irradiation. Secondly, fluctuations in oxygenation may drive tumour progression. Finally, clinical studies have reported that hypoxia might impact on the ability of tumours to form metastases.

1.4.1 Hypoxia and resistance to radiotherapy

It is an axiom in radiobiology that hypoxic cells are relatively resistant to sparsely ionising irradiation (Bristow and Hill, 1998). To achieve the same proportion of cell kill, approximately three times the radiation dose is required for hypoxic cells compared to the dose required for well-oxygenated cells. This ratio of doses for a given level of cell kill under anoxic versus oxic conditions is known as the oxygen enhancement ratio (OER) and is a measure of the amount by which oxygen will sensitise the cells. Maximal radiosensitization is generally believed to occur at oxygen tensions above about 20 mm Hg while for half-maximum radiosensitization (K_m value) oxygen concentrations of approximately 3-10 mm Hg are required. The mechanism believed to be responsible for oxygen-mediated radiation cell killing is described by the oxygen competition model (Hall, 1994). Ionising irradiation induces the formation of DNA radicals by either direct ionisation or indirectly by reaction with hydroxyl radicals produced from radiolysis of water. Due to its high electron affinity, oxygen will react with these DNA radicals to produce organic peroxides resulting in fixation of the damage. The reactions of oxygen with DNA radicals occur in competition with reducing species such as thiols (-SH) that can chemically repair

the DNA radicals by hydrogen donation. Thus in the absence of oxygen, there is less fixation of radiation-induced DNA damage and cell survival increases (Steel, 1993; Hall, 1994; Bristow and Hill, 1998).

Direct evidence supporting the hypothesis that hypoxia induces resistance to ionising irradiation has been obtained from survival assays of experimental tumours (Bristow *et al*, 1998). It has been more difficult in human tumours to establish the presence of hypoxia and to link it to decreased radiocurability. Here, indirect evidence that hypoxia compromises response to radiotherapy has come from the observation that anaemia adversely influences radiotherapy outcome (Bush *et al*, 1978; Bush, 1986; Dische, 1991). In addition, indirect evidence has resulted from clinical trials using hyperbaric oxygen (Henk *et al*, 1977; Henk, 1986) and hypoxic cell radiosensitisers like nimorazole (Overgaard and Horsman, 1996; Overgaard *et al*, 1998) where a therapeutic gain of strategies that selectively counteract hypoxia was reported.

Since the beginning of the 1990s, the advent of the Eppendorf pO_2 Histograph, has made direct measurements of tumour oxygenation *in vivo* possible. To date, several investigators have performed oxygen measurements with the Eppendorf needle probes in animals (Lartigau *et al*, 1992b; Nordsmark *et al*, 1995; Kavanagh *et al*, 1996; De Jaeger *et al*, 1998; Kavanagh *et al*, 1999a; Adam *et al*, 1999; Aquino-Parsons *et al*, 1999) and patients (Gatenby *et al*, 1988; Lartigau *et al*, 1993; Höckel *et al*, 1994; Brizel *et al*, 1995; Nordsmark *et al*, 1997; Fyles *et al*, 1998a; Sundfør *et al*, 1998). Studies in a number of accessible tumour sites like head and neck, cervix cancer and soft tissue sarcoma of the limbs have suggested that low oxygen levels in tumours prior to treatment correlate with poor local control and outcome following radiotherapy. In particular, Höckel *et al* (1996) have reported on a group of 81 patients with cervix cancer for which pre-treatment oxygenation, as measured with the Eppendorf pO_2 Histograph, was the strongest

independent prognostic factor followed by tumour stage in a multivariate analysis. The predictive value of hypoxia in cervix cancer patients treated by radiotherapy has been confirmed recently in a similar study conducted at the Princess Margaret Hospital (Fyles *et al*, 1998a). In figure 1.4 the disease-free survival (DFS) is plotted for 74 patients irradiated for cervical cancer according to the tumour oxygenation status prior to treatment initiation. For patients with hypoxic tumours, defined as tumours with percentage of pO_2 readings < 5 mm Hg > 50%, DFS was significantly worse (p = 0.02) as compared to DFS of patients with better-oxygenated tumours.

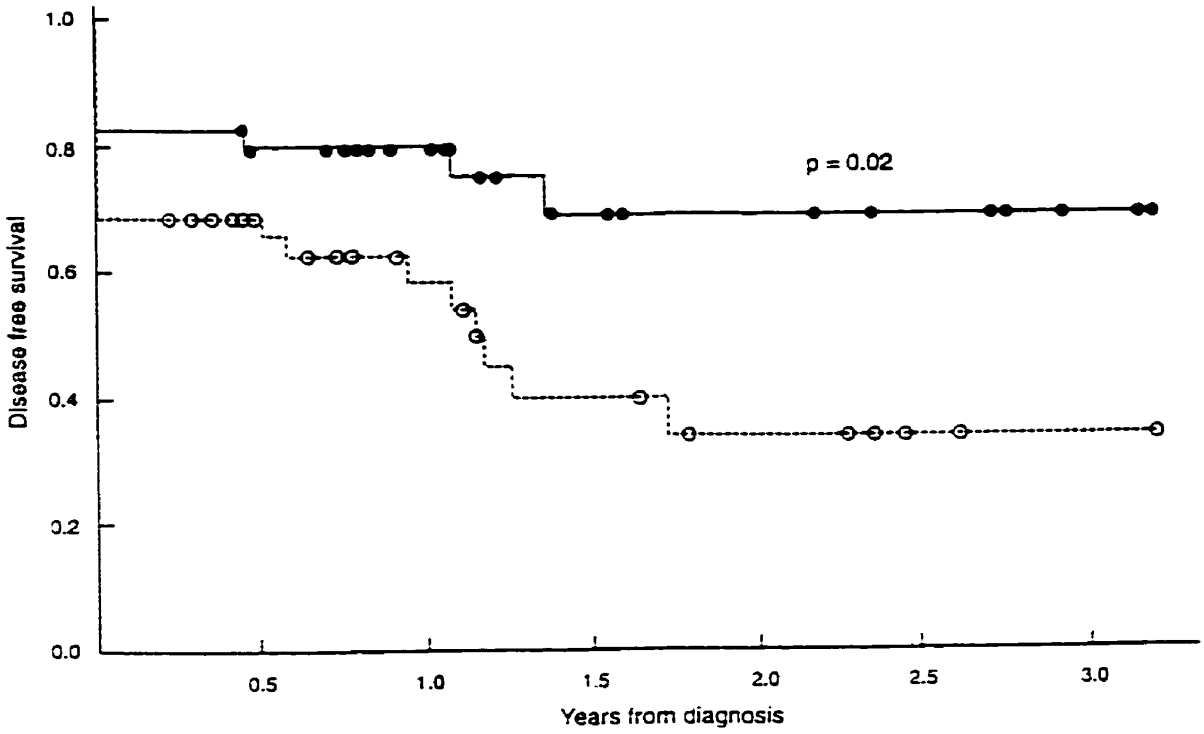

Figure 1.4: DFS as a function of hypoxic proportion (reprinted with permission from Fyles *et al*, 1998a)

1.4.2 Hypoxia and resistance to chemotherapy

The presence of chronic hypoxia may also affect chemotherapeutic drug action (Sartorelli, 1988; Sakata *et al*, 1991; Grau and Overgaard, 1992; McSheehy *et al*, 1998). Limited drug supply because of poor vascularization and/or local blood flow can lead to chemotherapy failure (Tannock, 1986). Moreover, most therapeutic drugs target specifically proliferating cells hence they will be less effective under hypoxia as cells exhibit decreased proliferation when the oxygen concentration drops to low levels (Bedford and Mitchell, 1974; Brown, 1990).

1.4.3 Hypoxia, malignant progression and metastasis

1.4.3.1 Clinical evidence

Several studies on direct measurements of oxygenation in human tumours using the Eppendorf technique have now provided evidence that pre-treatment hypoxia *in vivo* compromises locoregional tumour control and survival after radiation treatment (Gatenby *et al*, 1988; Höckel *et al*, 1993; Brizel *et al*, 1996; Höckel *et al*, 1996; Nordsmark *et al*, 1996; Brizel *et al*, 1997; Fyles *et al*, 1998a; Sundfør *et al*, 1998). Höckel *et al* (1996) also measured pre-treatment oxygenation in groups of patients that underwent either surgery or radiotherapy and found that patients with hypoxic cervix cancers have a poorer prognosis, because they are more likely to present locally aggressive disease and to develop distant metastasis, irrespective whether their initial treatment modality was radiotherapy or surgery. This increased propensity of hypoxic tumours to metastasize was also suggested in studies on head and neck cancer (Gatenby *et al*, 1988; Brizel *et al*, 1997) and soft tissue sarcoma (Brizel *et al*, 1996). Preliminary data from the clinical study of

oxygenation of cervical cancer ongoing at the Princess Margaret Hospital is consistent with this hypothesis. In a group of 81 patients treated a trend was found towards increased nodal metastases in patients with more hypoxic tumours (Fyles, 1998b). Similarly, patients with relatively better-oxygenated tumours were more likely to present with a negative nodal status (Fisher exact test, $p = 0.10$).

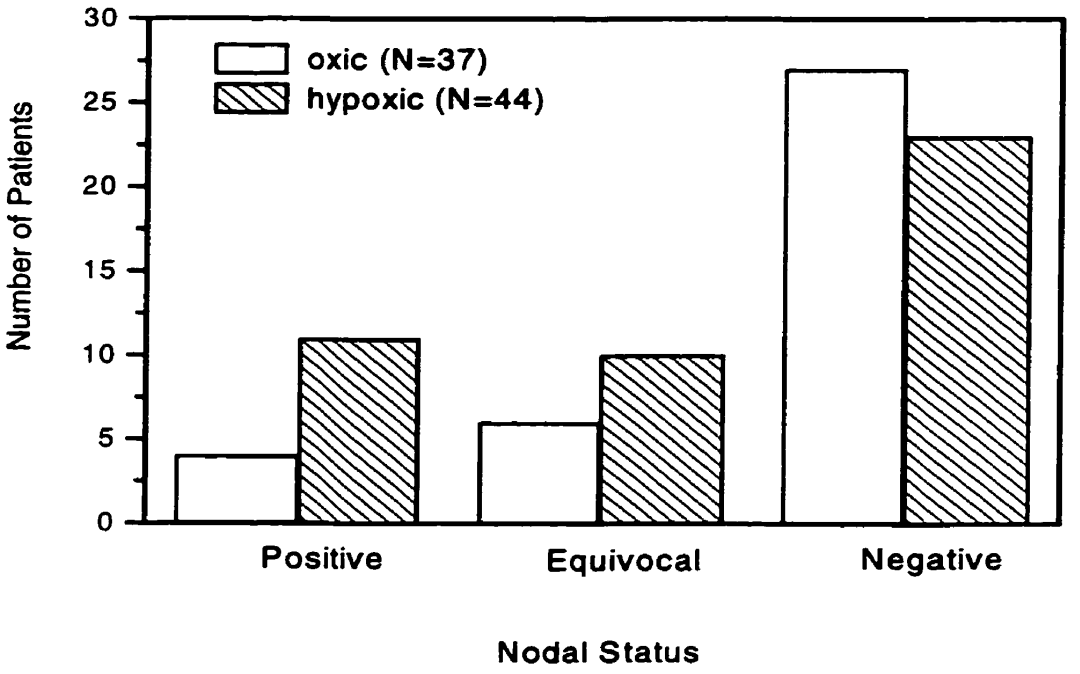

Figure 1.5: Nodal status as a function of tumour oxygenation in 81 patients irradiated for cervix cancer (Fyles, 1998b).

These clinical studies have led to a change in thinking about hypoxia, not only is it a mediator of radioresistance but it may also act as a potential marker of more aggressive disease. In fact, these studies have shed a new light on earlier *in vitro* research in our lab exploring the impact of hypoxic exposure on the behaviour of tumour cells.

1.4.3.2 Experimental data

Young *et al* (1988) were the first to demonstrate that hypoxia could modify the metastatic ability of tumour cells. They incubated rodent tumour cells (KHT-C, B16 and SCC-VII) under hypoxia *in vitro* and found enhanced pulmonary metastasis formation when cells were reoxygenated and injected intravenously back into recipient mice. They demonstrated that the increased metastatic potential of the cells depended on the length of exposure to hypoxia and the length of the reoxygenation period. Recently, similar findings in human melanoma cell lines have been reported (Rofstad and Danielsen, 1999).

There is growing evidence that hypoxia may have a short-term effect on the metastatic potential of cells, through its ability to alter the expression of specific genes. Rofstad and Danielson (1999) have demonstrated that an angiogenesis factor, vascular endothelial growth factor (VEGF) is such a candidate gene that may be implicated in enhanced metastatic efficiency following hypoxic exposure. Our lab has shown hypoxia-induced changes in mRNA levels of various genes including VEGF (Jang and Hill, 1997; Chiarotto and Hill, 1999) but could not show parallel changes in metastatic potential (Jang and Hill, 1997). Numerous other genes and gene products are affected by exposure of the cells to hypoxia and may be involved in this process. These include various transcription factors (HIF-1[2], AP-1[3], NF-κB[4] p53), angiogenic and other growth factors (VEGF, ANG[5], PDGF[6]), invasive and metabolic enzymes (Stoler *et al*, 1992; Dachs and Stratford, 1996; Jang and Hill, 1997; Rofstad and Danielson, 1998; Sutherland, 1998; Dachs and Chaplin, 1998; Graham *et al*, 1999; Hartmann *et al*, 1999). Although the up-regulation of key genes

[2] Hypoxia-inducible factor-1
[3] Activator protein-1
[4] Nuclear factor-κB
[5] Angiogenin
[6] Platelet-derived growth factor

such as HIF-1 and VEGF is fairly well understood (Semenza, 1998), the complex network of interactions between the hypoxia-induced genetic alterations still needs to be elucidated.

A number of investigators have postulated that hypoxia may also exhibit a more long-term effect on tumour development by stimulating tumour progression through increased genomic instability. Reynolds *et al* (1996) have shown that hypoxic exposure of mammalian tumour cells that carry a shuttle vector containing a reporter mutation gene gave rise to a 3-4-fold increase in mutation frequency. Graeber *et al* (1996), using transformed mouse embryo fibroblasts growing in SCID mice, have reported that cells with a pre-existing p53 mutation have decreased apoptotic potential and a mixed population of cells may undergo selective pressure resulting in establishment of a population which is primarily p53 mutated and likely to have a more malignant phenotype. Similarly, reduced sensitivity to hypoxia-induced apoptosis was demonstrated in human cervical epithelial cells transfected with HPV16 E6 and E7 genes (Kim *et al*, 1997).

1.5 Rationale for the experiments and outline of thesis

There is substantial evidence from *in vitro* experiments that hypoxic cells participate in the resistance of solid tumours to ionising irradiation and can affect the efficacy of chemotherapy. In addition, molecular investigations have revealed that an hypoxic tumour microenvironment may favour tumour aggressiveness and metastatic potential. Recently, corroborative evidence from clinical studies has emerged, suggesting that patients with hypoxic tumours respond poorly to radiotherapy and are more likely to develop distant metastases as compared to patients with well-oxygenated tumours. Hence, techniques that would accurately measure hypoxia are of significant interest as they might allow us to select patients for which hypoxia-directed treatment strategies could turn into a

therapeutic gain. In the past, the results of strategies that circumvent hypoxia have been of marginal significance. However, in these studies no procedures to determine the oxygenation of individual tumours were available and the presence of hypoxia was rather 'assumed'. In the early 90s, the Eppendorf polarographic pO_2 Histograph was introduced in the clinic, allowing direct measurements of tumour oxygenation *in vivo*. This technique has been widely used and has provided all the clinical data relating to tumour hypoxia and outcome. Along with this correlation, significant heterogeneity in oxygenation of human and rodent tumours was observed, emphasising the importance of making measurements in individual tumours. The goal of the work described in this thesis was two-fold:

In the first data chapter, chapter 2, we investigated whether the presence of hypoxia, as measured with the Eppendorf pO_2 Histograph, relates to increased pulmonary metastasis in individual KHT-C and SCC-VII murine tumours. In an earlier study of our lab (Young *et al*, 1988), a transient enhanced ability to form experimental lung metastases was observed in these two cell lines after exposure of tumour cells to hypoxia *in vitro* and i.v. injection of cells back into recipient mice. The advent of polarographic electrodes, allowing direct measurements of tumour oxygenation, and the clinical data, stimulated us to readdress the question of whether hypoxia affects metastatic ability in a spontaneously metastasizing rodent tumour model. The relationship of hypoxia to metastatic potential in such a model would allow evaluation of the effects of modulating oxygenation *in vivo* on tumour aggressiveness and *in vivo* testing of hypoxia-targeting strategies that could lead to effective treatments.

