Cancer and Metastasis Reviews 5: 313–341, 1987

Tumor hypoxia: its impact on cancer therapy

John E. Moulder[1], Sara Rockwell[2]
[1] *Department of Radiation Oncology, Medical College of Wisconsin, 8700 W. Wisconsin Avenue, Milwaukee, WI 53226, USA;* [2] *Department of Therapeutic Radiology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA*

Keywords: experimental tumors, oxygenation, hypoxic cells, radiotherapy, chemotherapy, radiation resistance

Abstract

The presence of radiation resistant cells in solid human tumors is believed to be a major reason why radiotherapy fails to eradicate some such neoplasms. The presence of unperfused regions containing hypoxic cells may also contribute to resistance to some chemotherapeutic agents. This paper reviews the evidence that radiation resistant hypoxic cells exist in solid tumors, the assumptions and results of the methods used to detect hypoxic cells, and the causes and nature of tumor hypoxia. Evidence that radiation resistant hypoxic cells exist in the vast majority of transplanted rodent tumors and xenografted human tumors is direct and convincing, but problems with the current methodology make quantitative statements about the magnitude of the hypoxic fractions problematic. Evidence that radiation resistant hypoxic cells exist in human tumors is considerably more indirect than the evidence for their existence in transplanted tumors, but it is convincing. However, evidence that hypoxic cells are a significant cause of local failure after optimal clinical radiotherapy or chemotherapy regimens is limited and less definitive. The nature and causes of tumor hypoxia are not definitively known. In particular, it is not certain whether hypoxia is a chronic or a transient state, whether hypoxic cells are proliferating or quiescent, or whether hypoxic cells have the same repair capacity as aerobic cells. A number of new methods for assessing hypoxia are reviewed. While there are still problems with all of the new techniques, some of them have the potential of allowing the assessment of hypoxia in individual human tumors.

Introduction

It has been known since the 1930's [1, 2] that hypoxic conditions protect cells from the lethal effects of ionizing radiation. It has been speculated ever since that time that the radioresistance of some solid neoplasms is due to the presence of hypoxic, and hence radiation resistant, tumor cells in areas distant from a patent vasculature [1, 3, 4]. The presence of hypoxic cells in solid tumors is presumed to result from transient deficiencies in blood flow through individual tumor blood vessels, areas of chronic vascular insufficiency, and regions of frank necrosis [3–5]. Evidence that solid rodent tumors normally contain a subpopulation of viable (clonogenic) tumor cells with a radioresistance similar to that of artificially hypoxic cells was first obtained in 1963 [6]; since that time, radiation-resistant, hypoxic tumor cells have been detected in the vast majority of the solid transplanted rodent tumor lines that have been examined [7, 8].

More recently, it has been suggested that hypox-

Address for offprints: John E. Moulder, Radiation Oncology, MCMC Box 165, Medical College of Wisconsin, 8700 W Wisconsin Ave, Milwaukee, WI 53226, USA.

ia also protects cells in solid tumors from some chemotherapeutic agents [9–11]. Hypoxic tumor cells may be resistant to chemotherapy because drugs do not penetrate into the avascular areas that contain hypoxic cells [12–14], because of differences in the uptake and metabolism of drugs by aerobic and hypoxic cells [11, 15], and because hypoxic tumor cells are not proliferating [16, 17] or are slowed in their progression through the cell cycle [18–20].

The hypothesis that the efficacy of therapy is limited by the presence of hypoxic tumor cells has resulted in a variety of experimental and clinical therapies designed to eliminate hypoxic areas, to kill hypoxic cells, or to eliminate the selective protection of hypoxic cells [21–23]. Irradiation schedules which minimize the importance of hypoxic cells have been proposed and tested [24–26]. Radiotherapy has been combined with hyperbaric oxygen [27–29], with oxygen or carbogen breathing [30–33], and with oxygenated perfluorochemical emulsions [34–36] in attempts to directly improve tumor oxygenation. Patients with low hemoglobin levels have been transfused prior to radiotherapy in attempts to improve tumor oxygenation [37–39]. Deliberate regional hypoxia has been used in attempts to make both tumors and the adjacent normal tissues equally hypoxic, and therefore uniformly radioresistant [40, 41]. Hypoxic cell radiosensitizers have been tested extensively in the laboratory and in the clinic [42–46]. High LET radiotherapy [47, 48] has been developed partially on the basis of the lower sensitizing effect seen with oxygen for these radiations. Adjunctive therapy with bioreductive alkylating agents [9, 10], certain nitroheterocyclic radiosensitizers [9], and hyperthermia [49, 50] has also been tested in an attempt to exploit the selective toxicity of these agents to hypoxic cells.

Despite the attention devoted to tumor hypoxia, evidence that hypoxic cells exist in solid human neoplasms and that these cells influence the outcome of conventional fractionated radiotherapy remains indirect. The histological pattern seen in many human tumors is compatible with the idea that tumor growth is limited by oxygen diffusion [4, 51]. Measurements of oxygen tensions in human tumors suggest that average oxygen tensions in tumors are lower than those observed in normal tissues [31, 52–54], but the techniques used in these studies cannot distinguish viable hypoxic areas from necrosis. Studies of the correlation between anemia and local control in solid tumors indicate that lowered oxygen carrying capacity is related to increased local failure after radiotherapy for certain tumors [37–39]. In addition, misonidazole, which binds selectively in metabolically active hypoxic cells, binds in regions of some, but not all, of the small number of human tumors that have been examined [55]. The strongest evidence that hypoxic cells exist and affect the outcome of radiotherapy is the limited success of certain controlled clinical trials with hyperbaric oxygen [28] or with radiosensitizers [37, 44, 46, 56].

Clinical data in this area are difficult to interpret, as many of the trials have not been well controlled, or have been too small to have statistical power. Some trials have also used adjuvant doses or radiation schedules that are known to be suboptimal. The existence of negative trials (i.e., ones showing no statistically significant gain with adjuvant therapy) cannot, therefore, be taken as proof that hypoxia does not influence the efficacy of treatment. Conversely, some 'positive' trials cannot be taken as definitive proof that hypoxia limits the outcome of optimally-designed conventional therapy.

Despite the limitations of the clinical data, there are certain neoplasms for which there are multiple lines of evidence, from different institutions, which suggest that hypoxic cells exist and have therapeutic importance. For example, in both carcinoma of the cervix and certain carcinomas of the head and neck, hemoglobin levels affect local control, histology suggests the existence of hypoxic areas, and both radiosensitizers and hyperbaric oxygen have been shown to improve the results of radiotherapy. It seems likely that hypoxia is important in these neoplasms; they are, therefore, ideal candidates for clinical trials directed against tumor hypoxia.

Measuring hypoxia in animal tumors

While it is difficult to prove the existence of hypoxic cells in human tumors, there are several well-developed radiobiological techniques for assessing hypoxia in animal tumors. All of these hypoxic fraction assay techniques are based on comparisons of tumors irradiated under 'normal' conditions and tumors irradiated under 'complete artificial hypoxia'. Artificial hypoxia is created by temporarily clamping off the blood supply to the tumor or by asphyxiating the tumor-bearing animal with nitrogen; the latter technique is applicable only when tumor cells are excised after irradiation and assayed for cell survival in vitro or in new hosts [57]. The comparison of the radiation response of aerobic tumors and artificially hypoxic tumors can be based on assays measuring the survival of excised tumor cells (the paired survival curve technique), on analyses of the doses needed to control tumors (the clamped TCD_{50} technique), or on analyses of post-irradiation tumor growth (the clamped growth delay technique). The details of each of these techniques have been reviewed by Moulder and Rockwell [8].

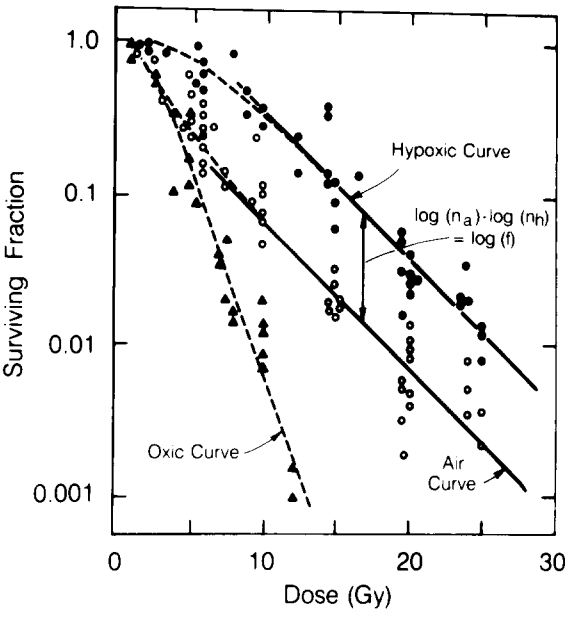

Fig. 1. Excision hypoxic fraction assay for EMT6 tumors. Cells irradiated in vitro under fully oxygenated conditions (▲). Cells irradiated in tumors in unanesthetized air-breathing mice (○). Cells irradiated under hypoxic conditions in situ and in vitro (●). Dashed lines are best fits to the individual data sets and assume multi-target single-hit kinetics. Solid lines are the best parallel lines that could be fitted to the air and hypoxic curves for doses of 10 Gy and above. Reprinted with permission from Moulder and Rockwell [8].