In the third chapter, the relationship between Eppendorf electrode measurements and binding of the relatively new hypoxic marker EF5 was examined in individual human cervix cancer xenografts. EF5 binding holds great promise for detection of hypoxia on a cell-by-cell basis and can be identified through invasive and non-invasive

techniques. Thus, it would allow measurement of hypoxia in many more tumour sites. The purpose of the study described here was to evaluate whether EF5 binding could serve as a surrogate technique for the Eppendorf pO_2 Histograph, which is currently considered the 'gold standard' for measuring hypoxia *in vivo*, but can only be applied in accessible tumours. The presence of necrosis as a potential explanation for discrepancies in measurement results was also investigated.

The fourth chapter contains a summary and discussion of the results from experiments presented in the data chapters. It concludes with outstanding questions and considerations for future work.

In the appendix chapter, the results of experiments in which we investigated the relationship between tumour oxygenation, as measured with the Eppendorf technique, tumour size and degree of tumour necrosis are described. This chapter also reports preliminary results on the assessment of lung metastasis in the KHT-C model, which has been addressed in detail in chapter 2.

CHAPTER 2

RELATIONSHIP OF HYPOXIA TO METASTATIC ABILITY IN RODENT TUMOURS

Katrien De Jaeger, Mary-Claire Kavanagh and Richard P Hill

This chapter is the text of a paper with similar title and authorship, conditionally accepted for publication in *British Journal of Cancer*

2.1 Summary

The relationship between tumour oxygenation *in vivo* and metastatic potential was investigated in two rodent tumour models, KHT-C fibrosarcoma and SCC-VII squamous cell carcinoma. The oxygen status in these rodent tumours transplanted intramuscularly in syngeneic mice was measured using the Eppendorf pO_2 Histograph. The results indicate a considerable heterogeneity in oxygenation between individual tumours within each tumour cell line. At different tumour sizes, animals were killed and lung lobes were examined for macroscopic and microscopic lung metastases. In the KHT-C tumours, a significant increase in early pulmonary metastasis formation was observed in mice with hypoxic primary tumours. Hypoxic SCC-VII tumours did not give rise to enhanced lung metastasis formation despite oxygenation in a range similar to the KHT-C tumours. However, the overall metastasis incidence in the SCC-VII model was very low. The results obtained in the KHT-C model, which show that hypoxic tumours are more likely to metastasize, are in agreement with recent clinical data suggesting that an hypoxic environment might be implicated in the metastatic ability of human tumours.

2.2 Introduction

It is well-documented that most human and rodent solid tumours contain a significant proportion of hypoxic cells (Rockwell *et al*, 1986; Rockwell and Moulder, 1990). From radiobiology studies, hypoxia is known to render tumour cells resistant to ionising radiation (Bristow and Hill, 1998). Methods to detect hypoxia might allow identification of patients with radioresistant tumours who would benefit from selective, hypoxia-targeting treatment strategies. Presently, the determination of oxygen concentration with polarographic electrodes is the only method of measuring hypoxia that has been extensively studied in patients. Decreased tumour oxygenation, as measured with polarographic electrodes, has been reported to be a predictor of poor local response following radiotherapy in cervix cancer (Höckel *et al*, 1993; Fyles *et al*, 1998a; Sundfør *et al*, 1998) head and neck cancer (Gatenby *et al*, 1988; Nordsmark *et al*, 1996; Brizel *et al*, 1997) and soft tissue sarcoma (Brizel *et al*, 1996). Recently, clinical data have emerged suggesting that hypoxia adversely affects locoregional control of cervical cancer, irrespective whether the initial treatment modality is radiotherapy or surgery (Höckel *et al*, 1996). For soft tissue sarcoma, poorer oxygenation has also been linked to increased likelihood of developing distant metastasis (Brizel *et al*, 1996). Thus, in addition to radioresistance, hypoxia may be implicated in local tumour aggressiveness and distant progression.

Our lab has previously demonstrated in a murine model that metastasis formation by rodent tumour cells can be increased by exposure to hypoxia (Young *et al*, 1988). When murine fibrosarcoma cells were exposed to hypoxia *in vitro* they acquired a transient, enhanced ability to form experimental metastases. We hypothesised that an hypoxic environment induces genomic instability, possibly through gene amplification. Other

investigators have reported hypoxia-mediated increased mutation frequencies in tumours (Reynolds *et al*, 1996) and selection of cells deficient in genes of normal regulatory pathways (Graeber *et al*, 1996; Kim *et al*, 1997). In addition, it is well-known that hypoxic stimuli can alter gene expression by up-regulating specific transcription factors (Dachs and Stratford, 1996; Sutherland, 1998; Iyer *et al*, 1998). There is also evidence that hypoxia can act at a post-transcriptional level by increasing messenger RNA stability (Ikeda *et al*, 1995). Intensive investigations are ongoing to elucidate the complex mechanisms underlying these epigenetic/genetic interactions.

In the previous study by Young *et al* (1988) metastasis formation was examined with rodent tumour cells after exposure to hypoxia *in vitro* and intravenous injection of tumour cells back into the animal. The availability of polarographic electrodes allowing direct measurements of tumour oxygenation stimulated us to re-address the question of whether hypoxia affects distant metastasis formation using a spontaneous metastasis model. In the current study we performed direct measurements of oxygenation in individual primary tumours *in vivo* and investigated their ability to form metastases in the lungs.

2.3 Materials and methods

2.3.1 Mice and tumour cell lines

Experiments were carried out with two murine tumour cell lines, KHT-C fibrosarcoma and SCC-VII squamous cell carcinoma. Their origin has been described previously (Bristow *et al*, 1990). Both cell lines were maintained in the present lab by alternate *in vitro* and *in vivo* passage. *In vitro* passage was done in plastic flasks, growing cells as monolayers in α-minimal essential medium (Gibco BRL, Burlington, Ontario) supplemented with 10% fetal bovine serum (FBS, Wisent, Quebec). Cells were removed

from the monolayer while in exponential growth with 0.05% trypsin for 5 minutes at 37°C. Tumour cells were used for experiments between their 2-4th passage *in vitro* and established in syngeneic 8-12-week-old C3H/HeJ male mice (The Jackson Laboratory, Bar Harbor, Maine). Approximately 2.5 x 10^5 cells, suspended in 30-50 µl growth medium were injected into the left gastrocnemius muscle. Tumour growth was followed by external measurement of the diameter of the tumour bearing leg. All animals were selected for oxygen measurements when this diameter reached a size of 9 ($\pm$ 0.5) mm (corresponding tumour weight 0.3-0.4 g). This generally occurred 8 days after injection. Animals were housed at the Ontario Cancer Institute animal colony and had access to food and water *ad libitum*. Experiments were performed according to the regulations provided by the Canadian Council on Animal Care.

2.3.2 Tumour oxygenation measurements

Direct oxygen measurements were made in individual tumours using a polarographic oxygen electrode (Eppendorf pO_2 Histograph, Kimoc 6650, Hamburg, Germany) as reported previously (Kavanagh *et al*, 1996 and 1999a). Calibrations were performed according to the manufacturer's recommendations. All pO_2 measurements were made approximately 15 minutes after induction of anaesthesia with intraperitoneally injected *Ketalean* (ketamine hydrochloride, 50 mg/kg) (M.T.C. Pharmaceuticals, Cambridge, Ontario) and *Rompun* (xylazine, 5 mg/kg) (Bayvet Division, Chemagro Limited, Etobicoke, Ontario). Anaesthetised mice were positioned on a heating pad. Core temperature was monitored and kept at 37 $\pm$ 2 °C. In each tumour, 8-12 measurements were made along each of 6 parallel tracks resulting in a total of 48-72 pO_2 values per tumour. The pO_2 data for each tumour were corrected for tumour temperature, which was measured at one point similar in position to an Eppendorf track, using a 25 gauge needle

thermocouple probe (Model #2300A, Fluke Electronics Canada Inc., Mississauga, Ontario). Oxygen measurements were performed in a total of 103 KHT-C tumours and 67 SCC-VII tumours at a tumour weight of 0.3-0.4 g. This was done in several experiments. In each experiment a number of mice were randomly allocated for cervical dislocation immediately after pO_2 measurements. Mice that were not sacrificed following oxygen measurements were monitored during recovery and kept under close surveillance until the tumour-bearing leg reached a diameter of 15 ($\pm$ 0.5) mm (corresponding tumour weight 1.6-1.9 g).

2.3.3 Metastasis assessment

As lungs are the primary site of metastasis formation from leg tumours for both KHT-C and SCC-VII cells, the development of lung metastases was assessed.

After oxygen measurements at a tumour weight of 0.3-0.4 g, a total of 86 tumours (40 KHT-C and 46 SCC-VII) randomly selected from the group KHT-C and SCC-VII tumours were grown until the tumour bearing leg had reached a tumour weight of 1.6-1.9 g. At this tumour size, the animals were killed by cervical dislocation, their lungs were removed, briefly washed with distilled water, cleaned of extraneous tissue, fixed in Bouin's solution overnight (BDH Inc., Toronto, Ontario) and stored in buffered formalin 10% (BDH Inc., Toronto, Ontario) until they were counted.

A total of 84 animals (63 bearing KHT-C tumours and 21 SCC-VII) was sacrificed immediately after oxygen measurements at a tumour weight of 0.3-0.4 g. Lungs were similarly fixed in Bouin's solution followed by storage in formalin. In both experiments, the five lung lobes of each animal were coded and examined.

Macroscopically visible metastases were counted using a dissecting microscope. In the absence of macroscopic lung metastases, lung lobes were embedded in paraffin.

Four histological sections at least 20 µm apart were cut through each lobe and stained with hematoxylin and eosin. The rationale for cutting 4 sections is based on work by Thrall *et al* (1997), who showed in tumour biopsies that, for quantification of hypoxic marker labelling, 4 randomly selected sections provide an accurate estimate of the truly labelled area. The presence of microscopic metastasis was evaluated at a 10x magnification using a transmitted light microscope. Lungs were classified as positive if at least one section revealed a micrometastasis. Likewise, lungs were scored as negative in the absence of any micrometastases.

2.3.4 Data evaluation

Hypoxic fractions, defined as the percentage of pO_2 values lower than 5 mm Hg, and median pO_2 values were computed from the histogram, calculated from the pooled needle track readings of each individual tumour, using the pO_2 pool software package (Eppendorf). A Mann-Whitney test was applied to test differences in oxygenation between the KHT-C and SCC-VII tumours (figure 2.1a and 2.1b) and differences in number of macroscopic lung metastases between hypoxic and non-hypoxic KHT-C tumours. A Spearman rank correlation coefficient was calculated for evaluation of the correlation between macroscopic lung metastases and oxygen status in the primary KHT-C tumours (figure 2.2). Pearson's Chi-squared test with Yates correction was applied to compare frequencies in the contingency tables. The level of significance was defined as $p < 0.05$ (two-sided).

2.4 Results

2.4.1 Tumour oxygenation measurements

The results of the oxygen measurements in mouse tumours of 0.3-0.4 g are plotted in figure 2.1a for 103 individual KHT-C tumours and in figure 2.1b for 67 individual SCC-VII tumours. In both figures, the percentage of pO_2 values lower than 5 mm Hg is plotted as a function of the median pO_2. The dashed lines indicate the median value for each parameter. The hypoxic proportion, represented by the percentage of pO_2 values lower than 5 mm Hg ranges from 25.3% to 100% (median 68%) and from 28.6% to 100% (median 72.7%) in KHT-C and SCC-VII respectively. There is no statistically significant difference in median hypoxic proportion between the two tumour cell lines ($p = 0.52$). For both tumour cell lines, a considerable inter-tumour heterogeneity in oxygenation is observed. The spectra of inter-tumour heterogeneity however are similar for both tumour cell types.

2.4.2 Metastasis assessment

2.4.2.1 Macroscopic lung metastasis at tumour weight 1.6-1.9 g.

For KHT-C, the number of macroscopically visible lung metastases as counted using a dissecting microscope is plotted versus the fraction of pO_2 values lower than 5 mm Hg at a tumour weight of 0.3-0.4 g in figure 2.2. This graph is updated from previously reported preliminary results (De Jaeger *et al*, 1998). Although there seems to be a trend suggesting increasing incidence of lung metastases with increasing hypoxic fraction, there is only a weak, non-significant correlation ($r_s = 0.19$, $p = 0.25$). Also, analysis of these data by dividing the tumours at the median value for the hypoxic fraction demonstrated that the

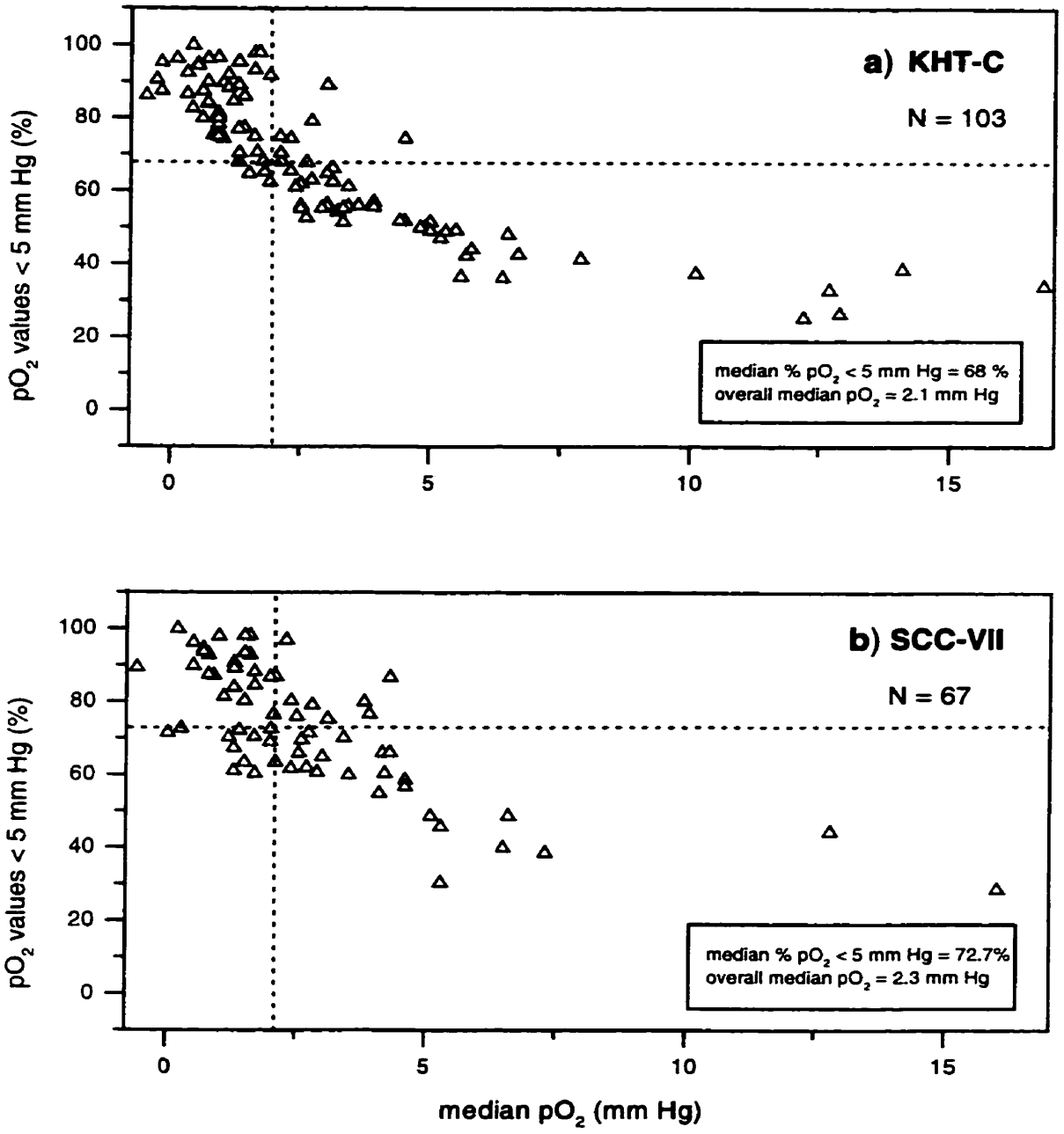

Figure 2.1a and 2.1b:
The percent of pO_2 values less than 5 mm Hg as a function of median pO_2 for a) 103 KHT-C and b) 67 SCC-VII tumours, measured at tumour weight 0.3-0.4 g. Each point represents the measurements from an individual tumour. The dashed lines indicate the overall median value of each paramenter for the group of KHT-C tumours (figure 2.1a) and SCC-VII tumours (figure 2.1b)

number of lung metastases is not significantly different for primary tumours with an oxygenation level above or below the median (median number of lung metastases 18.5 versus 29, p = 0.21). Macroscopic lung metastases were not detected in any of the 46 SCC-VII tumours analysed.