Paired survival curve (excision) assays

In paired survival curve assays, radiation survival curves are determined for cells from normally aerated and artificially hypoxic tumors (Fig. 1). The survival data are fitted with parallel lines [58] using Eqns (1) and (2):

$$\log(S_a) = \log(n_a) - \frac{[\log(e)][D]}{D_0} \quad (1)$$

$$\log(S_h) = \log(n_h) - \frac{[\log(e)][D]}{D_0} \quad (2)$$

where S_a and S_h are the survivals of cells from normally aerated and artificially hypoxic tumors; $\log(e)/D_0$ is the slope of the parallel lines; and n_a and n_h are the extrapolation numbers of the parallel lines. The hypoxic fraction, f, is derived from Eqn (3):

$$\log(f) = \log(n_a) - \log(n_h) \quad (3)$$

Clamped tumor control dose (TCD_{50}) assays

In clamped TCD_{50} assays, dose-response curves for local control of the tumors are determined for tumors irradiated under normally aerated conditions and for tumors irradiated with the blood supplies to the tumors clamped off (Fig. 2). Using probit analysis [59], the best parallel lines are fitted to the tumor control data, and the hypoxic fraction is calculated from Eqn (4):

$$f = e^{(D_{clamp} - D_{air})/D_{0,h}} \quad (4)$$

where $D_{air} - D_{clamp}$ is the difference between the parallel dose response curves and $D_{0,h}$ is the D_0 for naturally-occurring hypoxic cells.

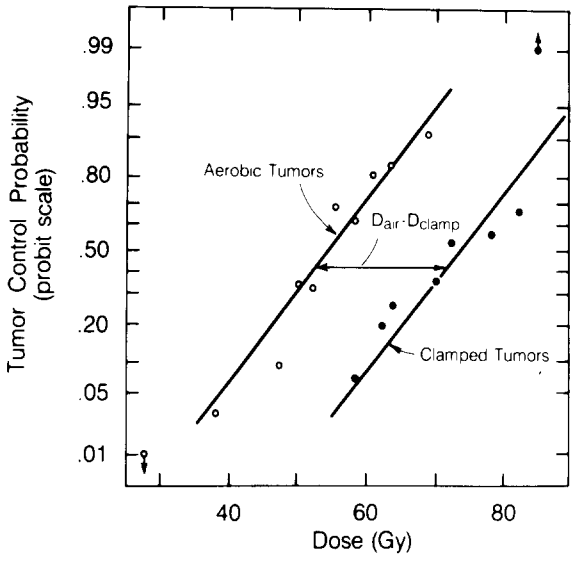

Fig. 2. Clamped tumor control hypoxic fraction assay for BA1112 tumors. Tumors were irradiated under normal aeration (○) or with the blood supply to the tumors clamped off (●). Solid lines are the best parallel probit [59] fit. Reprinted with permission from Moulder and Rockwell [8].

Clamped growth delay assays

In clamped tumor growth delay assays, tumors are irradiated under normally aerated and clamped conditions, and the size of each tumor is measured until it reaches a predetermined size (Fig. 3). The hypoxic fraction, f, can be determined by two different methods. In the 'delay difference' method, the hypoxic fraction is calculated from the increase in growth delay due to hypoxia using Eqn (5):

$$f = 0.5^{(T_{clamp}-T_{air})/T_d} \quad (5)$$

where $T_{clamp} - T_{air}$ is the delay (time) displacement of the growth delay curves, and T_d is the volume doubling time during the terminal portion of post-irradiation growth. The value of $T_{clamp} - T_{air}$ is determined by fitting parallel lines [58] to the dose-delay relationship for aerobic and clamped tumors using the dose range in which the dose-delay relationship is linear. In the alternative 'dose difference' method, the hypoxic fraction is derived from the increase in dose required to produce equal growth delay in normal and clamped tumors using Eqn (4), where $D_{air} - D_{clamp}$ is the dose difference between the parallel dose-response curves [58] and $D_{0,h}$ is the D_0 for naturally-occurring hypoxic cells. The choice of dose range used for the parallel fit and the choice of a delay or log(delay) relationship are based on minimizing the confidence interval on $D_{air} - D_{clamp}$.

Assumptions made in hypoxic fraction assays

All three of the standard radiobiological methods for measuring hypoxic fractions of animal tumors (paired survival curve, clamped TCD_{50}, and clamped growth delay) make numerous assumptions that may not be obvious from the equations described above. Some of the assumptions are implicit in all three techniques, while others are made in only some of the techniques. There are data which challenge the validity of almost every one of these assumptions.

Assumptions made in all hypoxic fraction assay techniques

The hypoxic fraction assay techniques all assume that the tumor irradiation techniques do not alter the hypoxic fraction. However, anesthesia is known to alter the radiation response in a number of tumors; the stress of restraining unanesthetized animals can also affect their radiation responses [60]. The effects of anesthesia and restraint can be tested or avoided using excision assays. However, the localized irradiation necessary for the in situ assays (TCD_{50} and growth delay) requires the use of either anesthesia or restraint; the effects of the two factors on in situ assays cannot, therefore, be assessed, except by observing whether the two procedures give the same results. Differential effects of restraint and anesthesia on hypoxic fractions have been found in most [61–64], but not all [61, 65], tumor systems tested.

All techniques assume that the methods used to induce hypoxia produce complete, uniform radiobiological hypoxia throughout the tumor mass. The production of complete artificial hypoxia can

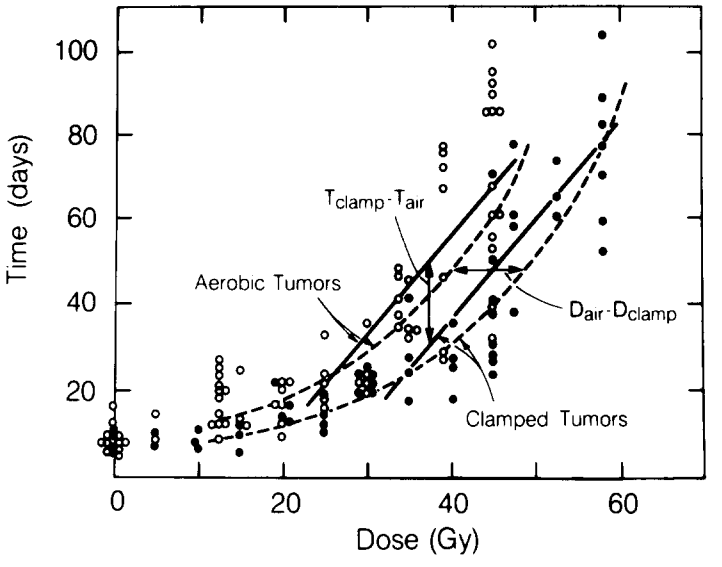

Fig. 3. Growth delay hypoxic fraction assay for BA1112 tumors. The time required for individual tumors to grow from 8 mm to 15 mm is shown for tumors irradiated under normal aeration (○) or with the blood supply to the tumors clamped off (●). Solid lines are the best parallel fit to the delay versus dose relationship; dashed lines are the best parallel fit to the log(delay) versus dose relationship. Adapted with permission from Moulder and Rockwell [8].

be a problem if the clamping or asphyxiation techniques are inadequate, if the duration of hypoxia prior to irradiation is too short, or if the tumors cannot respire oxygen to a level low enough to eliminate all oxygen sensitization [40, 41, 57, 66]. The adequacy of artificial hypoxia can be partially tested in excision assays by verifying that clamping and nitrogen-asphyxiation have the same effects [57, 60] and by comparing the in vivo survival curves obtained with these techniques with that obtained under maximum hypoxia in vitro. The adequacy of artificial hypoxia can be partially tested in all types of assays by determining whether altering the duration of artificial hypoxia has an effect on tumor response [67].

All techniques assume that the induction of artificial hypoxia does not alter cell viability, but producing complete artificial hypoxia without killing cells may present a serious problem. Significant cytotoxicity from artificial hypoxia has been demonstrated in tumors clamped for 2 hours or more [57, 68, 69]. In the Gardner lymphosarcoma [70], cell death occurs so rapidly in tumors of dead mice that it interferes with hypoxic fraction assays. The assumption that artificial hypoxia does not kill cells can be tested directly in excision assays, and can be tested indirectly in all assays by observing whether artificially hypoxic tumors become more radiosensitive as the duration of pretreatment hypoxia is increased.

In calculating a hypoxic fraction from radiation response data by any of these techniques, it is assumed that a tumor normally contains only two cell populations: aerobic cells with maximal radiosensitivity and hypoxic cells with maximal radioresistance. In fact, a spectrum of oxygen tension, and thus a spectrum of cellular radiosensitivity, should exist in solid tumors [71]. If a tumor contains cells with a range of radiosensitivities, the lower the radiation doses used in the assay, the greater the number of cells which will be measured as resistant. Because of this, excision assays (which use the lowest radiation doses) should measure more hypoxic cells than TCD_{50} assays (which use the highest doses), with growth delay assays (using intermediate doses) giving intermediate values.The two-compartment assumption has seldom been tested in either excision or in situ assays, as an adequate test requires that a wide range of doses, and an extensive data set at each dose, be available for analysis [72].

All assay techniques assume that naturally-oc-

curring hypoxic cells are hypoxic for a period of time that is long compared to the duration of irradiation; that is, they are chronically hypoxic. However, there is evidence for rapid fluctuations in blood flow in individual blood vessels within tumors, which could produce transient hypoxia persisting for only seconds or minutes [5, 73–76]. If tumor cells are hypoxic for periods of time shorter than the irradiation time, they will not be measured as fully resistant. There is, as yet, no direct evidence that transiently hypoxic cells exist in solid tumors, although there are experiments which provide indirect evidence for [5, 75–77] and against [62, 78, 79] their existence.

To calculate an accurate hypoxic fraction the survival of the oxic cells in the normally aerated tumors must be negligible at the radiation doses used in the experiment. This is not a problem for tumors with large hypoxic fractions. However, analysis of hypoxic fractions smaller than 0.2% by excision assay would require the measurement of surviving fractions of less than 0.005%; this cannot be achieved in many of the systems used to measure cell survival [8]. Similarly, this requirement cannot be satisfied in in situ assays if the hypoxic fraction is 0.01% or below, because of the statistical difficulty of distinguishing the measured dose-response curve for normally aerated tumors from the theoretical dose-response curve expected for a totally aerobic population [8].

The calculation of hypoxic fractions also presumes that the survival curves for naturally and artificially hypoxic cells are exponential at high radiation doses, and that they have the same slope and intercept. The requirement that the curves be exponential and parallel can be tested in excision assays, but there is seldom enough data over a wide range of doses for this test to be powerful [72]. The assumption that survival curves are exponential at high radiation doses is questionable, because there are cultured cell lines which do not show exponential survival curves [80–82]. The assumption that the survival curves have the same slope is questionable, because of the many in vitro studies showing that chronic hypoxia can alter cellular radiosensitivity [18, 83, 84]. The requirement that the survival curves for naturally and artificially hypoxic tumor cells have the same intercept in vivo cannot be tested directly, but there is in vitro [18, 85, 86] and in vivo [77, 87, 88] evidence that survival curves of chronically hypoxic cells have smaller shoulders than those of acutely hypoxic cells, presumably because of a reduced capacity to repair sublethal radiation damage. If the survival curves are not exponential or if naturally and artificially hypoxic tumor cells do not have the same intercept and slope, the calculated hypoxic fractions will be biased in a manner which depends on the actual shapes of the survival curves and on the dose range used in the assay.

An assumption made only in excision hypoxic fraction assays

Unique to excision assays is the assumption that the cell suspensions made from the normally aerated and artificially hypoxic tumors are representative of the initial tumor cell populations. Although tests have not been done to determine whether suspension selects for or against hypoxic cells, studies have shown that some dissociation procedures are selective for other tumor subpopulations [89]. Artificially high hypoxic fractions could be obtained if aerobic cells, located in a healthy stromal matrix, were harder to dissociate than were hypoxic cells located adjacent to necrotic areas, or if hypoxic cells which would have died if left in situ survive because they are removed from the tumor. Alternatively, hypoxic cells might be more fragile than aerobic cells and might therefore be selectively killed during the suspension process, or hypoxic cells might be less viable than aerobic cells under the relatively stringent growth conditions used to measure clonogenicity. The validity of this assumption can be tested indirectly by observing whether changes in the suspension method or the cell survival assay produce changes in the hypoxic fraction.

Assumptions made only in tumor control hypoxic fraction assays

To derive a hypoxic fraction from TCD_{50} data, one must assume that the same level of cell survival in a normally aerated tumor and a clamped tumor will give the same probability of tumor control. For this to be true, clamping must have no effects on the tumor, tumor bed, or vasculature that influence cell survival; this is more stringent than merely assuming that the induction of hypoxia kills no cells. Indirect tests of this assumption are to observe whether clamping affects the growth of an unirradiated tumor and to test whether clamping the blood supply before or after irradiation affects tumor control or post-irradiation tumor growth [57, 60].

The calculation of a hypoxic fraction from TCD_{50} data also requires the selection of a value for the D_0 of naturally hypoxic tumor cells remaining in situ after irradiation. It has been argued that this value can be derived from the slope of the tumor control dose-response curve [90]. However, this slope places only an upper limit on the hypoxic D_0, as experimental and biological factors contribute variability that decreases the slope of the dose-response curve [91, 92]. Where there are tumor control data for tumors of different sizes, it may be possible to derive the hypoxic D_0 by an indirect method [93–95]; however, such data are rarely available. For some tumors, hypoxic D_0 values can be estimated from excision assays or in vitro studies. This approach requires a consideration of the potential effects of differences in population structures and intracellular contacts in vivo and in vitro, and of repair of potentially lethal damage in cells remaining in situ, as neglecting these factors could lead to inaccurate estimation of the D_0 in vivo.

All in situ assays assume that the post-irradiation conditions have no differential effect on the survival probabilities of naturally-occurring and artificially hypoxic cells. This is a more stringent assumption than that required for excision assays, because artificially and naturally hypoxic cells remaining in situ are in different environments after, as well as before irradiation. Of particular concern is the possibility that post-irradiation hypoxia may influence the repair of potentially lethal damage [96]. If potentially lethal damage were repaired only in chronically hypoxic cells, then TCD_{50} assays would overestimate the hypoxic fraction [97]. If repair were inhibited in chronically, severely hypoxic cells, the hypoxic fraction would be underestimated [77, 88, 98].

Assumptions made only in growth delay hypoxic fraction assays

Any discussion of hypoxic fraction assays based on growth delay data is complicated by the lack of consensus on how such data should be analyzed [8]. One approach, the 'delay difference' method, analyzes the difference in growth delays for normal and clamped tumors given the dose of radiation. The other approach, the 'dose difference' method, analyzes the difference in the doses required to produce equal growth delays in normally aerated and clamped tumors. The two methods use different assumptions, and generally yield significantly different hypoxic fractions [8].

Both methods for analyzing growth delay data assume that clamping has no effects on cell proliferation and that differences in the post-irradiation environments of naturally and artificially hypoxic cells have no differential effects on the proliferation of these cells. These assumptions are more stringent than those made during TCD_{50} hypoxic fraction assays, as there must be no effects on cell proliferation, as well as no effects on cell viability. The assumption that clamping has no effect on tumor cell proliferation cannot be tested by direct observation, because an effect of clamping on a small tumor subpopulation, such as the hypoxic cells, might not be observed in tumor growth or cell proliferation studies. An indirect test of this assumption would be to observe whether clamping tumors after irradiation affects their growth. The assumption that the post-irradiation environment has no differential effects on cell proliferation cannot be tested in situ. There are, however, in vitro studies that show that chronic hypoxia, and also the low pH and nutrient depletion which probably accompany natural hypoxia in

vivo, can inhibit or prevent cell proliferation [18, 83, 99–101]. If naturally hypoxic cells proliferate more slowly in vivo after irradiation than do artificially hypoxic cells, the hypoxic fraction will be overestimated.

'Delay difference' analyses are based on the assumption that growth delay is determined solely by the level of cell kill. This assumption is brought into question by observations that equal cell survival (determined by excision assays) does not always produce equal growth delay [102] and that the density of cells within tumors changes during post-irradiation tumor growth [47]. For growth delay to be determined solely by cell survival, the tumor growth rate must be independent of the radiation dose. However, irradiation of the tumor bed can alter the growth of a tumor implanted after irradiation [103–107], and there are tumor systems in which the post-irradiation tumor growth rate has been shown to vary with the radiation dose [108–109].

One of the consequences of the assumptions underlying delay difference analysis is that the growth delay vs. dose curves for normal and clamped tumors should be linear and parallel at high doses; this is not true in some tumor systems [61, 110]. Another consequence of the assumptions underlying delay difference analysis is that the slope of the delay vs. dose curve can be used to calculate a D_0 for the hypoxic cells. While some growth delay studies yield D_0 values that are reasonable for hypoxic cells, others give values that are unrealistically high [8].

'Dose difference' analyses are based on the assumption that equal levels of cell survival in normal and clamped tumors will produce equal growth delays. This is a less stringent requirement than that underlying delay difference analyses, as the delay does not have to be proportional to cell kill, but must only be a continuous function of cytotoxicity. The assumption is more stringent than the requirement inherent in the TCD_{50} analysis that equal levels of survival give equal probabilities of tumor control, because of the added requirements concerning cell proliferation. Any effect of clamping on the tumor, tumor bed, or vasculature which alters cell viability or proliferation will compromise the validity of this assumption. Because the radiation doses for equal survivals in clamped and normal tumors are different, dose-dependent radia-

Table 1. Comparison of hypoxic fractions assayed by different techniques[a].

Tumor system	Excision assay	Tumor control assay	Growth delay assay		References
			Delay difference	Dose difference	
RIB5	15 (6–42)%	–	8 (2–34)% 8 (5–12)%	2 (1–20)% 0.05 (0–0.2)%	109, 111, 140
RIB5C	13 (7–24)%	–	0.1 (0.02–1)%	0.05 (0–3)%	102
MT	7 (4–10)%	>51%	–	–	116
EMT6 (lrg) EMT6 (sml)	20 (14–28)% 5 (4–8)%	–	72 (63–83)% 49 (42–59)	23 (11–42)% 7 (3–14)%	231
BA1112	21 (15–64)%	1 (0.1–9)%	2 (0.4–71)%	10 (5–19)%	62, 122
FSaII	17 (10–29)%	–	43 (27–71)%	7 (2–24)%	115, 237
RIF-1	1.2 (0.6–2)% 1.6 (1–2)%	–	20 (12–31)%	1 (0.4–2)%	5, 130, 131, 238
NR-S1	47 (28–76)%	–	1.4 (0.2–14)%	6 (2–15)%	239
'mamm ca'	–	3 (0.1–100)	22 (6.7–76)%	3 (0.2–60)%	112

[a] Hypoxic fraction with 95% confidence interval, see text and Moulder and Rockwell [8] for method of calculating hypoxic fractions.

tion effects on the tumor bed or vasculature will also affect the validity of this assumption. The assumption can be tested indirectly, by observing whether survival (measured by an excision assay) is the same under different conditions giving equal growth delays. This test has been performed in only a few systems; it can be satisfied in some cases [62], but not in others [111].