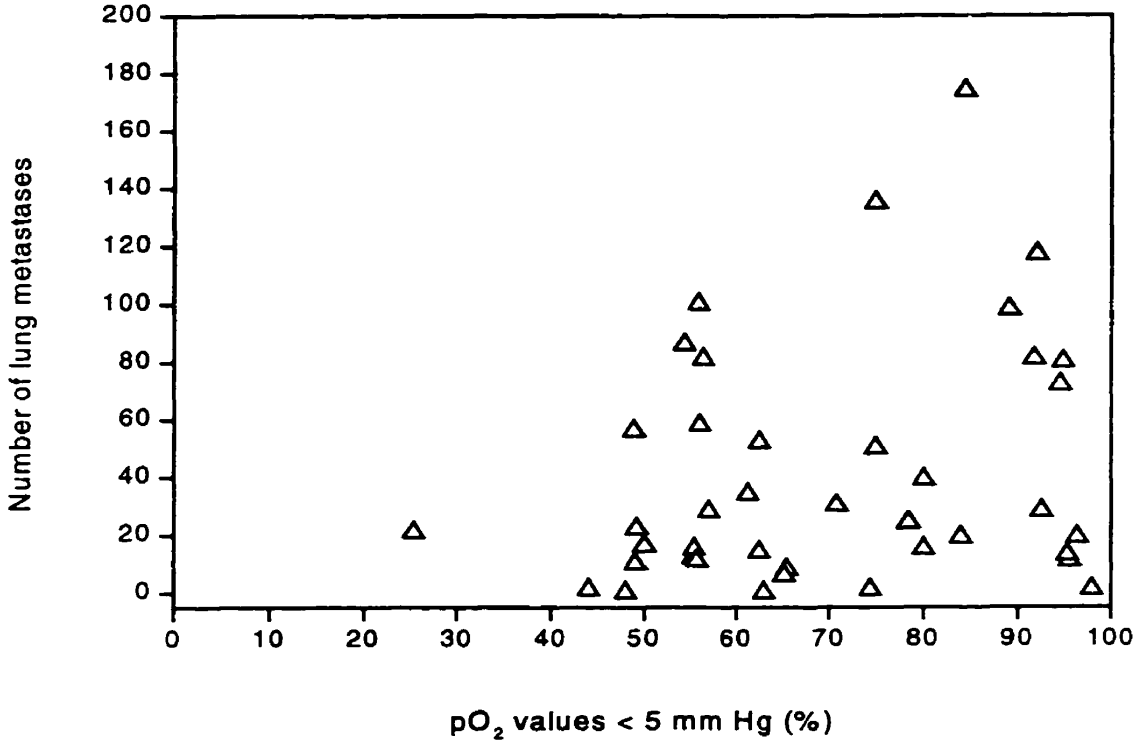

Figure 2.2: The number of macroscopic lung metastases in each of 40 mice bearing KHT-C tumours as a function of the percentage of pO_2 values < 5 mm Hg measured at tumour weight 0.3-0.4 g. The mice were killed for assessment of macroscopic lung metastases when the tumours reached a weight of 1.6-1.9 g.

2.4.2.2 Microscopic lung metastasis at tumour weight 0.3-0.4 g.

Because all but two of the animals with KHT-C tumours examined at a primary tumour weight of 1.6-1.9 g had metastases and many had a large number of metastases, we also examined the extent of metastases at an earlier stage of tumour growth.

Table 2.1 summarises the results of the evaluation of microscopic lung metastasis for KHT-C when the tumour-bearing animals were assessed for hypoxic fraction and then killed at a tumour weight of 0.3-0.4 g. Each individual animal was classified in this 2 x 2 table according to whether the hypoxic fraction in the tumour (at tumour weight 0.3-0.4 g) was above/equal to or below the overall median percentage of values lower than 5 mm Hg, and whether it was positive or negative for lung metastases, based on the evaluation of 4 independent histological sections. In 45 of 63 lungs, at least one microscopic metastasis was present. In 32 of these 45 mice with lung metastases, measurement of oxygen level in the primary revealed a hypoxic proportion above or equal to 68%, representing the overall median percentage of values < 5 mm Hg. Thus, hypoxic tumours seem to metastasize at an earlier stage of growth more frequently as compared to better-oxygenated tumours. Likewise, a higher proportion of negative lungs was observed in mice with relatively better-oxygenated tumours. These proportions are significantly different ($X^2 = 6.178$, $p = 0.0143$).

% pO_2 values < 5 mm Hg	Lung metastasis		Total
	positive	negative	
≥ 68	32	6	38
< 68	13	12	25
Total	45	18	63

Table 2.1: Classification of 63 animals with KHT-C tumours according to % pO_2 values < 5 mm Hg ≥ or < the overall median of 68%, and positive or negative score for microscopic lung metastasis.
Oxygen measurements and lung metastases were both evaluated at tumour weight 0.3-0.4 g.

A similar series of studies was undertaken with SCC-VII tumours. Table 2.2 shows that the incidence of spontaneous metastasis at 0.3-0.4 g is very low in this tumour cell line with detectable metastasis development in only 3/21 animals. The numbers are very small and do not suggest any correlation between oxygenation status and metastasis formation in SCC-VII. Following the observation of low metastatic incidence at tumour weight 0.3-0.4 g in SCC-VII, we also examined the lungs from mice killed at a primary tumour weight 1.6-1.9 g for microscopic metastasis. These results are summarised in table 2.3. Even at a larger tumour size, only 6/46 tumours demonstrated detectable lung metastasis. Again, there was no correlation with the oxygen status of the primary tumour at 0.3-0.4 g.

% pO_2 values < 5 mm Hg	Lung metastasis		Total
	positive	negative	
≥ 72.7	2	8	10
< 72.7	1	10	11
Total	3	18	21

Table 2.2: Classification of 21 animals with SCC-VII tumours according to % pO_2 values < 5 mm Hg ≥ or < the overall median of 72.7%, and positive or negative score for microscopic lung metastasis.
Oxygen measurements and lung metastases were both evaluated at tumour weight 0.3-0.4 g.

% pO_2 values < 5 mm Hg	Lung metastasis positive	negative	Total
≥ 72.7	5	19	24
< 72.7	1	21	22
Total	6	40	46

Table 2.3: Classification of 46 animals with SCC-VII tumours according to % pO_2 values < 5 mm Hg ≥ or < the overall median of 72.7%, and positive or negative score for microscopic lung metastasis.
Oxygen measurements were performed at tumour weight 0.3-0.4 g. Lung metastases were evaluated at tumour weight 1.6-1.9 g.

2.5 Discussion

In the present study the effect of hypoxia *in vivo* on the formation of distant metastases was examined in two murine tumour cell lines. We utilised the Eppendorf Histograph to measure oxygen concentrations because it is currently the only clinically applicable technique whose strong predictive value in terms of radioresistance, tumour progression and metastasis has been extensively documented in patients (Gatenby *et al*, 1988; Höckel *et al*, 1993; Brizel *et al*, 1996; Höckel *et al*, 1996; Nordsmark *et al*, 1996; Brizel *et al*, 1997; Fyles *et al*, 1998a; Sundfør *et al*, 1998).

Data on the relationship between direct measurements of oxygenation in rodent tumours and radiation curability has been reported (Nordsmark *et al*, 1995) but the relationship to metastasis formation has, to our knowledge, not been previously addressed in a murine model.

We observed substantial intra-tumour heterogeneity in oxygenation of KHT-C and SCC-VII tumours. Both tumour types, measured at tumour weight 0.3-0.4 g show variation within an almost identical range (figure 2.1a and 2.1b). This range is in agreement with our preliminary data (De Jaeger *et al*, 1998) and with data obtained in our lab in a different group of KHT-C tumours (Kavanagh *et al*, 1999a). We and others have postulated earlier that variations in pO_2 values, measured in individual tumours from the same cell line, at the same size and transplanted in identical hosts, are likely to be a consequence of differences associated with local tumour growth and stochastic development of vasculature rather than intrinsic genetic differences (Rockwell and Moulder, 1990; De Jaeger *et al*, 1998). Similar heterogeneity in oxygenation, but over a wider range has been reported in human tumours (Gatenby *et al*, 1988; Höckel *et al*, 1993; Brizel *et al*, 1995; Brizel *et al*, 1996; Höckel *et al*, 1996; Nordsmark *et al*, 1996; Brizel *et al*, 1997; Fyles *et al*, 1998a; Sundfør *et al*, 1998).

The SCC-VII and KHT-C tumours were found to be quite dissimilar in terms of metastases formation, despite the comparable oxygenation status *in vivo* of both tumour types. For SCC-VII, the overall incidence of lung metastases was very low. There was no difference whether the lungs were examined at a tumour weight 0.3-0.4 g or 1.6-1.9 g, either for macroscopic or microscopic metastases. Also, there was no correlation with oxygenation status in the primary tumour. However, when pooling the results of tables 2.2 and 2.3 a slight trend for a relationship between metastasis formation and oxygenation status was observed (7/9 mice with lung metastasis had hypoxic primary tumours) but the correlation was not significant.

In the KHT-C model metastases were much more frequent. At a tumour weight 1.6-1.9 g, macroscopic metastases were present in the majority of the lungs with only 2/40 mice not showing macroscopically visible pulmonary metastases. As depicted in figure 2.2

only a weak non-significant correlation was observed between oxygenation of the primary tumour at tumour weight 0.3-0.4 g and the number of lung metastases observed at weight 1.6-1.9 g. We hypothesised that a possible relationship could be obscured by the presence of massive lung metastases when the primary tumours reach a weight of 1.6-1.9 g and by the fact that the tumours become increasingly hypoxic as their weight increases above 0.3g (De Jaeger *et al*, 1998). In a previous study (Hill *et al*, 1986) we showed that KHT-C fibrosarcoma starts seeding metastases into the lungs at a tumour weight of 0.25 g (corresponding leg diameter 7.5-8 mm).

Consequently, we decided to examine whether hypoxia in the primary tumour correlates with metastasis formation in the lungs at an earlier time point (tumour weight 0.3-0.4 g) in the process of seeding. Table 2.1 represents the results for 63 KHT-C tumours and clearly illustrates that hypoxic KHT-C tumours, defined as tumours with a percentage of pO_2 readings < 5 mm Hg above or equal to the median percentage of 68%, gave rise to significantly more positive lungs as compared to better-oxygenated tumours. This result, suggesting that hypoxic KHT tumours are more likely to be metastatic is consistent with the clinical data obtained in head and neck cancer, cervix cancer and soft tissue sarcoma (Brizel *et al*, 1996; Höckel *et al*, 1996). Preliminary analysis from the clinical study of oxygenation of cervix cancer being conducted at the Princess Margaret Hospital (Fyles *et al*, 1998a) has indicated a trend to increased nodal metastases in the patients with more hypoxic tumours (Fyles, 1998b).

There is growing evidence from laboratory studies supporting the clinical observations that the significance of hypoxia in cancer may extend far beyond the traditional scope of radioresistance. Young *et al* (1988) have shown that exposure of murine KHT fibrosarcoma cells to hypoxia *in vitro* results in a transient enhancement of their ability to form lung metastases. They suggested that gene amplification, associated

with DNA overreplication, was responsible for the enhanced metastatic potential. Further studies of the expression of a number of metastasis-related genes following hypoxic exposure did not identify a gene whose altered expression correlated with the increased metastatic potential of the cells, although vascular endothelial growth factor (VEGF) was up-regulated (Jang and Hill, 1997). In similar experiments with human melanoma cells, other groups found that exposure to hypoxia promotes metastasis formation (Rofstad and Danielsen, 1999; Hartmann *et al*, 1999). They demonstrated a correlative up-regulation of the expression of VEGF (Rofstad and Danielsen, 1999; Hartmann *et al*, 1999) and angiogenin (Hartmann *et al*, 1999), both potent angiogenic factors.

Reynolds *et* al (1996) studied the impact of fluctuating hypoxia on the frequency of mutations arising in a shuttle vector carried in a tumorigenic mouse cell line. They detected a 3-4-fold increase in mutation frequency under severe hypoxic conditions. Their results indicate that the environmental conditions within solid tumours can be mutagenic and suggest that hypoxia mediates tumour progression by induction of genetic instability. Graeber and colleagues (Graeber *et al*, 1996; Kim *et al*, 1997) found that hypoxia can mediate the selection of cells deficient in genes of normal regulatory pathways. This group demonstrated that hypoxia provides a selective pressure for cells mutant in the p53 tumour suppressor gene resulting in decreased apoptotic potential and establishment of a more malignant phenotype. Furthermore, it has been well documented that hypoxic stimuli can alter the expression of a myriad of genes, transcription factors, growth factors, cytokines, metabolic and invasive enzymes (Stoler *et al*, 1992; Dachs and Stratford 1996; Jang and Hill, 1997; Dachs and Chaplin, 1998; Sutherland, 1998; Graham *et al*, 1999). Despite tremendous progress in understanding fundamental mechanisms of hypoxia-induced genetic, metabolic and chemical alterations in cells, it still remains unclear how and whether these alterations act in concert. Also, oxygenation is not a binary physiological condition

and the contribution of transient and chronic changes on these interactions remains to be determined. The results in this paper establish the KHT fibrosarcoma as a model system for such studies.

Moreover, it should be pointed out that hypoxia is not the only tumour microenvironmental condition affecting tumour progression. Other factors, such as pH and low glucose may play a role (Schlappack *et al*, 1991). Also, hypoxia *per se* does not necessarily imply metastatic ability. This is clearly illustrated in the SCC-VII model where, despite severe hypoxia in the primary tumours, cells fail to metastasize. Limitations related to the model, such as heterotopic implantation could have contributed to the metastatic inefficiency of SCC-VII cells. It is well known from the work of Fidler (1990) that orthotopic models give rise to higher metastatic rates. Therefore, the i.m. transplanted KHT model is likely to be a more relevant representative of natural tumour behaviour than i.m. transplanted SCC-VII tumours.

In summary, this is the first report investigating the relationship between direct measurements of tumour oxygenation *in vivo* and metastatic behaviour of rodent tumours. In the KHT-C model, early metastasic ability was found to be enhanced in hypoxic tumours. The present results are consistent with previous clinical and laboratory findings indicating that hypoxia may contribute to malignant progression. The availability of this model allows *in vivo* testing of hypoxia-directed strategies leading to potentially effective treatment. However, as demonstrated in the SCC-VII model, there are factors other than hypoxia which affect the metastatic ability of tumour cells.

CHAPTER 3

RELATIONSHIP BETWEEN pO_2 MEASUREMENTS AND EF5 BINDING IN HUMAN CERVICAL CANCER XENOGRAFTS

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A paper describing the results presented here with similar title and authorship will be submitted to *Clinical Cancer Research*

3.1 Abstract

The relationship between two methods of assessing tumour oxygenation, namely oxygen electrode measurements and binding of the hypoxic cell marker EF5 was investigated in two human cervical cancer xenograft models, Me180 and HeLa. All measurements were made in individual tumours. Oxygen electrode measurements were performed using the Eppendorf pO_2 Histograph. EF5 binding was assessed by semi-quantitative image analysis of immunostained sections. The results show considerable heterogeneity in tumour oxygenation, as assessed by the oxygen electrode technique and EF5 labelling, within as well as between individual tumours. A significant correlation between the two techniques was observed in Me180 tumours, but not in HeLa tumours. Possible explanations for the disparate results such as the presence of histopathological characteristics are discussed.

3.2 Introduction

It has been recognised for many years that the presence of hypoxia is implicated in the resistance of solid tumours to ionising irradiation and chemotherapy (Gray *et al*, 1953; Moulder and Rockwell, 1987; Sartorelli, 1988; Bristow and Hill, 1998). Several investigators have shown in a number of easily accessible tumour sites that decreased tumour oxygenation, as measured with the Eppendorf pO_2 Histograph, is correlated with poor clinical outcome after radiotherapy (Höckel *et al*, 1993; Nordsmark *et al*, 1996; Brizel *et al*, 1997; Nordsmark *et al*, 1997; Fyles *et al*, 1998a). Recently, clinical data has emerged suggesting that hypoxia adversely affects prognosis, independent of whether the initial treatment modality was radiotherapy or surgery (Höckel *et al*, 1996). In addition, it has been reported that patients with hypoxic sarcoma (Brizel *et al*, 1996) and cervix cancer (Höckel *et al*, 1996; Fyles, 1998b; Sundfør *et al*, 1998) have a poor prognosis because they are more likely to present locally aggressive disease and to develop distant metastasis. Based on these observations, it has been postulated that hypoxia implies more than just radioresistance and might serve as a marker for aggressive disease.

In parallel to the studies in patients, many laboratories have started investigating hypoxia-induced molecular changes in an attempt to elucidate the potential link between tumour hypoxia and a more malignant phenotype. There is growing evidence from *in vitro* experiments that hypoxic stimuli lead to alterations in the expression of numerous genes, transcription factors, cytokines, growth factors, enzymes and stress proteins (Stoler *et al*, 1992; Dachs and Stratford, 1996; Cuvier *et al*, 1997; Sutherland, 1998; Dachs and Chaplin, 1998; Graham *et al*, 1999; Hartmann *et al*, 1999). Probing of hypoxia and co-localisation with detectable molecular changes could contribute to

understanding the mechanisms underlying the development of hypoxia-induced tumour aggressiveness.