In all the discussions of growth delay assays which follow, we have chosen to make exclusive use of the dose difference method of analysis. There are three reasons for this choice. First, in cases where the same tumor line has been assayed at the same site and size by both an excision assay and a growth delay assay, the dose difference analysis agrees somewhat better with the results of the paired survival curve assays than does the delay difference analysis (Table 1). Excision assays and delay difference growth delay assays agree adequately only in RIB5 and FSaII, while excision assays and dose difference growth delay assays agree adequately in EMT6, BA1112, FSaII, RIF-1, and spontaneous mouse mammary carcinomas [112]. 'Adequate agreement' in this context means that the hypoxic fraction estimates disagree by less than a factor of 3. Neither method of analyzing growth delay data agrees with excision assay results in RIB5C and NR-S1. Second, some growth delay

Table 2. Additional[a] hypoxic fraction determinations for solid tumors.

Tumor system	Type of assay	Tumor size and site	Hypoxic fraction[b]	Reference
RIF-1[c]	excision	7 mm, id	0.8 (0.4–1.8)%	130, 131
	excision	7 mm, id	1.6 (0.8–3.2)%	
	delay	7 mm, id	0.9 (0.4–2.1)%	
MT[c]	excision	0.5 g, sc	6.2 (1.9–20)%	125
	excision	0,5 g, sc	7.9 (3.4–18)%	
R-1[c]	excision	12 mm, sc	43 (27–69)%	142
KHT[c]	excision	1.1 mm, pulm	4.3 (2.8–6.6)%	242
	excision	1.5 mm, pulm	14 (8.6–22)%	
C22LR[c]	excision	5–7 mm, sc	14 (10–20)%	240
MCA4[c]	TCD50	8 mm, im	25 (11–59)%	191
FSaII[c]	TCD50	4 mm, im	0.26 (0.08–0.75)%	115
	delay	7 mm, im	6.8 (1.8–2.4)%	
Ca G	TCD50	25 mg, id	3.6 (1.1–12)%	128
	TCD50	25 mg, id	7.6 (2.5–23)%	
	TCD50	25 mg, id	0.6 (0.2–1.8)%	
CA775	excision	0.1 g, sc	22 (10–48)%	226
	excision	1.1 g, sc	54 (31–92)%	
	excision	2.9 g, sc	52 (31–88)%	
NR-S1	excision	8–10 mm, sc	47 (28–76)%	239
	delay	8–10 mm, sc	5.7 (2.1–15)%	
'mammary ca'	TCD50	4 mm, im	40 (25–69)%	115
	delay	7 mm, im	28 (11–69)%	
'mammary ca'	TCD50	5–10 mm	3 (0.1–100)%	112
	delay	spontaneous	3 (0.2–60)%	

[a] Not covered in Moulder and Rockwell [8]; [b] Calculated as described in the text, with 95% confidence intervals; [c] Other determinations for this tumor system are included in Moulder and Rockwell [8].

data [43, 110] do not meet the requirement that the dose vs. delay curves for normal and clamped tumors be linear and parallel; these data sets cannot be analyzed by the delay difference method. Third, delay difference analyses yield hypoxic D_0 values of greater than 10 Gy in some tumor systems [8], implying that factors others than those assumed in the analysis are influencing the response of the tumors. The tenous nature of some of the assumptions required in the delay difference analysis and the problematic nature of the results obtained with this analysis therefore suggest that this analytical approach is inferior to the dose difference analysis.

A survey of hypoxic fractions of transplanted rodent tumors

In 1984 we surveyed existing data on the hypoxic fractions of transplanted solid rodent tumors [8]. That review covered 92 hypoxic fraction determinations in 42 tumor systems. Since that review was written, we have collected data on 12 more hypoxic fraction assays on tumor lines that were included in the review, plus 10 hypoxic fraction assays on 4 additional tumor systems (these are listed in Table 2). These 114 hypoxic fraction determinations in 46 transplanted rodent tumor systems form the basis of the analyses to follow.

Fig. 4 shows the distribution of hypoxic fraction values for macroscopic subcutaneous (sc) and intradermal (id) tumors. Tumors assessed by in situ assays (tumor control or growth delay) show a wider range of hypoxic fractions than those assessed by excision assays. For both excision and in situ assays, the most common value for the hypoxic fraction is 10%, with 65% of the excision assays and 50% of the in situ assays compatible with such a value. Relatively few data sets are compatible with hypoxic fraction values greater than 50%. Very few excision data sets are compatible with hypoxic fraction values below 1%, but a substantial number of the in situ data sets are compatible with hypoxic fraction values as low as 0.5%.

There is evidence for the presence of severely hypoxic cells in all but 11 of the 114 determinations (Table 3). Three of the 11 negative determinations are for very small tumors from tumor lines that had significant hypoxic fractions at larger sizes. Two of the 11 negative determinations are for growth delay

Table 3. Determinations not showing a significant hypoxic fraction.

Tumor	Reference	Assay type	Tumor size and site	Maximum[a] hypoxic fraction	Comments
'Mammary ca'	230	Tumor control	0.13 mm pulm	23%	Significant hypoxic fraction at larger sizes
Lewis Lung	224	Excision	1 mm pulm	0.5%	
B16	228, 229	Excision	1.2 mm pulm	6.0%	
RIB5	140	Growth delay	8–10 mm sc	0.24%	Sigificant hypoxic fraction by excision assay
RIB5C	102	Growth delay	8–10 mm sc	3.3%	
9L	113	Excision	0.05 g ic	0.35%	Significant hypoxic fraction in another assay and in sc sites
CBA leuk	241, 242	Excision	Liver	1.5%	Not true solid tumors; selected on the basis of histologic evidence of good oxygenation
Leuk Th	153	Excision	infiltrate	0.90%	
Sarcoma S	43	Growth delay	8.5 mm sc	5.5%	
'slow'	[b]	Tumor control	8–10 mm sc	0.5%	Only a lower limit could be placed on the clamped TCD_{50}
S102F	[b]	Tumor control	8–10 mm sc	0.01%	

[a] Largest fraction of severely hypoxic cells that is statistically compatible with the data; [b] Personal Communication, Lyle Dethlefson, December 10, 1979.

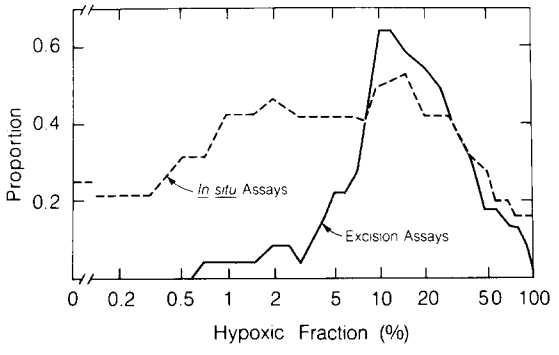

Fig. 4. Proportion of tumors for which measured hypoxic fractions are statistically compatible (95% confidence intervals) with a given value. Values are for macroscopic subcutaneous and intradermal tumors from Moulder and Rockwell [8] and Table 2. Separate distributions are shown for excision assays (solid line) and in situ (dashed line) assays. For tumors assayed more than once, the most extreme confidence interval was used. When tumors were assayed by both excision and in situ techniques, these were included in both distributions.

assays of tumors that showed significant hypoxic fractions in paired survival curve assays. The 9L gliosarcoma lacked detectable hypoxic cells in one intracerebral assay [113] but had significant numbers of hypoxic cells in another intracerebral assay [114] and in subcutaneous sites [65].Thus 41 of the 46 tumors were found to contain significant numbers of hypoxic cells in at least one study. Two of the five exceptions are leukemic liver infiltrates; it can be argued that they are not true solid tumors. Another system for which there is no evidence of hypoxic cells is Sarcoma S [43], but a hypoxic fraction as high as 5% would be statistically compatible with the existing data on this tumor. The remaining lines for which there is no evidence for hypoxic cells are the mammary carcinoma lines of Dethlefson; the 13 other transplanted mouse mammary carcinoma lines surveyed all show evidence for hypoxic cells. It would therefore appear that radiobiologically hypoxic cells are present in the vast majority of solid, macroscopic, transplanted rodent tumors.

Multiple measurements of hypoxic fraction in the same tumor system

When hypoxic fractions determined from excision assays are compared to those determined by 'dose difference' growth delay assays, the values from growth delay assays are significantly lower in some tumor systems, and the two values are compatible in the remaining systems (Table 1). A direct comparison of growth delay and tumor control assays has been performed only with BA1112, where the tumor control assay gives a significantly lower hypoxic fraction. FSaII and a mouse mammary carcinoma [115] have been assayed by both growth delay and tumor control, but different tumor sizes were used in the two assays (Table 2). The two assays are in reasonable agreement for the mammary carcinoma, but for FSaII, the tumor control assay gives a significantly lower hypoxic fraction. Excision and tumor control assays have been compared only in MT and BA1112 (Table 1); in both tumor systems the two values are significantly different, but the differences are in opposite directions in the two systems.

There is no obvious pattern to the disagreement between the different types of hypoxic fraction assays, except for the tendency of growth delay assays to give lower hypoxic fractions than excision assays. Disagreements between tumor response endpoints have also been seen for measurements of oxygen enhancement ratios [102], enhancement by hypoxic cell radiosensitizers [116], neutron relative biological effectiveness [47, 117, 118], cancer drug sensitivity [119, 120] and radiation repair capacity [117, 121]. Clearly more comparisons of assay techniques are needed if we are to hope to explain the disagreements.