Several methods are currently available to measure hypoxia in tumours (Stone *et al*, 1993; Raleigh *et al*, 1996; Horsman *et al*, 1998). Binding of 2-nitroimidazoles, such as pimonidazole (Arteel *et al*, 1995; Varia *et al*, 1998) and EF5, a pentafluorinated derivative of etanidazole (Lord *et al*, 1993) is unique in that it provides information on oxygenation of individual cells. Monoclonal antibody detection of 2-nitroimidazole adducts can be done on tumour sections using immunohistochemical techniques (Koch *et al*, 1995; Kennedy *et al*, 1997; Varia *et al*, 1998). Recently ^{18}F-EF5 and its analogue ^{18}F-EF1 (Hustinx *et al*, 1999) have been successfully synthesised allowing detection of drug-adduct distribution by positron emission tomography imaging non-invasively but with loss of cellular resolution. The use of EF5 has now been extensively tested in animal tumour models (Lord *et al*, 1993; Evans *et al*, 1995; Koch *et al*, 1995; Lee *et al*, 1996; Laughlin *et al*, 1996; Siim *et al*, 1997; Evans *et al*, 1997; Koch *et al*, 1998; Kavanagh *et al*, 1999a) and studies have indicated stable binding rates and absence of toxicity to normal tissue. Studies on the use of pimonidazole as a hypoxia marker in patients have been reported (Kennedy *et al*, 1997; Varia *et al*, 1998) and recently testing of EF5 has started in a clinical phase I trial (Evans *et al*, 1999).

The value of hypoxia as a prognostic factor for radiation response and outcome is, however, based on data obtained with the Eppendorf pO_2 Histograph which is considered to be the 'gold standard' for measuring hypoxia in patients. This is an invasive technique which is only applicable in accessible tumour sites. Moreover, the needle probe measures an average oxygen level in small tumour volumes rather than in individual cells and has a low signal-to-noise ratio. Binding of EF5, like other 2-nitroimidazoles, provides detection of decreased oxygenation on a single cell basis. It holds great promise for clinical

application. Raleigh *et al* (1999) reported recently on a good correlation between pimonidazole binding and Eppendorf pO_2 measurements in groups of C3H mammary tumours when oxygenation was deliberately modified.

In the present study we addressed the question of whether direct measurements of tumour oxygenation using the Eppendorf pO_2 Histograph relate to EF5 binding. All comparisons were made in individual human cervical cancer xenografts. The rationale for this approach is based on earlier observations that there is a considerable amount of tumour-to-tumour heterogeneity in rodent (Rockwell *et al*, 1984; Rockwell and Moulder, 1990; Kavanagh *et al*, 1996 and 1999a; Evans *et al*, 1996; De Jaeger *et al*, 1998; Adam *et al*, 1999) as well as in human tumours (Brizel *et al*, 1994; Nordsmark *et al*, 1994; Brizel *et al*, 1995; Rasey *et al*, 1996; Olive *et al*, 1996; Wong *et al*, 1997; Adam *et al*, 1999). Comparisons of groups of tumours will average out this heterogeneity and may not reflect individual tumour differences which could occur in a clinical setting.

3.3 Materials and methods

3.3.1 Animals and tumour cell lines

The tumour cell lines used for the experiments were HeLa and Me180. Both are established cell lines derived from human cancer of the uterine cervix. The Me180 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). HeLa cells were kindly provided by Dr. M. Rauth, Ontario Cancer Institute. The cells were maintained as monolayer cultures in α-minimal essential medium (α-MEM) (Gibco BRL, Burlington, Ontario) supplemented with 10% fetal bovine serum (FBS) (FBS, Wisent, Quebec) in a humidified 5% carbon dioxide/air incubator at 37°C. Cells were grown to about 70%

confluence, then trypsinized and resuspended in α-MEM plus 10% FBS. The cells were alternately passaged *in vivo* and *in vitro* for a minimum of 3-times prior to the initiation of these studies. Cells used to initiate tumours were no more than 2-4 passages *in vitro* from an *in vivo* passage. Xenografts were established in 8-10-week-old inbred male severe combined immunodeficient (SCID) mice. Tumours were initiated by injection of $1x10^6$ cells in 30-50 µl growth medium into the left gastrocnemius muscle. Animals were housed at the Ontario Cancer Institute animal colony in a humidified and aseptic environment and had free access to food and water. All experiments were performed according to the regulations provided by the Canadian Council on Animal Care.

3.3.2 EF5 binding

Animals were selected for the experiments when the combined leg plus tumour diameter reached 12 ± 0.5 mm. EF5 was administered according to the protocol outlined by Koch *et al* (1995). The tumour-bearing SCID mice were given two injections each of 10 mM EF5 (100 mg/kg) dissolved in 0.9% saline. The first dose was given via the tail vein and this was followed by a second injection intraperitoneally 15 minutes later.

3.3.3 pO_2 measurements

Three and one half hours after EF5 administration direct tumour oxygen measurements were performed using a polarographic oxygen needle electrode (Eppendorf pO_2 Histograph, Kimoc 6650, Hamburg, Germany). All measurements were made in anaesthetised mice. *Innovar-Vet* (fentanyl-droperidol, 0.1mg/kg) (Janssen Pharmaceutica, Mississauga, Ontario) was injected intraperitoneally. Approximately five minutes later,

Ketalean (ketamine hydrochloride, 50mg/kg) (M.T.C. Pharmaceuticals, Cambridge, Ontario) was administered i.m. Anaesthetised mice were positioned on a heating pad. Core temperature was monitored and kept at 37 $\pm$ 2 °C. pO_2 measurements were made as reported previously (Kavanagh *et al*, 1996 and 1999a). Briefly, the Eppendorf needle probe was inserted a minimum of 2 mm in the tissue through a hole, created in the mouse skin using a 20 gauge needle. The needle probe, driven by a stepping motor, was then automatically advanced and measurements of oxygen concentration were taken at 0.7 mm steps. On average, 8-14 measurements were made along each of 6-8 parallel tracks. Before and after pO_2 measurements in each individual tumour-bearing animal, the machine was calibrated using air and nitrogen, according to the manufacturer's instructions. All pO_2 data was corrected for tumour temperature, which was measured at one point similar to an Eppendorf track, using a 25 gauge needle thermocouple (Model #2300A, Fluke Electronics Canada Inc., Mississauga, Ontario). After the oxygen measurements, the tumours were rapidly excised from live anaesthetised mice. Half of the tumour was embedded in 0.5 ml Tissue-Tek®OCT Compound (Sakura Finetek USA Inc., Torrance, CA) containing vials, snap frozen and stored at -80 °C until sectioning for immunohistochemical analysis. The other half was processed into a single cell suspension for flow cytometry analysis as described previously (Kavanagh *et al*, 1999a,b). Immediately after dissection of the tumour, the mice were killed.

3.3.4 Immunohistochemical detection of EF5 adducts

The frozen tumour halves were cut to provide twelve 4 µm-thick cryostat sections approximately 200 µm apart. Two sections (first and last) were stained with hematoxylin and eosin as controls to confirm the presence of tumour. The tumour sections

were air dried, fixed for 10 minutes in 3.7% paraformaldehyde, and rinsed three times in Dulbecco's phosphate buffered saline (PBS). Endogenous peroxidases were quenched using 0.3% hydrogen peroxide for 10 minutes. After triple rinsing with PBS, sections were blocked for 20 minutes with 20% skim milk, 5% normal mouse serum in PBS with 1.5% albumin and 0.3% Tween 20. Following the blocking, immunohistochemical staining for EF5 adducts was done using the Level 2 Multispecies Ultra Streptavidin Detection System HRP kit, by Signet Laboratories, Dedham, MA. Sections were incubated for one hour with a biotinylated monoclonal antibody ELK3-51 (2mg/ml, diluted 1:30 in PBS) which binds to EF5 adducts in the cell. After rinsing, the kit link and labelling reagents were used according to the manufacturer's instructions. Sections were then treated at room temperature with AEC (3-amino-9-ethylcarbazole) chromogen for 5 minutes, counterstained 45 seconds with Mayer's hemalum solution (BDH, Poole, UK) and rinsed with deionised water. Finally slides were sealed with Crystal Mount (Biomeda, Foster City, CA). Most, but not all the sections were suitable for analysis. On average the number of quality sections available per tumour was 8.4 (range 5-10) for HeLa and 8.7 (range 3-11) for Me180.

3.3.5 Image analysis

The sections stained for EF5 adducts were analysed semi-quantitatively, using a MicroComputer Image Device (MCID; Imaging Research Inc., St. Catharine's, Ontario) linked to a colour CCD camera (Sony DXC 970 MD) mounted on a transmitted-light microscope (Zeiss Axioskop) fitted with a Ludl Biopoint motorised stage. Using a 10x objective an automated mini program produced a microscopic, field-by-field, digitised, tiled image of the entire tumour section. On average 80 fields were digitised for each section. All tumour image files were coded and examined in a blind fashion. On each section the

tumour area was delineated using drawing tools. Acellular spaces or artefacts and areas of necrosis were included in the total tumour area, but also outlined separately. Semi-automatic thresholding according to colour, hue and intensity was performed on zoomed areas of intense red AEC chromogen staining. For each section, thresholding was inspected visually and settings were adapted if necessary. The proportion of EF5 staining was computed as a percentage of the total tumour area minus acellular spaces and potential artefacts. For comparison of percentage EF5 staining with pO_2 Eppendorf measurements, no correction for necrosis was made. To evaluate reproducibility of this semi-quantitative analysis consecutive threshold settings were performed on a number of randomly selected sections by the same observer with 6 months interval and by 2 different observers independently.

3.3.6 Data evaluation

Hypoxic fractions, defined as the percentage of pO_2 values less than 5 mm Hg were calculated from the frequency histograms of the pooled pO_2 measurements of each individual tumour, using the pO_2 pool software package (Eppendorf). Spearman rank correlation coefficients were computed to examine the correlation between the percentage of EF5-adduct staining on sections and the percentage of pO_2 values less than 5 mm Hg. Analysis of variance was used to assess the significance of the differences among observers. The intra-tumour and inter-tumour components of variance were estimated using the Restricted Maximum Likelihood (REML) Method (Searle *et al*, 1992). To determine the number of sections needed to obtain reliable results, we considered that in our hospital the clinical use of the hypoxic measurements is to classify patients into one of two equal sized groups depending on whether the measurement exceeds a threshold (Fyles *et al*, 1998a).

Increasing the number of sections results in a more precise estimation of the average hypoxic proportion in a tumour, thus increasing the probability that the tumour would be correctly classified as having a high or low average hypoxic proportion. We estimated the probability of misclassification as a function of number of sections, by assuming that each cell line produces tumours whose average hypoxic proportion can be characterized by a Gaussian distribution and that for each tumour, the hypoxia measurements in each section have another Gaussian distribution.

3.4 Results

Fields of digitised tumour sections, immunohistochemically stained for EF5 adducts are represented in figure 3.1a and figure 3.1b. Figure 3.1a shows a poorly differentiated HeLa carcinoma, consisting of solid confluent sheets of neoplastic cells. HeLa tumours typically present central areas of necrosis delineated by rims of EF5 positive cells. The empty spaces in the necrotic areas are a mix of tissue loss and some freezing artefact that occurred during sample processing. Figure 3.1b is a representative image of a Me180 tumour section. The tumour is a well-differentiated squamous cell carcinoma. The keratin-producing tumour cells are well organised in nests and sheets which are surrounded by desmoplastic stroma. There was no obvious necrosis in Me180 tumours within the size range examined. Some regions of keratinization show positivity for EF5.

A computer-aided image analysis system was used to calculate the proportion of tumour areas immunohistochemically labelled for EF5 adducts. This procedure is not entirely automated and operators need to establish threshold values on the AEC chromogen for each section. To evaluate the inter- and intra-observer reproducibility of this method, EF5-adduct binding was assessed in 4 randomly selected HeLa tumours. For each tumour, 5 sections were individually thresholded twice by the same observer, with an

interval of 6 months (observations 1A and 1B), and once by a second observer (observation 2). The results are compiled in figure 3.2. Table 3.1 displays the mean EF5-adduct labelling resulting from the evaluation of 5 sections per tumour, for 4 different HeLa tumours as assessed in the 3 different observations. In general, there was good agreement between the 3 sets of observations. Statistical analysis showed no obvious bias between the 3 sets of observations. However, the variance between the 3 sets of data on the same section was similar in magnitude to the variance between different sections of the same tumour.

Figure 3.3 shows the percentage of the tumour area binding EF5, for a series of 10 sections taken from each of 4 Me180 and 4 HeLa tumours. The different panels illustrate that there is considerable heterogeneity in EF5 staining between the various sections from any one tumour. However, the heterogeneity in EF5-adduct binding between the different tumours is clearly greater than the heterogeneity between the different sections from the same tumour. For Me180 and HeLa tumours, the percentages of the total variance due to differences between the tumours are 83% and 75% respectively.

The percentage of EF5-adduct staining versus the percentage of pO_2 values < 5 mm Hg as measured with the Eppendorf, is plotted in figure 3.4a for 15 individual Me180 tumours and in figure 3.4b for 11 individual HeLa tumours. The solid squares represent the mean percentage ($\pm$se) of EF5 staining for each tumour, which was calculated from the percentage of EF5 staining on each of the sections that were available for analysis. For Me180 tumours a strong correlation ($r_s = 0.889$, $p < 0.002$) was observed between the hypoxic fraction as computed from Eppendorf measurements and the hypoxic fraction as determined by the mean percentage of EF5 binding on tumour sections. However, as seen in figure 3.4a, the Eppendorf measurements seem to consistently overestimate the hypoxic fraction, as determined by EF5 binding.

For the HeLa tumours there was no correlation between percentage of EF5

staining and percentage of pO_2 values < 5 mm Hg (r_s = -0.164, p = 0.631). In general, HeLa tumours tend to be less hypoxic as compared to Me180 tumours. This is also illustrated in figure 3.5a and 3.5b where the mean percent of EF5 labelling and the mean percent of necrosis are graphed for Me180 and HeLa tumours respectively. HeLa tumours clearly contain more necrotic areas and in all but three tumours there was detectable necrosis, whereas only one Me180 tumour contained a necrotic area (figure 3.5a and 3.5b).

HeLa Tumour #	Mean EF5-adduct labelling (%) (± sd)		
	Observation 1A	Observation 1B	Observation 2
1	18.5 (±4.1)	17.2 (±3.1)	15.8 (±1.2)
2	14.3 (±2.6)	13.1 (±3.2)	15.1 (±4.1)
3	6.5 (±3.8)	8.5 (±1.7)	7.1 (±2.8)
4	18.0 (±3.3)	19.5 (±5.0)	25.9 (±4.8)

Tabel 3.1: Inter- and intra-observer variability in the assessment of EF5 labelling. For each HeLa tumour the mean % of EF5-adduct labelling is calculated from the % EF5 labelling on 5 different sections. The analysis was performed in 3 different observations (1A, 1B, 2).