While hypoxic fraction measurements done with different techniques often disagree, replicate excision assays generally do agree, even when different techniques are used to measure cell survival. Agreement between different types of survival assays has been seen in comparisons of endpoint dilution assays with in vitro assays in RIB5 [111] and BA1112 [62, 122], in a comparison of the endpoint dilution assay with the lung colony assay in KHT [123, 124], in a comparison of the lung colony assay with an in vitro assay in Lewis Lung [88], and in a comparison of in vitro monolayer and soft agar techniques in MT [125]. In addition, when replicate hypoxic fraction determinations on the same tumor

line are conducted using the same cell survival assay, even in different laboratories, the results are generally compatible [126]. Replicate hypoxic fraction measurements with TCD_{50} assays have shown some variability [62, 127, 128], but the variability is less than that generally observed when different types of assays are compared.

Correlation of hypoxia with tumor and host characteristics

Despite the problems associated with hypoxic fraction assay techniques, it is possible to draw some general conclusions about the relationship between hypoxic fraction and tumor and host characteristics from an examination of 114 hypoxic fraction determinations listed in the previous review [8] and in Table 2. Because of the possible dependence of the hypoxic fraction on the type of assay, however, different types of assays must be assessed separately.

Hypoxic fraction dependence on tumor size and growth site

In 7 tumor systems, hypoxic fractions have been determined for tumors of different sizes, using the same type of assay (Fig. 5). In 6 of these 7 tumor systems, the hypoxic fraction increases with size; only in the 9L gliosarcoma [65] is hypoxic fraction independent of tumor size. In the six tumor systems that show a relationship between hypoxic fraction and tumor size, doubling the hypoxic fraction requires an increase in the tumor diameter by a factor of about 1.5, but the range is broad (1.2 to 3.0). This increase in diameter corresponds to a volume increase of a factor of 3, with a range of 1.6 to 35.

Most hypoxic fraction determinations have been performed on subcutaneous (sc) or intradermal (id) tumors; surprisingly, there have been no direct comparisons of these two sites. Direct comparisons in RIF-1 [5] and indirect comparisons in EMT6 [8] suggest that intramuscular (im) tumors may have lower hypoxic fractions than id or sc tumors. For EMT6 tumors in nude mice, lymph node tumors have higher hypoxic fractions than sc tumors of the same size [129]. In 9L, intracerebral tumors have lower hypoxic fractions than sc tumors of the same size [113, 114]. Tumors growing as pulmonary nodules are impossible to compare with tumors in sc, id, and im sites, because the tumors differ in size as well as site (Fig. 5). To further complicate matters, there is evidence for variation of the hypoxic fraction for sc [62] and id [130, 131] tumors grown in different anatomical sites. In short, there is strong evidence that the growth site influences the hypoxic fraction, but there are insufficient data to identify sites where hypoxic fractions are consistently high or consistently low.

Hypoxic fraction and host anesthesia

Three tumor systems have been directly tested to assess the effect of barbiturate anesthesia of the host on the hypoxic fraction. BA1112 [62] shows a nonsignificant decrease in hypoxic fraction with anesthesia in survival curve assays, and a significant increase with anesthesia in TCD_{50} studies. Both EMT6 [132] and 9L [65] show nonsignificant increases in hypoxic fraction with anesthesia. In other tumor systems, barbiturate anesthesia has been shown to increase tumor radioresistance [61, 63, 64], to decrease tumor radioresistance [61] or to have no effect [61]. It is easy to understand how anesthesia could have variable effects on hypoxic fractions in different experimental systems, as anesthetics have a variety of effects, including lowering host and tumor temperature, altering regional blood flow, and modifying cell proliferation patterns [60, 133].

Hypoxic fraction and tumor histology

No significant difference in the hypoxic fractions of sarcomas and carcinomas is observed when differences due to the type of assays and the tumor size and site are taken into account. This comparison lacks statistical power, however, as few sarcomas have been analyzed using in situ assays and few carcinomas have been analyzed using excision as-

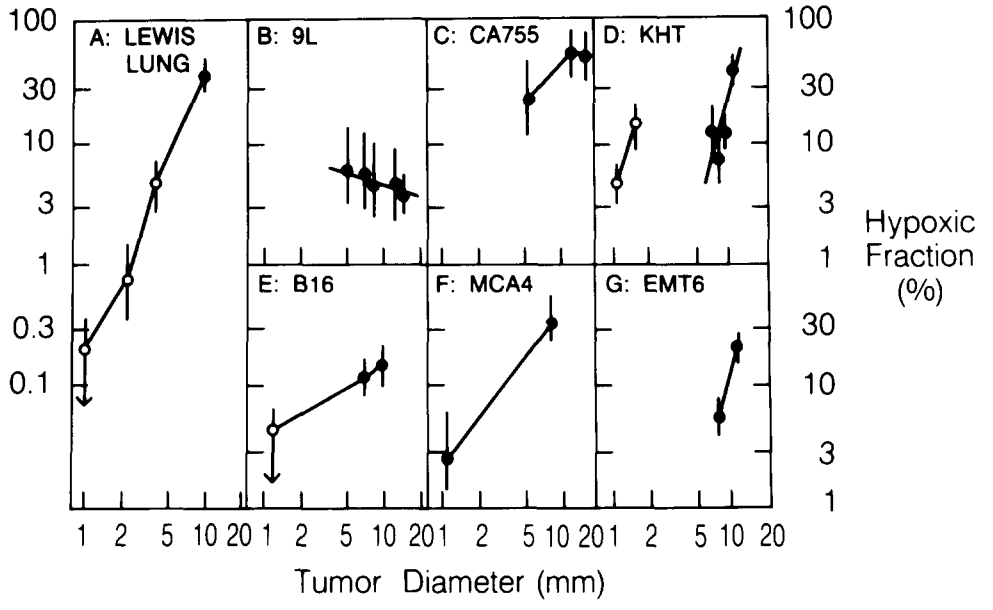

Fig. 5. Hypoxic fraction as a function of tumor size for:
A: Lewis Lung tumor as sc [88] tumors (●), and pulmonary [224, 225] nodules (○); B: 9L gliosarcoma [65] as sc tumors; C: CA775 mouse mammary tumors [226]; D: KHT sarcomas [67,123, 227] as sc tumors (●), and [242] pulmonary nodules (○); E: B-16 melanomas [228, 229] as sc tumors (●), and pulmonary nodules (○); F: Mouse mammary carcinomas [230] as sc tumors; G: EMT6 tumors [231] as im tumors.

says. There is a suggestion in the total data that well-differentiated tumors have higher hypoxic fractions than less differentiated tumors, but the number of well-differentiated tumors examined is sufficiently small that the difference is not statistically significant. Thorndyke *et al.* [134] have reported the reverse pattern in a pair of rat prostatic adenocarcinomas, with the well-differentiated tumor appearing to have the lower hypoxic fraction. The estimates of hypoxic fraction in the prostate adenocarcinomas, however, are based on differences in aerated tumor radiosensitivity, sensitization by a hypoxic cell radiosensitizer, and misonidazole binding, rather than by paired oxic/hypoxic response studies. An examination of the hypoxic fractions of mouse mammary tumors as a function of their transplant history (Fig. 6) provides no evidence that the hypoxic fractions of primary tumors and early transplants (which are generally more differentiated) differ from those of long transplanted and culture adapted lines (which are generally less differentiated). It would be extremely valuable if hypoxic fractions were determined for additional sets of well-differentiated and poorly-differentiated tumors, particularly if the determinations were done by the same technique and at the same site and size.

Hypoxic fraction and tumor origin, immunogenicity and transplant history

There is no significant difference in the spectrum of hypoxic fractions seen for tumors of truly spontaneous origin [135] and for tumors originally induced by chemical carcinogens. Similarly, there is no difference between the hypoxic fractions of mouse mammary carcinomas induced by mammary tumor virus and those of mouse tumors of spontaneous origin. The nature of the initial carcinogenic process seems to have no significant influence on the oxygenation of the resulting tumor lines. There is also no difference between the hypoxic fractions of immunogenic and non-immunogenic tumors. Thus while immunogenic tumors are unsuitable for certain types of studies [135–137], immunogenicity does not appear to influence oxygenation.

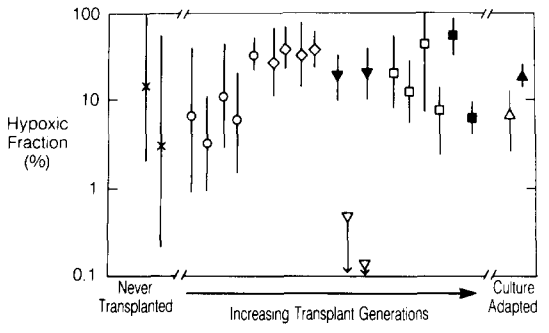

Fig. 6. Hypoxic fraction assays of mouse mammary carcinomas as a function of transplant history. In situ assays are shown as open symbols, excision assays as closed symbols. ×: autochthonous tumors [112, 158, 159]; ○: 1st generation tumors [127, 185, 186, 230]; ◇: 2nd through 5th generation tumors [115, 191, 232, 233]; ▽, ▼: 10th through 100th generation tumors ([68, 138] and L. Dethlefson, personal communication); □, ■: tumors passaged for more than 100 generations or more than 10 years [110, 116, 226, 234, 235]; △, ▲: a culture adapted line [139, 231].

When the entire data base is examined, there is no significant trend in the hypoxic fractions of tumors as a function of transplantation history. The hypoxic fraction does not differ systematically among tumors transplanted only once or a few times, tumors transplanted dozens of times, tumors transplanted for hundreds of generations, and tumor lines adapted for growth in vitro. The influence of transplant history can also be assessed by examining the 23 hypoxic fraction studies on mouse mammary carcinomas (Fig. 6). Although there is considerable variation among the hypoxic fractions of these tumors, there is clearly no trend wih transplantation history.

The impact of culture adaptation on the hypoxic fraction can also be assessed by comparing the hypoxic fractions of tumor lines before and after culture-adaptation. KHJJ [138] and its culture-adapted derivative, EMT6 [139] have the same hypoxic fraction, as do RIB5 [109, 111, 140] and its culture adapted version, RIB5C [102]. BA1112 [62, 93, 122] has the same hypoxic fraction as the Rijswijk subline of culture-adapted R-1 [47, 141], but other R-1 sublines [142, 143] have higher hypoxic fractions. It appears that the selection of a culture-adapted tumor cell line can result in a change in the hypoxic fraction, although this need not be true and no trend towards increasing or decreasing hypoxic fraction with culture-adaption is apparent. Culture-adapted tumors must therefore be treated as separate lines from their parent tumors in hypoxic fraction studies. This is not surprising, as the cloning and selection procedures associated with adaptation to growth in vitro can also select cell lines with growth rates, cell proliferation patterns, immunogenicities, and drug sensitivities different from those of the parent tumor line [60, 144].

Hypoxic fraction and tumor doubling time

Fig. 7 shows the relationship of hypoxic fraction to tumor volume doubling time. Analyses of the relationship of hypoxic fraction to tumor volume doubling time revealed no significant correlation, either when the entire data base was analyzed, or when the excision, growth delay and tumor control assays were analyzed separately. These analyses provide no evidence that tumor oxygenation varies with tumor growth rate. It should be noted, however, that the range of doubling times encompassed by these transplanted tumors is small (95% are between 1.5 and 6 days). Studies of slowly-growing transplanted tumors with doubling times of greater than 1 week would strengthen these analyses and facilitate extrapolation to human tumors which may have doubling times of months or even years [145].

Hypoxic fraction assays of xenografted human tumors

In principle, any of the techniques used to measure hypoxic fractions of solid rodent tumors could be applied to human tumors grown as xenografts in immune deficient animals. In practice, however, we have found only paired survival curve studies used with xenografts. We have found hypoxic fraction determinations published for six human melanomas [146–150], three colorectal tumors [148] and one pancreatic tumor [151]. All of these xenograft hypoxic fraction determinations were performed on subcutaneous tumors 5–8 mm in diameter. The median hypoxic fraction reported for the xeno-

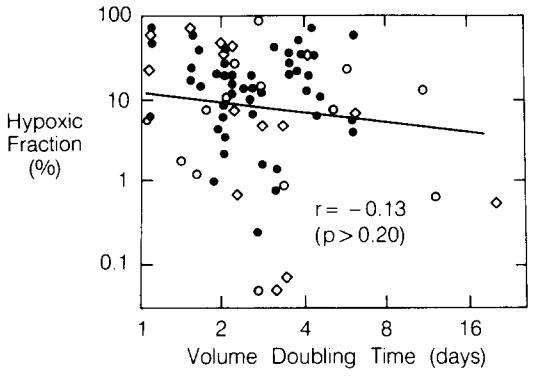

Fig. 7. Relationship of tumor volume doubling time to hypoxic fraction for excision (●), tumor control (◇) and growth delay (○) assays. Data are from Moulder and Rockwell [126] and Table 2. The solid line is the best fit to a log (doubling time) vs. log (hypoxic fraction) relationship; the r value is the correlation coefficient for this relationship.

grafts was 30%, with a range of 7.5% to 85%. The transplanted rodent tumors in our survey which were assayed by paired survival curve assays in subcutaneous sites at 5–10 mm in diameter have a median hypoxic fraction of 18%, with a range of 4% to 76%. While the xenografts tend towards slightly higher hypoxic fractions, the difference is not significant.

Hypoxic fraction assays of xenografted human tumors are interesting, but do not provide strong evidence for the existence of hypoxia in true human tumors, as hypoxia may be more a function of the stroma, the tumor-host relationship, and the hematologic characteristics of the host than an inherent characteristic of the malignant cells [4, 71, 152, 153]. As the vasculature and stroma in xenografts is derived from the host mouse and the tumor is supported by a mouse hematologic system [154, 155], the oxygenation of human xenografts in immune-deficient mice may be typical of mouse tumors rather than human malignancies. The use of the athymic nude mouse *per se* does not appear to affect hypoxic fraction assays, as shown by Guichard et al. [129] who studied the EMT6 tumor in athymic nude (BALB/c nu nu) mice and found a hypoxic fraction similar to that measured for EMT6 tumors in the normal immune-intact BALB/c host.

Hypoxic fractions of autochthonous rodent tumors

Only limited data are available on the radiation responses of autochthonous tumors in mice. Mottram's classic histologic studies on tar warts in mice [2, 3] showed regional variations in regrowth within irradiated tumors, with tumors regrowing from areas thought to be poorly oxygenated. Radiation growth delay studies of autochthonous mouse mammary tumors [156] and of methylcolanthrene-induced tumors in mice [157] show that the radiosensitivity of these tumors resembles that of transplanted rodent tumors with significant hypoxic fractions. It is not possible to derive hypoxic fraction estimates from these growth delay and tumor control studies, because data on the response of artificially hypoxic tumors are not available. This limitation does not apply to the work of Hawkes *et al.* [112], who assessed tumor cure and tumor growth delay in both normal and artificially hypoxic mammary tumors of C3H mice. The latter data imply hypoxic fractions of about 3% (Table 2); the confidence limits of the data are wide, but a value of zero can be excluded (i.e., these tumors do contain hypoxic cells).

An estimate of the hypoxic fraction of primary mouse mammary tumors can also be made by comparing the TCD_{50} for normally-aerated C3H mouse mammary tumors determined by Kallman and Tapley [158] with the TCD_{50} for clamped C3H mouse mammary tumors determined by Suit and Shalek [159]. This comparison yields a hypoxic fraction of 14 (2–89)%. The validity of this comparison is limited by the assumption that the experimental materials and techniques used in these experiments, which were run in different laboratories at different times, were sufficiently similar that the data are truly comparable. The limited radiobiological data on autochthonous rodent tumors supports the concept that these tumors do contain significant numbers of viable hypoxic cells.

The nature and effects of tumor hypoxia

Hypoxic fraction determinations on transplanted rodent tumors, xenografted human tumors, and

autochthonous mouse tumors provide evidence that tumors commonly contain hypoxic areas. The studies do not, however, tell us very much about the nature of tumor hypoxia, or about the effects of hypoxia on tumor cells. Hypoxia may have a variety of effects on cells other than the production of radiation resistance, and these effects may alter the response of the cells to treatment.

Chronic versus transient hypoxia

The rapid growth of malignant cells relative to the vasculature may lead to the development of avascular areas with chronic perfusion deficits. In the human bronchogenic carcinomas examined by Thomlinson and Gray [4], the tumors grew as cords in the well vascularized lung, with little or no vasculature within the tumor. The viable tissue within these avascular tumor cords never exceeded a radius of 200 microns, a distance similar to the distance that oxygen could diffuse from blood vessels at the edge of the cord through normally respiring tissue; beyond this distance the tumor was necrotic. Thomlinson and Gray [4] hypothesized that cells in the periphery of the cord were well oxygenated, those in the necrotic center were hypoxic, but dead, and those in the tumor cord near the edge of the necrosis were viable and hypoxic. Tannock *et al.* [160] studied the inverse situation in C3H mouse mammary tumors. These tumors developed extensive central necrosis. Individual blood vessels ran through the necrotic areas, each of them surrounded by a cord of viable tumor tissue. In this case, the cells in the center (near the vessel) would be well oxygenated, and those at the periphery, hypoxic. Either of these corded patterns would produce areas of chronic hypoxia. Most tumors show neither of these corded patterns; however, many do contain areas without functional vasculature, where necrosis develops. Analogy to the tumor cord situations suggests that the viable cells on the edge of these necrotic areas should be chronically hypoxic.

There are other mechanisms which may lead to hypoxia in tumors. Reinhold *et al.* [74, 75] found fluctuations in blood flow through individual tumor blood vessels which could lead to transient perfusion deficits lasting seconds to minutes. Sutherland [76] and Brown [5] suggest that such blood flow fluctuations could produce transient hypoxia in areas of tumor which appear to have adequate vasculature. There are data which support [5, 115] and which argue against [78, 79] the presence of acutely hypoxic cells in specific tumors. Acutely hypoxic cells would not be exposed to the microenvironmental inadequacies and low pH that accompany chronic hypoxia in vivo or to extended hypoxia before and after irradiation, and might not show some of the metabolic, kinetic, and radiosensitivity changes discussed in the next two sections. Acutely hypoxic cells could have a radioresistance similar to that of chronically hypoxic cells if hypoxia lasted throughout the duration of irradiation, but they might not always have the same chemosensitivity as chronically hypoxic cells, and they would not have the same implications for fractionated therapy. If some tumor hypoxia is due to chronic hypoxia and some to transient hypoxia, particularly if both types of hypoxia are present in the same tumor at the same time, some of the conflicting results obtained with different hypoxic fraction measurement tehniques (e.g. Table 1) could be explained.