Figure 3.1: **Fields of digitised sections showing immunostaining for EF5 binding in a HeLa tumour (panel a) and a Me180 tumour (panel b)**

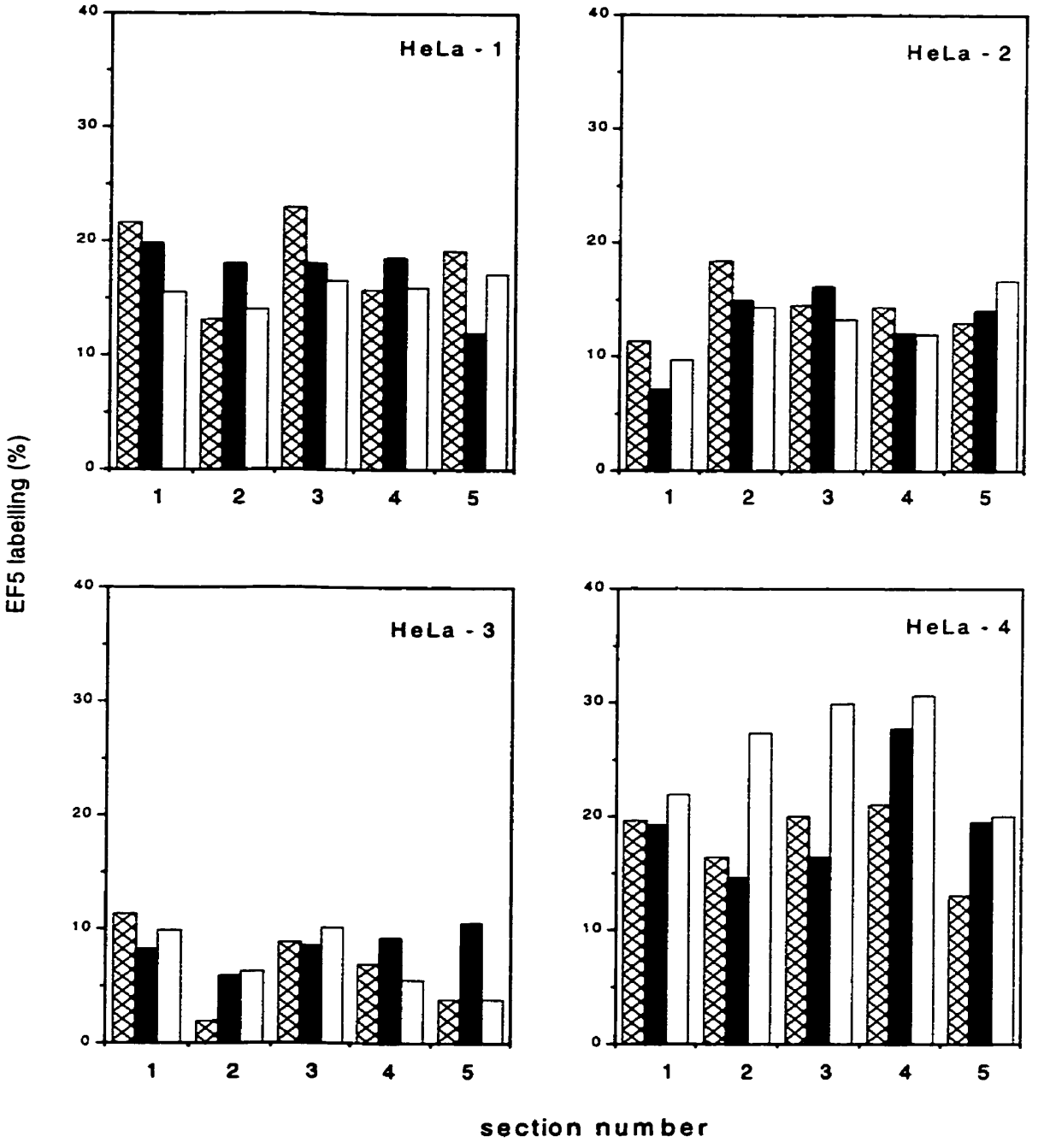

Figure 3.2: Inter- and intra-observer variability in the quantification of EF5-adduct binding in 4 HeLa tumours. For each tumour, 5 sections were analysed in 3 different observations: observation 1A (hatched bars), observation 1B (black bars) and observation 2 (open bars).

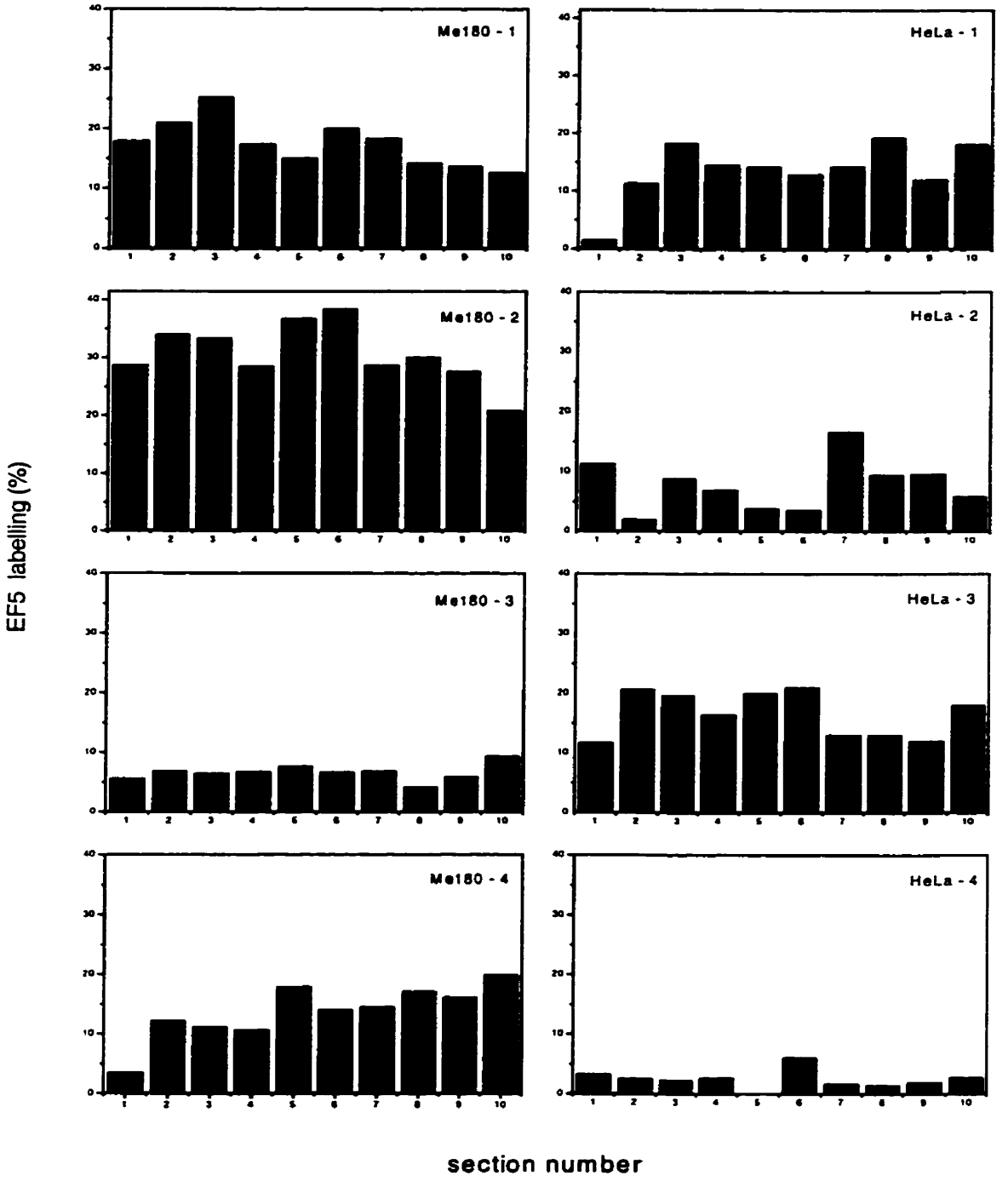

Figure 3.3: Inter- and intra-tumour heterogeneity in EF5-adduct labelling in 4 Me180 and 4 HeLa tumours. Each panel shows the percent EF5 labelling for a series of 10 sections taken from each tumour.

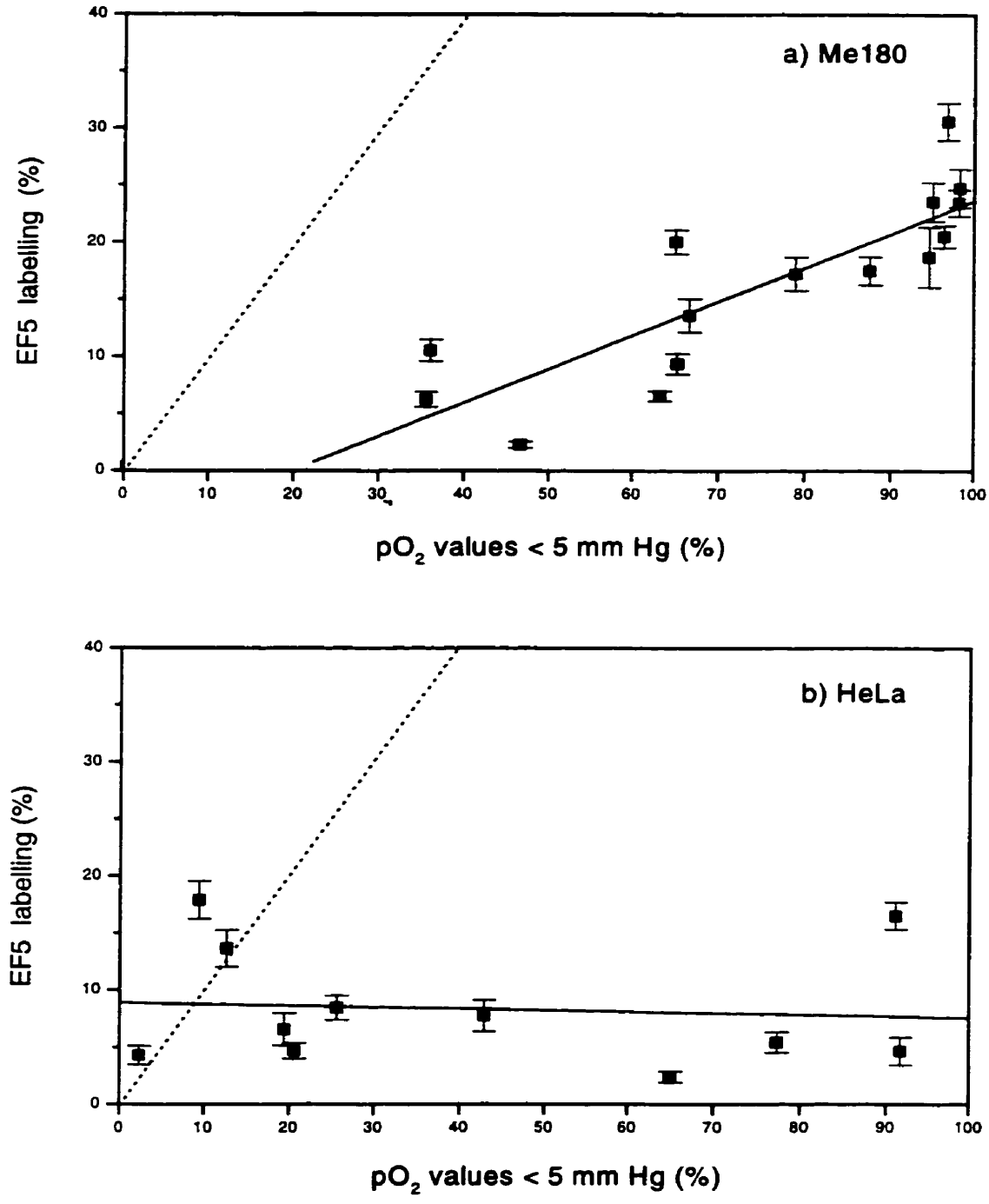

Figure 3.4: Correlation between EF5 labelling and Eppendorf pO_2 measurements (percentage of readings < 5 mm Hg) in Me180 (a) and HeLa tumours (b). Data for EF5 labelling are means ± se. The dashed lines represent the line of identity.

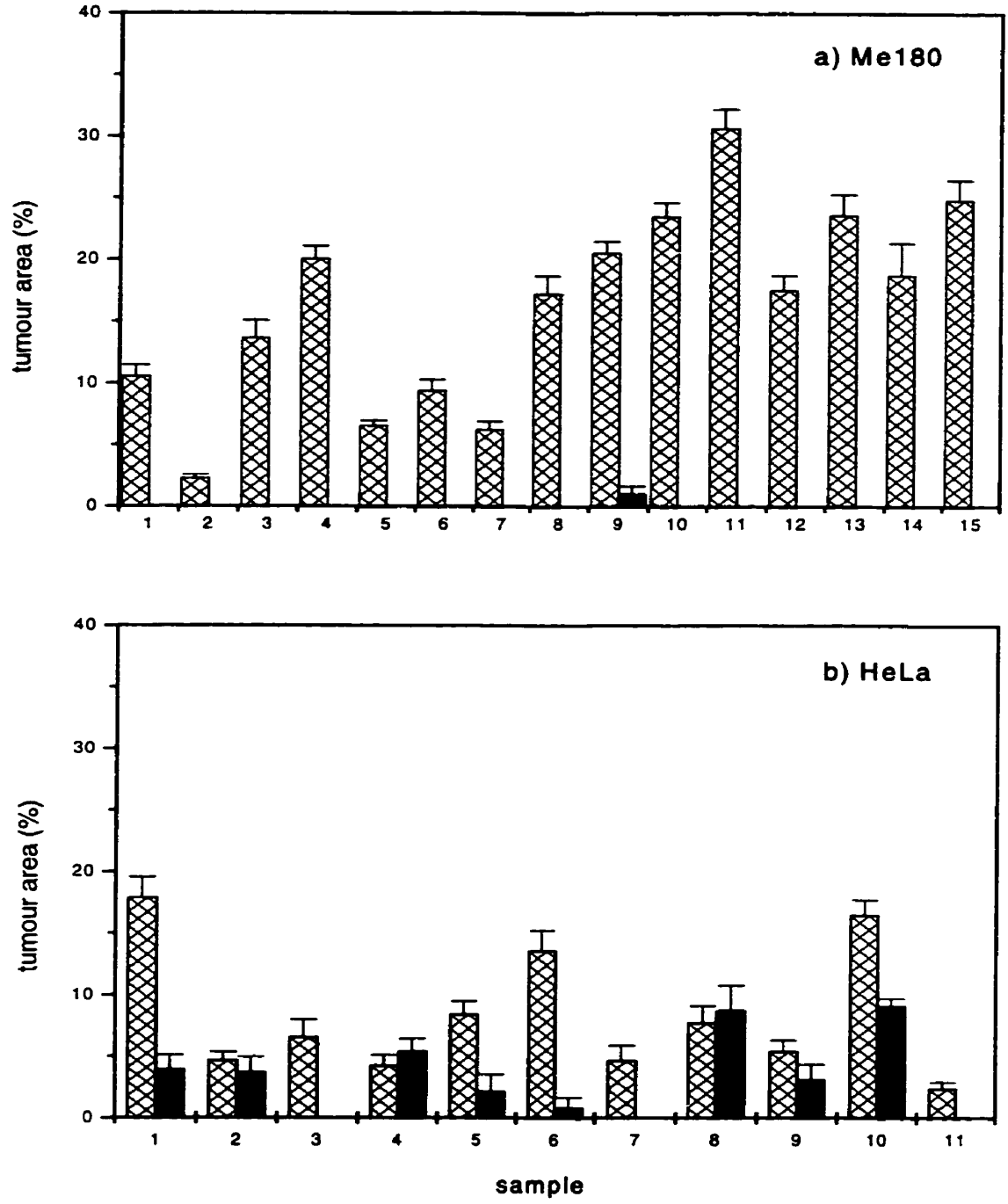

Figure 3.5: Percentage EF5 labelling (hatched bars) and percentage necrosis (black bars) in 15 Me180 tumours (a) and 11 HeLa tumours (b). The data are means ± se.

3.5 Discussion

The presence of hypoxia has been implicated in the resistance of solid tumours to various cancer therapies (Moulder and Rockwell, 1987; Sartorelli, 1988) and in the induction of malignant progression and metastasis (Brizel *et al*, 1996; Höckel *et al*, 1996; Fyles, 1998b; Sundfør *et al*, 1998; De Jaeger *et al*, 1999). Hence, methods that measure hypoxia would allow selection of patients in which hypoxia-directed strategies could translate into a therapeutic gain. The aim of this study was to examine in individual tumours the Eppendorf pO_2 Histograph technique, which provided strong clinical data regarding the predictive value of oxygen measurements, and binding of the relatively new and promising hypoxic cell marker EF5. Experiments were performed using the Me180 and HeLa cervical cancer cell as much of the clinical data has been obtained in studies in patients with cervix cancer.

A multiplicity of studies in rodents comparing the Eppendorf technique with other methods of measuring hypoxia has been reported (Horsman *et al*, 1993; Kim *et al*, 1993; Sasai and Brown, 1994; Horsman *et al*, 1994a,b; Kavanagh *et al*, 1996; Aboagye *et al*, 1998; Siemann *et al*, 1998; Raleigh *et al*, 1999). Most investigators, using groups of tumours, have given mice different treatments to change oxygenation and have found a significant correlation of the group mean, as calculated from the Eppendorf pO_2 measurements, with paired survival assay and nitroimidazole binding, under different oxygenation conditions. However, in studies on measurements and comparisons in individual tumours, the Eppendorf technique has failed to correlate with most other techniques. Only one group reported, in a study of cervix cancer patients and SCC-VII murine tumours, a moderate correlation with the comet assay (Aquino-Parsons *et al*, 1999). Kavanagh *et al* (1999a) found in KHT-C murine fibrosarcoma a trend indicating a relation

between Eppendorf and EF5 binding, but the correlation did not reach statistical significance.

In previous studies with experimental tumours, we have observed considerable heterogeneity in oxygenation within groups of ostensibly identical tumours and similar results have been published by other investigators (Horsman *et al*, 1994a,b; Yeh *et al*, 1995; Kavanagh *et al*, 1996 and 1999a; Milross *et al*, 1997; Adam *et al*, 1999; Aquino-Parsons *et al*, 1999; De Jaeger *et al*, 1999). Comparisons of groups of tumours will average out this heterogeneity and may not reflect individual tumour differences. Therefore, evaluation of techniques for measuring hypoxia necessitates making measurements and comparisons in individual tumours.

Figure 3.3 illustrates the heterogeneity of EF5-adduct binding in representative Me180 and HeLa tumours. The individual panels display the variation in EF5 labelling on 10 different sections cut from the same tumour. Comparisons among the panels clearly indicate that the inter-tumour variability in oxygenation is greater than the intra-tumour variability.