The effect of hypoxia on cell proliferation

Many investigators have shown that hypoxia alters the proliferation of Chinese hamster cells in vitro, but the findings are somewhat contradictory. Decreasing the O_2 tension to 500 ppm has little effect on the population doubling time of V79 S171 cells [20, 161]. A further decrease to 200 ppm increases the doubling time; the cell population increases 2–4 fold, and then becomes quiescent. Severe hypoxia (<25 ppm O_2) causes the cells to accumulate in a state with G_1-like DNA content by flow cytometry, but with other characteristics (e.g., radiation dose-response curve and [^{3}H]-thymidine uptake after replating) resembling an asynchronous culture. In another line (CHL-F), hypoxia increases the cell cycle time, with a disproportionate lengthening of G_1 leading to a decrease in the labeling index; the

magnitude of the proliferative perturbation depends on the O_2 concentration [18]. In B-14-FAF-28 cells, hypoxia results in a gradual cessation of cell proliferation, with a 4–5 fold prolongation of S, and a 9-fold prolongation of G_1 [99]. In contrast, V-79-379A cells incubated in severe hypoxia are arrested immediately in all phases of the cell cycle; re-aerated cells have reduced rates of progression for many hours after re-aeration [162]. In another V79 subline, severe hypoxia inhibits progression through G_1 and S, but G_2/M cells complete division; hypoxic incubation was also found to kill S phase cells selectively [83]. In another CHO line, hypoxia has been reported to cause viable cells to accumulate in S phase [84].

In Ehrlich ascites mouse tumor cells [163, 164], hypoxia results is an accumulation of cells in G_1; this may reflect a lack of precursors for DNA and RNA synthesis secondary to respiratory insufficiency. In EMT6/SF cells [101], the induction of severe hypoxia does not alter the number of cells with S phase DNA content by flow cytometry, but suppresses the rate of DNA synthesis and the mitotic index. Protein synthesis is perturbed, cell number remains constant, and cell viability falls, but glucose utilization and lactic acid production remain constant. The proliferative and metabolic perturbations become more severe as the hypoxic incubation time increases from a few hours to 3 days. Hypoxic G_1 human NH1K 3025 cells are inhibited at the G_1/S border, while S phase cells are inhibited from progression both during hypoxia and after re-aeration; the degree of inhibition is dependent on the O_2 concentration [165].

While there is abundant evidence that hypoxia alters the proliferation of mammalian cells in vitro, data defining the proliferative status of hypoxic cells in vivo are more limited. It is clear that cells in solid tumors do not proliferate at their maximum possible rate, because malignant cells explanted from solid tumors and cultured under optimal conditions in vitro grow with shorter cell cycle times and higher growth fractions that those measured for the same tumor lines in vivo [144, 166]. Regional variations in labeling indices, mitotic indices, and other indices of cell proliferation have been observed in many tumors and may reflect microenvironmental effects on proliferation [166]. Cell cycle times in the center of tumors are generally longer and more variable than those on the peripheries of the neoplasms [166]. In corded mouse mammary carcinomas there is a progressive decrease in the mitotic index and the [^{3}H]-thymidine labeling index with increasing distance from the capillaries in the centers of the cords; the cell cycle time changes only slightly with distance, but the growth fraction decreases [167, 168]. These studies also suggest a non-random pattern of cell loss, with a continuous migration of cells towards the necrosis and a short lifetime for cells near the necrosis. After irradiation, more degenerative cells were found near the blood vessel than on the hypoxic periphery of the cord. In EMT6 tumors, which do not show a corded structure, the labeling and mitotic indices are lower, and the cell cycle longer, on the edges of necrotic regions than in areas without necrotic features [169]. A major limitation of these morphological studies is that they identify and examine only cells which are chronically hypoxic because they reside in areas of severe vascular insufficiency. The proliferative status and characteristics of transiently hypoxic cells, which are not assessed by such studies, could be quite different.

Several investigators have attempted to exammine directly the proliferative status of the hypoxic tumor cells which are clonogenic and survive irradiation. Kallman [170] examined the labeling index of microcolonies derived from EMT6 tumors which had been perfused with [^{3}H]-thymidine for 24 hours prior to explant. The changes in the labeling indices of these microcolonies after irradiation suggest that proliferating tumor cells are more radiosensitive than quiescent cells. This could reflect intrinsic differences in the radiosensitivity of proliferating and quiescent cells (e.g. because of cell cycle position) or could imply that quiescent tumor cells are more likely to be hypoxic. Similar studies using R-1 rhabdomyosarcomas show that cells surviving radiation are more likely to be quiescent than are clonogenic cells from unirradiated tumors [171].

In contrast, other data suggest that the population structures of hypoxic and aerobic cell populations are similar. Bateman and Steel [172] used a

hydroxyurea suicide technique to show that the proportion of clonogenic Lewis lung carcinoma cells in S phase is similar in irradiated and unirradiated tumors. Using the same approach, Rockwell *et al.* ([173] and unpublished data) found similar proportions of S phase clonogenic cells in irradiated and unirradiated EMT6 tumors. Pallavicini et al. [174] used Hoechst 33342 and flow cytometry techniques to sort and clone KHT tumor cell populations from different positions in the cell cycle. The primarily hypoxic cells which survived irradiation included a greater proportion of G_1 cells than did the viable population from unirradiated tumors. However, there were also significant numbers of hypoxic cells in S and G_2/M. Interpretation of all these data is complicated by the finding that solid tumors contain cells which have S phase DNA contents by flow cytometry, but which do not incorporate [^{3}H]-thymidine. This implies that tumors contain cells in S or G_2/M which are proliferating slowly or which are arrested in these phases [19, 87, 175]. If such cells proliferate rapidly upon resuspension and exposure to a better environment, they might rapidly acquire sensitivity to hydroxyurea and be measured as proliferating in the assay used by Bateman and Rockwell.

It is clear that hypoxia can inhibit cell proliferation in vitro. In addition, the environmental inadequacies which may accompany hypoxia in vivo (e.g. low pH, nutrient deficiencies) can also inhibit proliferation [100]. The proliferative perturbations depend on a variety of factors, including the nature, severity, and duration of the environmental inadequacy. Data on tumors in vivo are limited and ambiguous. It seems certain that some solid tumors contain viable hypoxic cells in S and G_2/M; it is not clear whether such cells are proliferating relatively normally, proliferating slowly or are arrested in these phases. The proliferative status of the hypoxic cells will affect their radiation response, because intrinsic cellular radiosensitivity, oxygen radiosensitization, and the ability to repair sublethal and potentially lethal damage all vary with cell age and population structure [18, 80, 82, 83, 98, 176, 177]. The proliferative status of hypoxic cells could similarly affect their chemosensitivity [9]. More studies in this area are needed.

Effects of hypoxia on cellular radiosensitivity and repair

The effects of hypoxia on cellular radiosensitivity and the repair of radiation damage are complex. The induction of acute hypoxia shortly before irradiation produces classical hypoxic radioprotection by removing O_2 and eliminating the radiosensitizing effect of this electron-affinic molecule. The amount of radioprotection depends on the concentration of residual oxygen at the time of irradiation [178, 179]. Survival curves for most mammalian cell lines irradiated in vitro under conditions of acute hypoxia are similar in shape to the corresponding survival curves for the same cells irradiated under aerobic conditions [18, 80, 83, 85, 139, 180, 181]. However, there are also reports that cells made exceedingly hypoxic in vitro have survival curves with smaller shoulders and accumulate less repairable damage than cells rendered only severely hypoxic [182].

Prolonged hypoxic incubation has additional effects which modulate cellular radiosensitivity. Incubation of cells in hypoxia for prolonged periods prior to irradiation has been reported to decrease the shoulders and increase the slopes of radiation dose-response curves [18, 83, 181, 183]; but increasing radioresistance has also been reported [84]. Changes in radiosensitivity persist, in some cases, after the cells are re-aerated [181, 183]. These changes may be due in part to alterations in the population structure during the hypoxic incubation and in part to thiol depletion during hypoxic incubation.

The oxygenation status of the cells after irradiation also influences their response to irradiation. Under certain circumstnces in vitro, post-irradiation hypoxia inhibits the repair of sublethal damage, the repair of potentially lethal damage, and biochemical repair processes [18, 85, 86, 98, 182, 184]. The effects of post-irradiation hypoxia are complex; the duration and degree of hypoxia, the proliferative status of the cells, the nutrient milieu, the glucose level, and the cell line all appear to influence the degree of repair inhibition. Under conditions of extreme hypoxia and glucose depletion, repair may be completely inhibited; less se-

vere deficits may allow some repair to occur [85, 86, 98].

The extrapolation of these in vitro data to predict the response of tumor cells in vivo is problematic. First, it is not clear whether hypoxic cells in vivo ever achieve the severe levels of oxygen depletion which produce the greatest effects on repair and radiosensitivity in vitro. Second, although poor nutritional status and low glucose levels would be expected to accompany hypoxia in vivo [71, 167], the severity of the actual deficits has not been well defined. In addition, transiently and chronically hypoxic cells would have different nutritional states and 'preincubation periods', and therefore different radiation responses. Moreover, the duration of post-irradiation hypoxia in vivo is unknown. As there is evidence in some tumors for rapid changes in blood flow and oxygenation after irradiation [74, 75, 130, 185–188], and for transiently hypoxic cells [5, 76], it is unclear whether hypoxic cells remaining in situ after treatment remain severely hypoxic or whether they become aerobic soon enough to influence repair processes.

It is difficult to define the radiation response of naturally hypoxic cells in solid tumors. The terminal slopes of the radiation dose-response curves for normally aerated tumors, which reflect the radiosensitivity of naturally hypoxic cells, are generally (but not always) compatible with the slopes of artificially hypoxic cells [8]. However, it is the shoulder of the survival curve which is most sensitive to hypoxic preincubation, and the shoulder of the dose-response curve for naturally hypoxic cells in vivo is difficult to determmine. Grdina [87] examined the radiation response of density-gradient separated cells from a fibrosarcoma, and found that the radiation survival curve of the naturally hypoxic cells had a smaller shoulder than the survival curve for aerobic cells made acutely hypoxic. Analogous studies in other tumor systems are clearly needed.