Several investigators have studied hypoxia marker labelling on multiple sections or biopsies per tumour. Cline *et al* (1994) assessed the variation of CCI-103F (another 2-nitroimidazole compound) labelling within and between canine tumours. They found that within and between tumour variation were essentially equal (respectively 27% and 30% of the total variation). Thrall *et al* (1997) examined the distribution of labelled CCI-103F within various individual biopsy samples of dog tumours. Mean CCI-103F-labelled areas ranged from 5% to 49%, with less inter-biopsy variability than inter-tumour variability. Comparable observations were published by Raleigh *et al* (1998) who evaluated pimonidazole binding on sections of cervix and head and neck cancers. They reported mean percentages of

staining in the range 0%-26%. This is in agreement with the range (2.3%-24.8%) we observed in the human cervical cancer xenografts.

Assessment of EF5 binding on immunohistochemically stained sections is a semi-quantitative method with thresholding on the AEC chromogen probably representing the most critical step. To evaluate the contribution to the variability of observer variations in threshold setting, a number of randomly selected sections of HeLa tumours were analysed, twice by the same observer with an interval of 6 months and once independently by a second observer. The percentages of EF5 staining, as assessed by the different observers are represented in figure 3.2. These results demonstrate no systematic differences between the sets of observations but the variance in the observations on the same section was similar in magnitude to that observed between sections of the same tumour. This suggests that some of the intra-tumour heterogeneity may be introduced by the analysis procedure.

Substantial intra- and inter-tumour heterogeneity in oxygenation has also been reported using the Eppendorf technique, in rodent (Nordsmark *et al*, 1995; De Jaeger *et al*, 1998; Adam *et al*, 1999; Aquino-Parsons *et al*, 1999) as well as in human tumours (Brizel *et al*, 1994; Nordsmark *et al*, 1994; Brizel *et al*, 1995) although the absolute pO_2 values in human tumours were higher (Fyles *et al*, 1998a; Adam *et al*, 1999). The results of the Eppendorf pO_2 measurements performed in the current study are plotted in figure 3.4a for Me180 tumours and in figure 3.4b for HeLa tumours and illustrate the considerable variability in oxygenation status of xenografted cervix cancers as assessed by both techniques, EF5 binding and Eppendorf pO_2 measurements.

Given the tremendous heterogeneity in tumour oxygenation, it is obvious that results from sampling multiple tumour sites will be more likely to represent the 'true' oxygen status. Our group (Wong *et al*, 1997) has reported that five needle tracks with 20-30 measurements per track are optimal to sample the oxygenation status of cervix cancers.

For measurements in rodent tumours, it is generally believed that 50-60 oxygen measurements give an acceptable estimate of the true oxygenation. (Stone *et al*, 1993). To evaluate hypoxic marker binding, Cline *et al* (1994) and Thrall *et al* (1997) recommended analysis of 4 random sections per tumour. This results in 90% of estimates of labelled area being within 5% of the true-labelled area.

Our evaluation was based on the clinical practice of classifying tumours as being above or below the mean or median hypoxic proportion. If there is high inter-tumour variability and low intra-tumour variability, then a low number of sections would suffice to ensure that tumours are correctly classified. In this way, Me180 tumours are less prone to missclassifications than HeLa tumours. With 4 sections, 7.0% of the Me180 tumours would be missclassified while 8.7% of the HeLa tumours would be incorrectly assigned in such a binary classification. Reducing the number of sections to 2 would increase the missclassification proportion to 10.1 % for Me180 and 12.4% for HeLa, while using 8 sections would result in missclassifications of 5.1% and 6.4% for Me180 and HeLa respectively. Thus analysis of 4 sections appears to be a good compromise.

The major goal of this study was to evaluate the relationship between Eppendorf pO_2 measurements and EF5 binding in two xenografted cervical cancer cell lines. A significant correlation ($p < 0.002$) between the two methods was observed for Me180 tumours but not for HeLa tumours (figure 3.4). There are several reasons that could explain the disparity between these results.

One obvious difference between the Me180 and HeLa tumours is the presence of necrosis in the HeLa tumours. In figure 3.5a, the proportion of necrosis, as outlined on digitised tumour sections, is represented for the 11 HeLa tumours that were analysed. In only 3 tumours, was there no detectable necrosis. The typical architecture of this histopathological feature in HeLa tumours is illustrated in figure 3.1a. The Eppendorf needle

probe can not distinguish these necrotic zones from viable, clonogenic cells. Hence, one would expect a consistent overestimation of the true proportion of hypoxic cells in the presence of necrosis. Several investigators have proposed methods to correct for the necrotic fraction (Khalil *et al*, 1995; Lyng *et al*, 1997; Milross *et al*, 1997). However, in a series of KHT-C tumours, we found little evidence for a relationship between Eppendorf pO_2 measurements and percent necrosis (De Jaeger *et al*, 1998, Appendix). Even if the Eppendorf measurements were corrected for necrosis in the HeLa tumours, it is not obvious that this would lead to an improvement of the correlation, since the presence of necrosis should also result in an underestimation of the hypoxic proportion using the EF5 binding technique. We calculated a corrected EF5-labelled area by subtracting the necrotic area from the total tumour area, but the results of both corrected measurement techniques remained discordant (data not shown).

It is conceivable that the presence of solid necrotic regions hindered the probe measurements which could have influenced reliability of this data. Furthermore, it is possible that loss of necrotic tumour tissue could have occurred during sample processing for the EF5 analysis and that regions of necrosis were lost from the slides. In part, tissue loss could also explain why in general HeLa tumours seem to be less hypoxic as compared to Me180 tumours (figures 3.4 and 3.5). Other factors, such as differences in nitroreductase levels between the cell lines, which might influence levels of EF5 binding, could have played a role as well (Workman, 1992).

Although there is a strong correlation between Eppendorf measurements and EF5 binding in Me180 tumours, there is a lack of correspondence in absolute values as indicated by the line of identity (see figure 4a). The results suggest that the Eppendorf technique consistently overestimates the degree of hypoxia in Me180 tumours. Raleigh *et al* (1999) published similar findings on the correspondence between Eppendorf and

pimonidazole binding in C3H mammary tumours and found that this consistent overestimation is true for all pO_2 data independent of whether the cut-off for pO_2 readings is defined at < 10mm Hg, < 5 mm Hg or < 2.5 mm Hg. Also the study by Aboagye *et al* (1998) comparing the Eppendorf technique with magnetic resonance spectroscopy of the fluorinated 2-nitroimidazole SR-4554 suggests consistent overestimation of hypoxia by the Eppendorf technique. Although necrosis was rarely observed in Me180 tumours within the size range examined, this overestimation could reflect the presence of other acellular and non-viable tissue such as stroma which was rather abundant in Me180 tumours (see figure 3.1b). One could also speculate that EF5 is more likely to bind to chronically hypoxic cells while the Eppendorf probe would measure both chronic and acute hypoxia.

This is the first study on comparisons of the Eppendorf technique and EF5 binding in individual xenografted cervix tumours. The lack of correlation between the two techniques in HeLa tumours stresses the importance of taking into account histopathological characteristics. In the current study, EF5 binding was analysed by immunohistochemistry, using the ELK3-51 antibody visualised by the immunoperoxidase method. This approach provides a positive/negative type of staining. In fact, oxygenation is not a binary environmental condition. The availability of a fluorescently-tagged ELK3-51 antibody offers the unique opportunity to detect EF5-adduct binding, over a continuum of oxygen levels (Lord *et al*, 1993; Evans *et al*, 1995). Cells at specific oxygen levels can be identified by thresholding on different fluorescence levels. This is a promising approach as it has been postulated that cells at intermediate oxygen levels are more troublesome in terms of treatment response and outcome (Wouters and Brown, 1997).

CHAPTER 4

DISCUSSION

The therapeutic success of non-surgical cancer treatments mainly depends on their ability to specifically attack tumour cells, thereby sparing normal cells. The presence of hypoxic cells is one property that distinguishes most solid tumours from healthy tissue and is exploitable in the design of effective cancer therapies. The current thinking is that hypoxia might be involved in three different aspects of tumour behaviour namely radioresistance, malignant progression and metastasis. Therefore, therapies aiming at circumventing hypoxia have the potential to improve clinical outcome. A potential difficulty is that the presence of hypoxia is tremendously heterogeneous between different tumours. Hence, evaluation of reliable methods that measure hypoxia in tumours is needed to ascertain optimal selection of patients that could benefit from hypoxia-directed strategies.

4.1 Summary and discussion

In chapter 2 of this thesis, the relationship between tumour oxygenation *in vivo* and metastatic potential was investigated in two rodent tumour models, KHT-C and SCC-VII. Two observations preceded this study and have influenced its design. Firstly, almost a decade ago, our lab (Young *et al*, 1988) performed experiments in which KHT-C and SCC-VII cells were exposed to hypoxia *in vitro*. After reoxygenation, cells were injected i.v. back into mice and the formation of lung metastasis was quantified. The results indicated enhanced metastasis formation in animals injected with tumour cells that were previously exposed to hypoxia as compared to the control group. Secondly, a number of clinical reports have recently emerged suggesting that tumour hypoxia, as measured directly using the Eppendorf pO_2 Histograph, has an adverse impact on outcome. In particular, patients with hypoxic cervix (Höckel *et al*, 1996; Fyles, 1998b) and sarcoma tumours (Brizel *et al*, 1996) were found to be more likely to develop distant metastasis.

Based on these observations a spontaneous rodent metastasis model was designed to test the hypothesis whether direct measurements of tumour oxygenation relate to metastatic potential. KHT-C and SCC-VII (the cell lines that were used in Young's experiments) tumours were established i.m. in mice, direct measurements of oxygenation in unmanipulated tumours were performed using the Eppendorf technique (the method that was used in the clinical studies) and macroscopic and microscopic lung metastases were counted. This spontaneous experimental model is most relevant to study tumour hypoxia and metastasis *in vivo* as it represents as closely as possible the natural behaviour of tumours.

The results of this series of experiments have indicated considerable heterogeneity in oxygenation between individual tumours within each cell line but overall the oxygenation range in KHT-C and SCC-VII tumours was quite similar. In KHT-C, a significant increase in early pulmonary metastasis formation was found in mice with hypoxic tumours. In SCC-VII tumours however, the overall metastasis incidence was low and although a similar trend was observed there was not a significant correlation with oxygenation status of the primary tumour. The results obtained in the KHT-C model are consistent with the clinical and experimental observations, suggesting that hypoxia might relate to metastatic ability. The underlying mechanisms are not entirely clear. There is growing evidence that a complex network of hypoxia-induced genetic alterations could mediate the process of tumour aggressiveness and metastasis but the precise interactions and how these act in concert are the subject of ongoing research. Upregulation of a key transcription factor HIF-1 (Semenza, 1998) and subsequent induction of vascular endothelial growth factor (VEGF), a potent angiogenic factor has been proposed as a possible mechanism that could link hypoxia and metastasis. However, research investigating this area has not led to unequivocal results. Rofstad and Danielson (1999)

could demonstrate VEGF involvement in the development of metastasis in low VEGF-expressing melanomas. Raleigh *et al* (1998) analysed co-localization of the hypoxic marker, pimonidazole and VEGF protein immunohistochemically and concluded that the relationship between hypoxia and metastasis can not be established by measuring hypoxia and VEGF expression in squamous cell carcinoma, at the time of clinical presentation. This is conflicting with the results of clinical studies correlating VEGF expression, vascular density and tumour prognosis (Takahashi *et al*, 1995; Obermair *et al*, 1996).

The results observed in the SCC-VII model, where there was a low number of metastases and no significant increase in the metastasis rate was found, suggest that hypoxia is certainly not the only factor affecting tumour aggressiveness. Other environmental conditions such as low glucose and pH may play a role as well (Schlappack *et al*, 1991). Furthermore, based on the work of Fidler (1990) we have postulated that the metastatic inefficiency observed in the SCC-VII model could in part have resulted from the heterotopic transplantation of this cell line (as opposed to KHT-C which was transplanted orthotopically).

We concluded from the experiments presented in this chapter that hypoxia may mediate metastatic ability but, as shown in the SCC-VII model, there are certainly other factors that contribute to the induction of this process. The KHT-C model is a valid model for further *in vivo* testing of modulation of tumour oxygenation and its impact on metastatic ability.

In chapter 3, we compared the results of oxygen measurements obtained with the Eppendorf technique and immunohistochemical detection of EF5-adduct binding. Experiments were performed in two human cervical cancer cell lines since much of the clinical data on tumour hypoxia and outcome has been obtained in this tumour type. As described in the introductory chapter, the Eppendorf technique has provided all the clinical

data. This method has, however, several drawbacks e.g. is invasive and can only be applied in accessible tumour sites. Therefore, a surrogate technique that would allow expansion of measuring hypoxia to many more tumour sites and by many more clinics would be of interest. We postulated that EF5 binding would be a good candidate because it identifies hypoxia on a cell-cell basis and can be detected using various invasive as well as non-invasive techniques. The measurement techniques are based on different physical principles but a correlation between EF5 binding and Eppendorf measurements would suggest that EF5 binding could be capable of providing the same prognostic information as the Eppendorf technique.

The results of these experiments show that there is considerable heterogeneity in oxygenation of individual tumours for both cell lines, but that the variability is less within than between individual tumours. In the Me180 tumours, a strong correlation between the results of both measurement techniques was observed. However, there was no correlation in the HeLa tumours. Several groups (Khalil *et al*, 1995; Lyng *et al*, 1997; Milross *et al*, 1997) have reported that Eppendorf measurements should be corrected for the necrotic fraction in tumours. As the Eppendorf technique undoubtedly measures in necrotic regions of tumours (if they exist) it could overestimate the degree of hypoxia in viable tissue. Therefore, we included assessment of the necrotic fraction in these experiments. We had previously investigated the relationship between tumour hypoxia, as measured with the Eppendorf needle probe and necrosis in a group of KHT-C tumours (see Appendix) but we could not show a correlation between necrotic fraction and hypoxia (De Jaeger *et al*, 1998). Even if one could correct approximately for necrosis in the HeLa tumours, this would not lead to an improvement of the correlation. Examination of figure 3.4b reveals that this would likely result in a leftward shift of the hypoxic Eppendorf measurements, most of which had low EF5 binding, and this would continue to result in a poor correlation between the two

measurements. However, it should be pointed out that a confounding difficulty could have been the loss of necrotic tumour tissue during preparation of tumour sections. The necrotic content was high in HeLa tumours and loss of necrotic material presumably also occurred during sectioning of the HeLa tumours. In addition, the solid consistency might have influenced the reliability of the Eppendorf measurements.

Interestingly, in Me180 tumours, there is a significant correlation between both techniques. However, the Eppendorf technique seems to consistently overestimate the hypoxic fraction. There was no obvious necrosis in the Me180 tumours so the presence of necrosis is unlikely to explain this systematic shift to higher hypoxic fractions measured by the Eppendorf probe. The reason for this overestimation is not clear. We have considered the possibility that it could reflect that different types of hypoxia are being measured by both techniques. Most investigators believe that binding of hypoxic markers primarily reflects diffusion-limited hypoxia (Raleigh *et al*, 1996) while Eppendorf Histograph would measure transient and chronic hypoxia. This is however a speculation. As tumour oxygenation is in a continuous state of flux the delineation between acute and chronic hypoxia might be less distinct than classically thought (Dewhirst, 1998).

In general, HeLa tumours seem to be less hypoxic as compared to Me180 tumours. It is certainly possible that the presence of necrosis has compromised our ability to obtain representative tumour samples. In addition, it might be that differences in nitroreductase levels (e.g. higher levels in Me180 tumours) could contirbute to different levels of EF5 binding in both cell lines, transplanted in identical hosts and measured at almost identical size. Currently, there is no data available on the 'portfolio' of nitroreductases in these tumour cell lines (Workman, 1992).

Finally, higher levels of EF5-adduct binding in Me180 could be the result of false positive labelling in areas of keratinization and stroma. This was observed to some

extent in a few sections and has also been reported by other investigators (Kennedy *et al*, 1997). To rule out this possibility, additional trichrome staining needs to be done. Areas staining positive for trichrome could than be outlined and subtracted from the EF5 labelled area.

We concluded that the results obtained with the EF5 binding technique in the Me180 tumours are encouraging. They are in agreement with earlier results from our lab showing a trend for a correlation between Eppendorf measurements and EF5 detected by flow cytometry (Kavanagh *et al*, 1999a). The data obtained in the HeLa tumours underlines the importance of studying histopathological characteristics. They can contribute to the disparate results between Eppendorf and other techniques but should not be neglected as it might be this specific property that strengthens the predictive value of the Eppendorf technique.

4.2 Additional considerations and future directions

Comparison of techniques of measuring hypoxia in individual patients

The results described in chapter three showing a strong correlation between EF5-adduct binding in individual Me180 tumours and the Eppendorf technique are interesting. They suggest that EF5 binding could provide the same prognostic information as the Eppendorf 'gold standard'. This is important if one would like to translate this technique to the clinic. A phase I trial will soon start within the scope of an ongoing project of oxygenation of cervix cancer at the Princess Margaret Hospital. Extensive experience with measuring tumour oxygenation using the Eppendorf technique has already been gained (Wong *et al*, 1997; Fyles *et al*, 1998a). The new study will be designed to test the

hypothesis whether EF5 binding can substitute for the Eppendorf technique as a predictive assay to select patients with hypoxic cervix tumours for treatments that specifically attack hypoxia (e.g. tirapazamine). The study presented in chapter three was a preliminary investigation that provided information on the feasibility and reproducibility of a semi-quantitative analysis of EF5 binding on sections and on the number of tumour sections that should be analysed in order to obtain an EF5 labelling index which is representative for the whole tumour.

Hypoxia imaging

As reported in the introduction, EF5 binding can also be detected non-invasively. This approach does not require tumour biopsies and could extend the use of EF5 to many more tumour sites, including non-accessible ones. Very promising is the successful synthesis of ^{18}F-EF5 and its analogue ^{18}F-EF1 (Hustinx *et al*, 1999). These compounds allow detection of adduct distribution by positron emission tomography (PET). There is a tremendous interest in metabolic imaging and development of tracers for PET use. Unfortunately, PET facilities are very expensive and this research is limited to a few centres. Also, the resolution of currently available PET scanners for experimental use is not better than 3 mm. Hence, this technique will provide information on oxygenation of groups of cells rather than individual cells. It might be interesting to use this technique for studying changes in oxygenation over time e.g. after experimental manipulation. In addition, information obtained using PET-imaging of other physiologic processes e.g. vessel perfusion could be mapped and might provide information on spatial and temporal changes in oxygenation in relation to vascular perfusion (Laubenbacher and Schwaiger, 1998).

Hypoxia-induced gene expression and metastasis

It has been well documented that hypoxic stimuli can upregulate a myriad of genes, transcription factors, growth factors, cytokines, metabolic and invasive enzymes in response to hypoxic stimuli (Stoler *et al*, 1992; Dachs and Stratford, 1996; Jang and Hill, 1997; Sutherland, 1998; Dachs and Chaplin, 1998; Graham *et al*, 1999). There are plausible hypotheses on how some of these changes might interact (see discussion) but fundamental mechanisms still need to be elucidated. Also, oxygenation is not an on/off physiological condition and it is not entirely clear how fluctuations in oxygen levels affect gene expression. It is very possible that the generation of oxygen radicals during reperfusion after transient hypoxia could also play an important role in this process.

In the study outlined in chapter 3, EF5 binding was assessed immunohistochemically, using the biotinylated ELK3-51 antibody. This type of analysis results in a positive/negative staining. It has been postulated that cells at intermediate oxygen levels might be more important in terms of treatment outcome (Wouters and Brown, 1997). Therefore, it would be interesting to use the available fluorescently-tagged ELK3-51 antibody, that offers the opportunity to detect EF5 binding, over a continuum of oxygen levels (Lord *et al*, 1993; Evans *et al*, 1995) by thresholding on different fluorescence levels. This approach would allow identification of cells at specific oxygen levels. New technologies such as laser capture microdissection (LCMD) are becoming within reach (Simone *et al*, 1998). LCMD allows isolation of individual cells from tissue sections. The gene expression of selected cells could then be analysed using PCR strategies and cDNA arrays (Pappalardo *et al*, 1998; Gerhold *et al*, 1999). Combination of these new technological innovations would thus allow screening of cells at different oxygen levels for gene expression. Differences in gene expression and comparisons with gene expression of cells

originating from metastatic deposits could help in understanding the complexity of mechanisms that underlie tumour hypoxia and aggressiveness. For this purpose, the KHT-C spontaneous metastasis model, would be a valid model.

4.3 Concluding remarks.

Hypoxia is classically believed to be a major cause of radioresistance. Recent clinical and experimental data have shed a new light on hypoxia as a general marker of tumour aggressiveness. Ongoing research aiming at elucidating underlying molecular mechanisms has gained momentum by enormous technical innovations. In parallel, promising hypoxia detecting compounds and imaging techniques have been developed. The ultimate goal is to identify hypoxic tumours and to adapt cancer treatment for individual patients. The results of the work presented in this thesis support the concept that EF5 binding holds great promise but should be further tested in patients. The value of the Eppendorf technique to predict for metastatic potential was confirmed in a murine model. The challenge remains to identify the optimal technique for clinical application. As all techniques have their pros and cons, the use of a combination of techniques should not *a priori* be excluded.

APPENDIX

HETEROGENEITY OF TUMOUR OXYGENATION: RELATIONSHIP TO TUMOUR NECROSIS, TUMOUR SIZE, AND METASTASIS

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Abstract

Purpose: Measurements of oxygenation in the transplanted rodent KHT-C and SCC-VII tumours demonstrate significant heterogeneity from tumour to tumour as is observed in human tumours. This finding suggests that heterogeneity in oxygenation between tumours is likely related to factors associated with tumour growth rather than to intrinsic genetic differences. In this study we examined whether measurements of the oxygenation of individual KHT-C tumours were related to necrosis in the tumours or to tumour size and whether the more hypoxic tumours gave rise to more metastases.

Methods: Tumours were grown in the gastrocnemius muscle of C3H mice and tumour oxygenation was measured at defined sizes (approximately 0.35 g, 1.0 g, and 1.6 g) using an Eppendorf polarographic oxygen probe. Necrosis was assessed by examining histological sections cut from tumours used for the oxygen measurements. Metastasis was assessed by counting macroscopic lung nodules in mice sacrificed when their tumours reached a size of approximately 1.6 g.

Results: Tumour oxygenation in individual KHT-C tumours became poorer and necrosis became more extensive as the tumours grew larger but, at a size of 0.3-0.4 g, there was no relationship between oxygenation and extent of necrosis. In general, measurements of tumour pO_2 at a size of 0.3-0.4 g were predictive of tumour $p0_2$ in the same tumour at a tumour size of about 1.0 g, but by the time the tumours reached a size of about 1.6 g they were all very hypoxic. There was a trend suggesting a relationship between macroscopic metastases in the lung and and degree of hypoxia in the KHT-C tumours but this was not statistically significant.

Conclusion: The results indicate that the heterogeneity of oxygenation seen in KHT-C tumours is not explained by different degrees of necrosis in the individual tumours.

The lack of a correlation between increased metastasis formation and increased levels of hypoxia in the KHT-C tumours is not consistent with the results reported for human tumours.

Introduction

Measurements of oxygenation in tumours in patients have shown considerable heterogeneity from one tumour to another and it has been reported that tumours with higher levels of oxygenation have a better prognosis following treatment than tumours with poorer oxygenation (Gatenby *et al*, 1988; Vaupel *et al*, 1991; Höckel *et al*, 1996; Nordsmark *et al*, 1996; Brizel *et al*, 1996; Sundfør *et al*, 1997; Brizel *et al*, 1997; Fyles *et al*, 1998a,b). This improved outcome has been observed following treatment of cervical carcinoma with either radiation or surgery (Höckel *et al*, 1996) and, for soft tissue sarcoma, poorer oxygenation has been linked to the development of distant metastases (Brizel *et al*, 1996). These findings suggest that hypoxia may be a marker for more aggressive disease as well as reducing tumour response to radiation treatment.

Recent experimental studies have indicated that a hypoxic environment may select for tumour cells with a more unstable genome (Reynolds *et al*, 1996; Graeber *et al*, 1996). This, in turn, raises the possibility that hypoxia in tumours results in the development of more aggressive disease. Alternatively more aggressive cancers may be more likely to develop hypoxia. In either case the development of hypoxia might relate to genetic differences influencing the development of cancer. This issue can be addressed by considering the results obtained with transplanted animal tumours, because in this situation all the tumours are genetically very similar. In our previous studies with experimental tumours, we have observed considerable heterogeneity in oxygen tension (pO_2) values within a group of ostensibly identical tumours and similar results have been reported by

others (Horsman *et al*, 1994; Yeh *et al*, 1995; Kavanagh *et al*, 1996; Milross *et al*, 1997; Kavanagh *et al*, 1999a; Adam *et al*, 1999; Aquino-Parsons *et al*, 1999). In the current series of studies we have determined the range of variations in oxygenation observed between individual tumours with two experimental systems and have examined possible reasons for this heterogeneity in one of these systems. We have concentrated on measurements made in individual tumours since the heterogeneity of oxygenation makes it problematic to pool results from groups of tumours. Finally, we have assessed whether the number of metastases arising in the individual tumour-bearing mice is related to the oxygenation of the tumour growing in that mouse.

Methods

Animals and tumours

C3H/HeJ mice were house in the small animal colony of the Ontario Cancer Institute under conditions meeting the guidelines of the Canadian Council on Animal Care. The animals had access to food and water ad libitum. KHT-C or SCC-VII tumours (Bristow *et al*, 1990) were initiated by injection of 2.5×10^5 cells into the left gastrocnemius muscle of 8-12-week-old animals. When the leg diameter reached a size of 9 ($\pm$ 0.5) mm (tumour wt 0.3-0.4 g) the animals were selected for measurements of pO_2 as described below.

Oxygen measurements

Oxygen measurements were made in each tumour individually using an Eppendorf oxygen probe. The animals were anaesthetised with *Ketalean* (ketamine hydrochloride, 50 mg/kg) and *Rompun* (xylazine, 4 mg/kg) and placed on a warming pad. The oxygen measurements were made as described previously (Kavanagh *et al*, 1996 and 1999a) with a total of 6 parallel tracks (3 each in two planes) of 8-10 measurements each.

The data for each tumour was analysed separately after correction for the average temperature of the tumour (which was measured at various points similar in position to the Eppendorf probe tracks). Initial measurements were made when the tumours had grown to a leg diameter of 9 (± 0.5) mm (tumour wt 0.3-0.4 g) and some of the tumours were measured again when the leg reached a diameter of 12 (± 0.5) mm and 15 (± 0.5) mm (tumour weights of ~ 1.0 g and ~ 1.6 g respectively).

Assessment of necrosis

The fraction of necrosis in KHT-C tumours, in which oxygen measurements had been made, was assessed from two separate histological sections stained with hematoxylin and eosin (H & E) and cut through the approximate centre of the tumour, so that the cut surface of each section was approximately 3 mm apart. This region represented the approximate location of the oxygen measurements. The total area of the sections was scanned at 5x magnification using a Microcomputer Image Design (MCID; Image Research, Inc.) analysis system linked to a transmitted light microscope with a colour CCD camera attached. Each microscope field of the tumour section was digitised and semi-automatically thresholded according to colour hue and intensity to separate tumour from nontumour areas. The total tumour area in each field was calculated by the computer. Regions of necrosis were identified on the colour monitor screen and semi-automatically outlined and their areas calculated by the computer system. The total area of the two sections was analysed field-by-field and the proportion of the tumour necrosis in the field was calculated. Results for the two fields were averaged to give a single value of percent necrosis for each tumour. This strategy examines necrosis in the whole of two sections through the region of the tumour in which the pO_2 measurements were made and hence should be representative of the region of interest.

Assessment of metastasis

When the tumours reached a size of about 1.6 g the animals were killed and the lungs were removed, briefly washed to remove blood and then fixed in Bouin's solution. The number of macroscopic metastases that had developed in the lungs (which is the primary site of metastases for these tumours) was counted using a dissecting microscope.

Results and discussion

Oxygen measurements were made in groups of 46 KHT-C tumours and 20 SCC-VII tumours of size 0.3-0.4 g and the results are plotted in figure 1 as the median pO_2 versus the percentage of values < 5 mm Hg for each individual tumour. There is a considerable range of values of oxygenation for the individual tumours. This heterogeneity in tumour oxygenation is also observed in human tumours (see e.g. Fyles *et al*, 1998a), although the absolute pO_2 values in the rodent tumours are lower. The fact that a wide range of pO_2 values is observed in two different transplanted tumours suggests that the heterogeneity of oxygenation seen in human tumours is at least partly due to differences associated with tumour growth rather than to intrinsic genetic differences between the tumours.

It has been reported that percent necrosis plays an important role in the assessment of oxygenation in tumours of different size (Khalil *et al*, 1995; Milross *et al*, 1997; Lyng *et al*, 1997). Consequently we examined whether the wide range of pO_2 values that we had observed in small tumours (0.3-0.4 g) could be explained by the extent of necrosis in the tumours. A preliminary examination of H & E sections of KHT-C and SCC-VII tumours of this size suggested that there was significantly more necrosis in the KHT-C tumours. Hence we concentrated on this tumour type. The oxygen levels in the tumours

were measured and within two hours the animals were killed and the tumours were removed, fixed in 10% buffered formalin and embedded in paraffin for histological sectioning. The tumours showed multiple, mostly irregular, small areas of necrosis widely dispersed throughout the tumour sections. The results for 23 tumours are shown in figure 2 plotted as the percent necrosis versus the fraction of pO_2 values less than 5 mm Hg or 1 mm Hg.

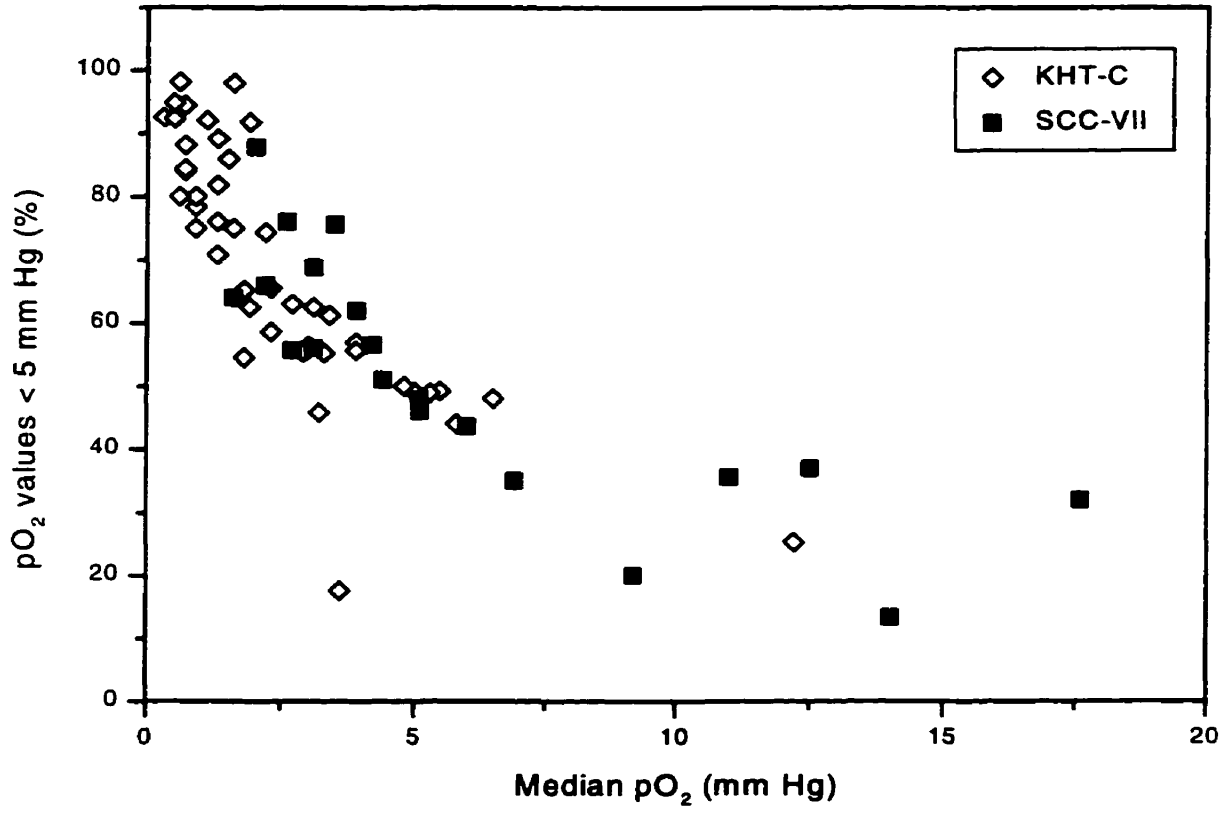

Figure 1: The percent of pO_2 values less than 5 mm Hg as a function of the median pO_2 for individual tumours. The figure shows results for 46 KHT-C tumours and 20 SCC-VII tumours of size 0.3-0.4 g.

There is little evidence of a relationship between the pO_2 values and the percent necrosis in the individual tumours. These measurements do not support the idea that the heterogeneity in measured pO_2 values can be due to differences in percent necrosis in the tumours.