In vitro, repair of potentially lethal damage (PLD) is limited to quiescent cells [86, 132, 189]. As tumors generally contain more quiescent cells than hypoxic cells [180], one would expect tumors to contain both aerobic and hypoxic cells capable of PLD repair. Many tumors have been shown to repair PLD in vivo [62, 96, 111, 117, 132, 180, 189, 190]; large tumors appear to repair more PLD than small tumors [189]. Since the radiation doses used in most of these in vivo PLD repair experiments are large enough that essentially all surviving cells must have been hypoxic at the time of irradiation, these studies imply that some naturally-occurring hypoxic cells must be capable of PLD repair. In some tumors oxic and hypoxic cells appear to have similar PLD repair capacity [190], in other tumors PLD repair seems to occur primarily among hypoxic cells [116], and some comparisons of in situ and excision endpoints have been interpreted as implying that naturally hypoxic cells might have a reduced capacity for PLD repair [102, 126]. Tumor cells in vivo also repair sublethal damage, and they do so after doses high enough that most of the surviving cells must be hypoxic. There is evience in some tumor systems [88, 191] that naturally hypoxic tumor cells repair less sublethal damage than do aerobic cells, but the generality of this finding has not been established.

In summary, in vitro data clearly demonstrate that prolonged exposure of cells to hypoxia has a variety of effects on cellular radiosensitivity and on the repair of radiation damage, which are not seen when cells receive only an acute exposure to hypoxia during irradiation. The role of these phenomena in vivo remain unclear, and it is quite possible that different phenomena are important in different tumor systems and under different experimental conditions.

New methods for measuring hypoxic fractions

A number of new methods for assessing tumor hypoxia are under development [192, 193]. These techniques avoid some of the assumptions implicit in the radiation response assays described earlier in this review; however, they may have other implicit assumptions that are equally open to challenge. Many of these techniques are theoretically applicable to the assessment of oxygenation in individual human tumors, and are therefore of considerable interest.

Labeling of hypoxic cells

One approach to identifying hypoxic cells in solid tumors is to examine the binding of agents which are enzymatically activated to very reactive, short-lived alkylating species by reductive processes that occur more readily under hypoxic conditions [194]. This approach is of special interest, as only metabolically active cells can activate the prodrugs; the technique therefore identifies viable hypoxic cells, rather than dead cells. Nitroimidazole radiosensitizers (e.g., misonidazole) are one such class of compounds. Cell culture studies show that hypoxic cells in culture bind more radiolabeled misonidazole than do aerobic cells [195]. Observations on spheroids show increased misonidazole binding in the rim of viable cells near the central necrosis, the area expected to contain viable hypoxic cells [196]. Mouse tumor studies suggest that the amount of misonidazole binding varies with the hypoxic fraction [197]. Autoradiographic studies of the distribution of labeled misonidazole in human [55] and in rodent [193, 198] tumors show spatial variations in labeling intensity which may correspond to aerobic and hypoxic areas.

Brominated misonidazole has also been tested as a hypoxic cell label; this raises the possibility that positron emission spectroscopy (PET scanning) could be used to assess human tumor hypoxia [199, 200]. An alternative to using radiolabeled nitroimidazoles is the use of fluoresent nitroheterocyclics [201] or fluorescent dyes which bind specifically to hypoxic cells [202–204]. A related technique assesses regional variations in tumor hypoxia by monitoring variations in the fluorescence of endogenous pyridine nucleotides [205].

These techniques are not without problems. Factors other than hypoxia (e.g., position in the cell cycle, intracellular and extracellular pH, and levels of specific cellular reductases) can modulate the metabolism of these compounds and affect the accumulation of label [203, 206–208]. The change in drug binding with oxygen concentration may not be the same as the change in radiosensitivity with oxygen concentration, so that the cell population which binds the drug may not be identical with the population that is radioresistant. Interestingly, some normal tissues, including liver and regenerating marrow, are highly labeled after treatment with labeled misonidazole [198, 207].

Oxygen microelectrodes

Oxygen electrodes have been used to examine pO_2 within solid tumors [209–211]. This technique is limited by the fact that it is invasive, measures average O_2 concentrations over a relatively large volume of the tumor, and cannot readily be used to identify or isolate hypoxic cells. The technique has the advantage, however, that it can be, and indeed has been, used to monitor oxygen within human tumors [31, 52]. There are many problems yet to be solved for oxygen microelectrodes to be of routine practical use; these include ensuring that the electrode itself does not alter oxygen levels, and developing sensitive systems capable of making accurate and reproducible measurements over the range of oxygen tensions producing different degrees of radioprotection.

Blood flow dyes and tracers

Studies with a variety of dyes, microspheres, and radiolabeled tracers have been used to assess regional deficiencies and variations in blood flow within tumors [12–14, 212]. Several of the tumors studied show striking intratumor inhomogeneities in the distribution of dyes such as Lissamine Green. Soon after injection some areas of the tumor are intensively stained and other areas totally unstained. Normal tissues, in contrast, show more uniform staining patterns. Some unstained areas in tumors appear to be composed of healthy tumor tissue rather than necrosis; one would expect that these areas would contain viable, hypoxic cells.

Resonance Spectroscopy

Nuclear magnetic resonance (NMR) measurements of ^{31}P are capable of determining intratumor pH, and of measuring cellular ATP and inorganic

phosphate levels, suggesting that NMR techniques might be used to determine the type of energy metabolism (oxidative or anaerobic) being used by a tumor [213–217]. It is also possible that ^{19}F spectroscopy could be used to detect the binding of fluorinated nitroimidazoles to hypoxic cells [218]. Currently, however, ^{31}P and ^{19}F NMR can be assessed only in relatively large regions (about 1–2 cm^3), and imaging is not yet possible.

Electron spin resonance (ESR) is capable of detecting and measuring oxygen tensions in cellular systems [219], but the cavities of standard ESR spectrometers are too small for practical animal experiments. It has been suggested that larger capacity ESR spectrometers could be developed, and that imaging of O_2would be possible with this technique [220].

Photometric techniques

A technique has been developed which uses cryomicrophotometry to measure the oxyhemoglobin saturation of individual erythrocytes in frozen tissue samples [221]. The technique has been used to assess oxygen tensions in unperturbed tumors and tumors of animals breathing hyperbaric oxygen [222, 223]. The technique requires the removal of a sample of the tumor, but it has been applied to human tumors [54].

Conclusions

1. Evidence that radiation resistant hypoxic cells exist in most transplanted rodent tumors is direct and convincing, but the quantitative accuracy of current techniques for assessing hypoxia in animal tumors is open to question.

2. Evidence for hypoxia in human tumors is indirect and qualitative, but convincing. There is nothing in the studies of hypoxia in transplanted and autochthonous rodent tumors or xenografted human tumors to suggest that tumor hypoxia is an artifact, that it is restricted to rodent hosts, or that it is unique to transplanted tumors.

3. Evidence that the efficacy of the radiotherapy and chemotherapy regimens currently used in the treatment of human tumors is adversely affected by the presence of tumor hypoxia is limited. Direct evidence, in the form of replicated, unambiguously successful, well-controlled trials of an 'anti-hypoxic cell' modality, has yet to be presented.

4. There are no proven techniques for assessing tumor hypoxia in individual human tumors. Some interesting techniques are under development, but many problems remain.

5. Some commonly made assumptions about the nature of tumor hypoxia are clearly wrong. The observation that replicate hypoxic fraction measurements using the same technique agree, but measurements using different radiobiological techniques disagree, forces us to conclude that some of the assumptions behind the techniques are faulty. The problem could be in an assumption common to all techniques, the failure of which affects different assays in different ways, or it could be in assumptions that are made in only some of the techniques.

6. The most questionable of the assumptions made in the radiobiological hypoxic fraction assays, and in some of the new assays under development, involve the nature and radiation response of the naturally-occurring hypoxic tumor cells.

7. Tests of more of the assumptions made in hypoxic fraction assays are needed in more tumor systems. Comparison hypoxic fraction studies are also needed in additional tumor systems, including comparisons among the three radiobiological assays and comparisons of the radiobiological hypoxic fraction assays with the newer techniques.

Key unanswered questions

1. What is the nature of naturally-occurring hypoxic tumor cells? Are they chronically or transiently hypoxic? Are they proliferating or quiescent? How does their microenvironment and metabolism differ from that of aerobic tumor cells?

2. How do naturally-occurring hypoxic tumor cells respond to radiation? Do they have the same ability to repair sublethal and potentially lethal damage as oxic cells? How does their radiosensitivity differ from that of cells artificially made acutely hypoxic?

3. What factors determine the hypoxic fractions of tumors? Does hypoxic fraction depend on tumor type, degree of tumor differentiation, tumor growth rate, tumor growth site, or other tumor and host characteristics?

4. Do hypoxic cells exist in any human tumors? Do they exist in all human tumors? What fraction of a human tumor is radiobiologically hypoxic?

5. Do hypoxic cells influence the outcome of conventionally fractionated radiotherapy regimens for some or all human tumors? If so, can this be overcome by appropriate adjunctive therapy?

Acknowledgements

Research in the authors' laboratories was supported by US National Cancer Institute grants CA27084 (JM) and CA35215 (SR) and by American Cancer Society Grant PDT145 (SR). Artwork was prepared by Carole Russell Hilmer.

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