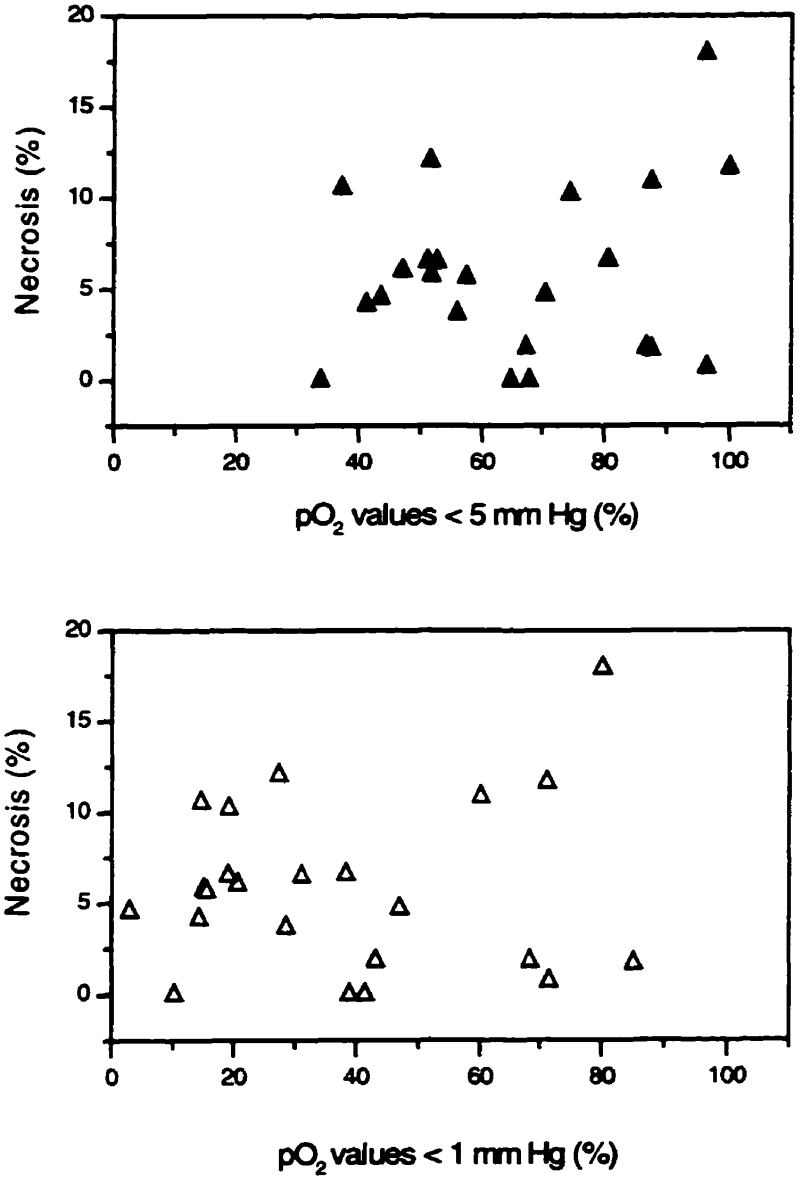

Figure 2: The percent necrosis in the tumour plotted as a function of the percent of pO_2 values less than 5 mm Hg or 1 mm Hg measured in 23 individual KHT-C tumours of size 0.3-0.4 g.

We next examined the effect of tumour size on oxygenation of KHT-C tumours. We had observed previously that the oxygen status of groups declined with tumour size as reported by Khalil *et al* (1995) and Milross *et al* (1997).

In particular, for a group of 44 tumours of size 0.3-0.4 g the percent of pO_2 values less than 5 mm Hg was 65.9% whereas in a group of 8 tumours of size 0.8-0.9 g this value was 73.9% (unpublished data). In the current studies we were interested in tracking changes in oxygenation in individual tumours, consequently we made measurements of pO_2 in a group of 46 tumours at size 0.3-0.4 g, then measured the same tumours again when they reached 1.0-1.1 g (about 3 days later) and then again when they reached a size of 1.6-1.9 g (about another 3 days later). The results of these studies are shown in figure 3 where the percentage of pO_2 values less than 5 mm Hg at the two larger sizes is plotted as a function of the value obtained at size 0.3-0.4 g. The data for each individual tumour is plotted, which allows a tracking of the changes in pO_2 as the tumours increase in size. It can be seen that there is some scatter in the data but there is a reasonable correlation between the values obtained at size 0.3-0.4 g and 1.0-1.1 g ($r = 0.33$, $p = 0.027$). Although some of the scatter observed in these studies may be due to measurement variation, we have observed that two groups of measurements made one immediately after the other in the same tumour gave good reproducibility of the results obtained (data not shown). Thus these results indicate that differences in oxygenation tend to persist in the tumours as they grow. A similar result was reported for the R-3327 rat tumour by Yeh *et al* (1995).

A trend to increased hypoxia is very pronounced by the time the tumours reach a size of 1.6-1.9 g, and no correlation is seen between the values obtained at size 0.3-0.4 g and those in the tumours at size 1.6-1.9 g. We have also found that there is a correlation between the extent of necrosis and tumour size in the KHT-C tumour (data not shown), thus these results are consistent with previous reports (Khalil *et al*, 1995; Milross *et al*, 1997).

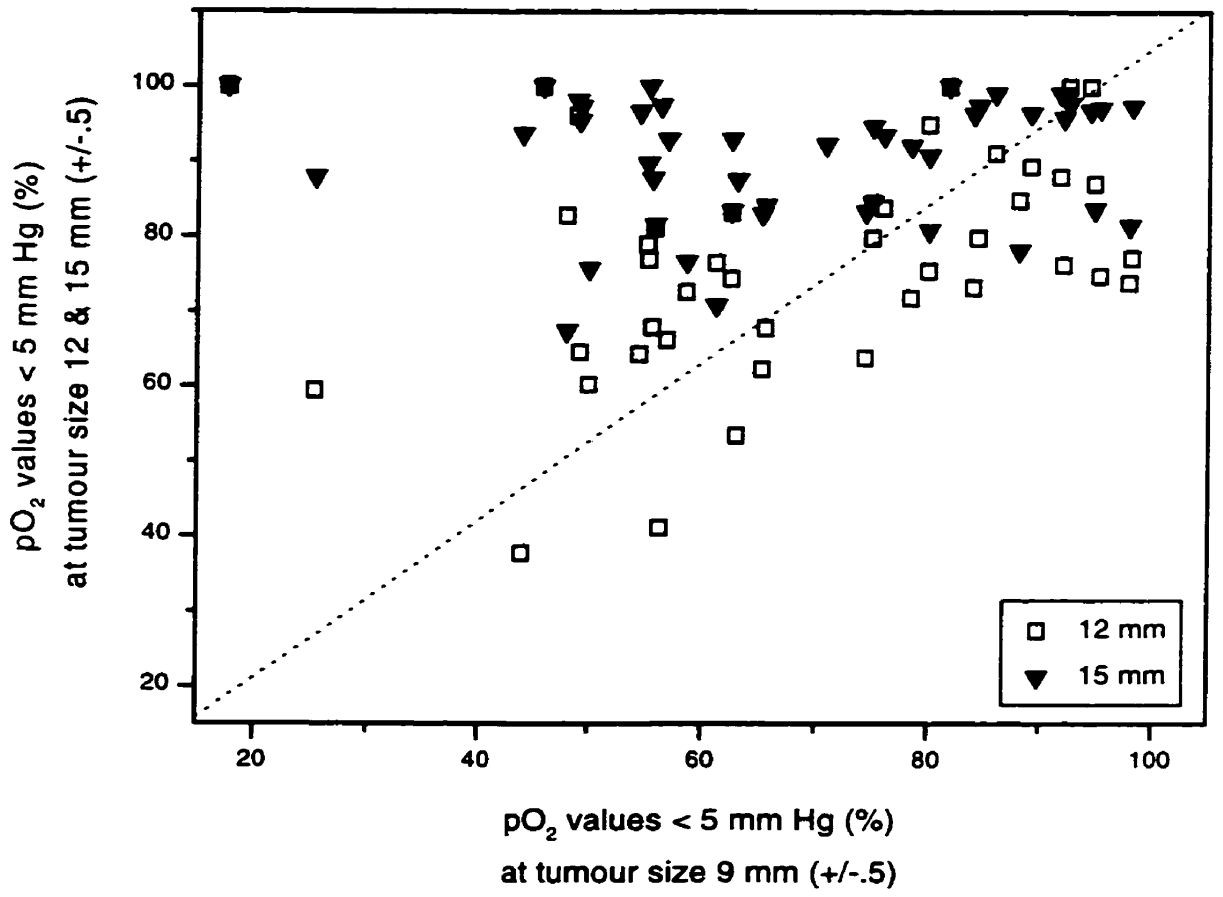

Figure 3: The percent of pO_2 values less than 5 mm Hg measured in 46 KHT-C tumours at different sizes. The measurements made in the tumours at a tumour size of 1.1-1.2 g (leg diameter of 12 ± 0.5 mm) and at a size of 1.6-1.9 g (leg diameter of 15 ± 0.5 mm) are plotted against the measurements made in the tumours at a size of 0.3-0.4 g (leg diameter 9 ± 0.5 mm)

The increase in hypoxia in very large tumours is also consistent with radiation survival studies which we have performed previously which indicate that tumours of this large size have an hypoxic fraction of close to 100% (data not shown). However our recent results indicate that, at size 0.3-0.4 g, there is no significant correlation between hypoxic fraction measured using a paired survival analysis and the measurement of oxygenation

using the Eppendorf probe (Kavanagh *et al*, 1996 and 1999a). Similar results have been reported by Horsman *et al* (1994). This finding initially seems to be inconsistent with the results obtained in the clinical studies of cervical and head and neck cancers, which have indicated that tumour oxygenation is predictive for the response of the tumour to radiation treatment (Höckel *et al*, 1996; Nordsmark *et al*, 1996; Brizel *et al*, 1996; Fyles *et al*, 1998a). However, the suggestion from the clinical data that hypoxia in tumours may be a marker for more aggressive disease and is predictive for an increased possibility of metastatic disease provides a possible explanation of this inconsistency (Höckel *et al*, 1996; Brizel *et al*, 1996 and 1997). Namely that radiation response is probably not the only factor which influences the predictive aspects of measurements of tumour oxygenation. Consequently, in the final part of the current experiments we addressed this question directly by examining whether there was a relationship between the pO_2 measurements made in the tumours and the extent of metastases observed in the lungs of the mice. In both tumours the lung is the primary site at which metastases arise.

Animals bearing tumours that had pO_2 measurements at size 0.3-0.4 g were maintained until the tumours had reached a size of 1.6-1.9 g. At this time they were killed and the number of lung metastases present was counted. We detected no macroscopic metastases in the lungs of mice bearing SCC-VII tumours of this size but there were a large number of metastases in lungs from mice bearing KHT-C tumours. The results of counting these metastases are shown in figure 4, with the number of macroscopic metastases per animal plotted as a function of the oxygenation status (percent of pO_2 values < 5 mm Hg) of the tumour at size 0.3-0.4 g. The results in figure 3 indicate that measurements of oxygenation at this size are a reasonable indicator of the oxygenation of the tumour as it grows, and our previous studies have shown that it is from size 0.25 g upwards that the KHT-C tumours start to seed metastases into the lung (Hill *et al*, 1986). The results in figure

4 show a slight trend for a relationship between the extent of tumour hypoxia and number of metastases in the lungs of the mice but there is not a statistically significant correlation ($p = 0.25$). This result is not consistent with the clinical findings discussed above and does not help explain the inconsistency between the experimental and clinical results discussed.

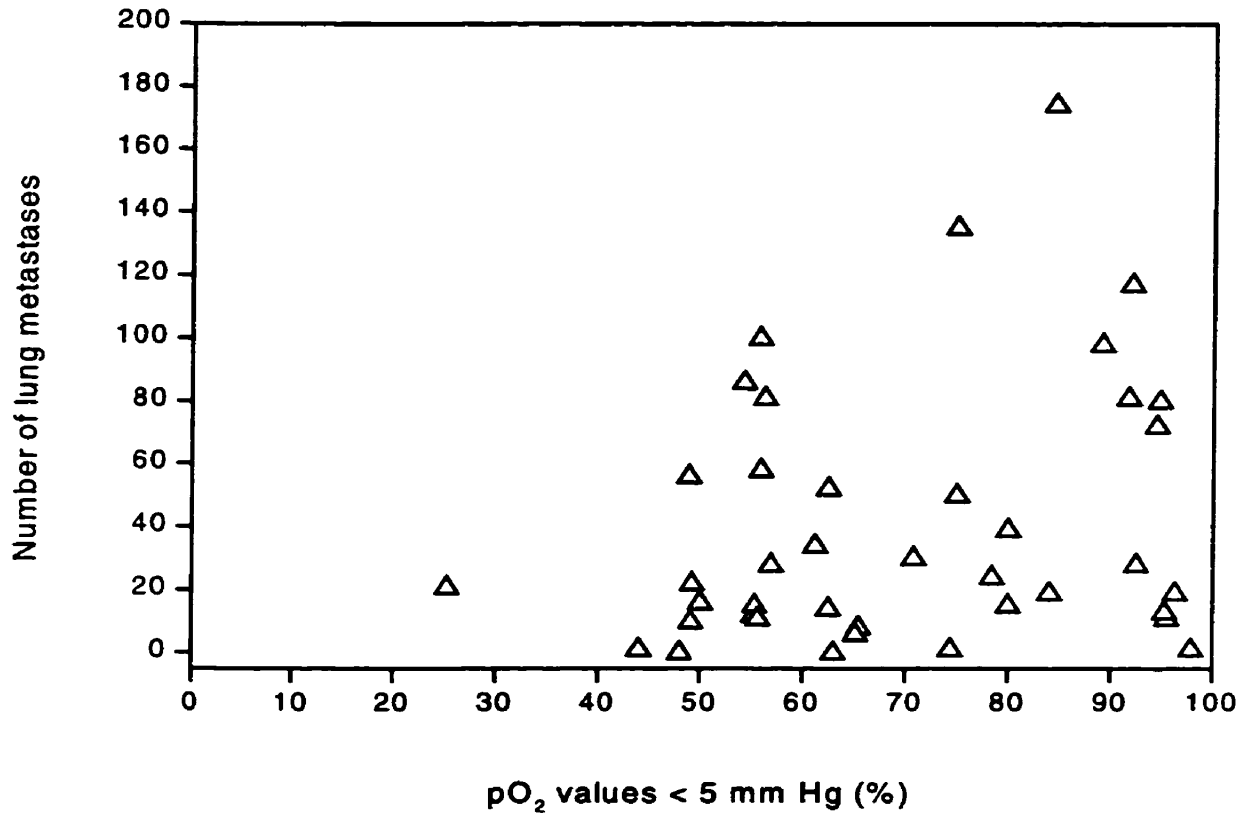

Figure 4: The number of macroscopic metastases present in the lungs of 40 tumour-bearing mice plotted in relation to the oxygenation status of the tumour at a size of 0.3-0.4 g (leg diameter of 9 ± 0.5 mm)

Furthermore, the fact that there was a large difference in the number of macroscopic metastasis in lungs from mice bearing KHT-C and SCC-VII tumours, despite the similar pO_2 values in these tumours (see figure 1), indicates that oxygenation status alone can not explain differences in the metastatic potential of different tumours.

Although a correlation was not observed between hypoxia and metastasis formation, it can be seen in figure 4 that, while mice with a large number of metastases always had hypoxic tumours, not all hypoxic tumours seeded large numbers of metastases. Thus hypoxia may be a factor that acts in concert with other cellular characteristics to promote metastasis formation in these KHT-C tumours. Our previous studies demonstrated that hypoxia can promote metastasis formation by KHT-C cells. In particular, we showed that if such cells are exposed to hypoxia *in vitro* they are more capable of forming experimental metastasis following intravenous injection (Young *et al*, 1988; Young and Hill, 1990). We also observed that such cells were more able to invade through Matrigel in an *in vitro* assay and that they produce increased levels of the mRNA for vascular endothelial growth factor (VEGF) which has been implicated in angiogenesis in tumours (Cuvier *et al*, 1997; Jang and Hill, 1997). It is not intuitively obvious why increased expression of VEGF at the early stage of seeding would influence metastasis formation. However, VEGF is not only capable of inducing new blood vessel growth but it also causes increased permeability of existing capillaries. Thus it may also act to increase the ability of a tumour cell which is producing it to extravasate by invading through the blood vessel wall of the lung and gain access to the interstitial space. These findings suggest that an hypoxic environment in the tumour may act to enhance the ability of the cells to form metastasis.

The ability of hypoxic tumour cells to access blood vessels and escape from the primary tumour is likely to be compromised by the fact that hypoxic cells are, of necessity, at a distance from functional blood vessels. Thus in tumours which contain largely chronically hypoxic cells there would likely be a need for the hypoxic cells to have increased motility and invasive ability to reach the blood vessels. Cells that are acutely hypoxic might not need these extra properties. Our studies have shown that upregulation of VEGF mRNA in human cervical carcinoma cells can be detected within 2 hours of exposure to hypoxia

and that the decay half-life on exposure to oxygen is about 45 min (Chiarotto and Hill, 1999). Thus a number of closely spaced cycles of acute hypoxia could well be sufficient to induce a significant increase in VEGF production. This raises the possibility that differences in chronic versus acute hypoxia might influence metastasis formation. Further studies are required to test the validity of this possibility.

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