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Hypoxic Fractions in Xenografted Human Colon Tumors[1]

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ABSTRACT

We investigated the percentage of radiobiologically hypoxic cells within 11 different xenografted human colon tumors using an *in vivo-in vitro* excision assay technique. Tumors were excised at average volumes of 750 mm^3, and it was found that hypoxic fractions varied from less than 1% (clone D) to over 80% (HCT-8). The geometric mean hypoxic percentage was 10.4% (95% confidence interval, 4.9 to 22.1%). Comparison of the percentage of hypoxia results from the xenografted human colon tumors to published data from xenografted melanomas suggests that transplanted colorectal tumors as a class contain significantly less hypoxia than do the melanomas.

INTRODUCTION

The presence of hypoxia cells within solid neoplasms has been extensively documented (1, 2). Hypoxic cells limit the curability from radiotherapy (3, 4) and possibly also from chemotherapy (5, 6). Consequently, much experimental research has been devoted to quantification of the extent of hypoxic cells within neoplasms. Many of these efforts have utilized *in vivo-in vitro* paired survival curve bioassays, wherein solid tumors are irradiated with graded doses in either air-breathing hosts or hosts that have been nitrogen gas asphyxiated immediately prior to exposure to create complete intratumor hypoxia. The clonogenic survival responses of the irradiated cancer cells are then assayed *in vitro* by disaggregating the tumors and plating single cells. If significant numbers of hypoxic cells are present in the neoplasms taken from the air-breathing hosts, a biphasic (concave upward) shape will be observed in the dose-response curve, representing the mixture of oxic (radiosensitive) and hypoxic (radioresistant) cells. The hypoxic (high dose) portion of the curve from the tumors in the air-breathing hosts will parallel the dose-response curve for cells taken from the nitrogen-gassed (100% hypoxic) tumors, and the percentage of hypoxic cells in the mixed tumors can then be estimated by the degree of displacement between the two survival curves (7–9).

A variation on this approach has been the study of hypoxic fractions of xenografts produced by human tumor cells transplanted into immunodeficient ("nude") mice or rats. The validity of this technique has been discussed and reviewed (9–12), and no compelling reasons not to accept results from such studies have been found, with the obvious caveat that one is investigating the physiology of human cancer cells growing in a murine host. Data exist on hypoxic fractions within xenografted human melanoma (13–21), colorectal (16, 22, 23), ovarian (24), bladder (25), lung (26), pancreatic (27), and squamous cancers of the oral cavity (28). The most extensive data exist for melanoma (10), where hypoxic cell percentages range from about 6 to 85% (geometric mean, about 30%; 95% confidence interval, about 14 to 62%).

With regard to human colorectal cancer, there have been 6 previous investigations of hypoxia within xenografted tumors (16, 22, 23). In these studies, hypoxic fractions ranged from less than 1% to about 80 to 85%, demonstrating that substantial variability exists in the expression of hypoxia within human colorectal neoplasms. To increase the data base on hypoxia in xenografted neoplasms, we have determined the hypoxic status of 11 different xenografted human colorectal cell lines. The results indicate that the distribution of hypoxic percentages within human colorectal tumors may be significantly less than that observed in human melanoma.

MATERIALS AND METHODS

Cells. The human colon tumor cell lines used in this work were obtained as frozen stock from the American Type Culture Collection, Rockville, MD, with the exceptions of clone A and clone D which have been in our laboratory for a number of years. For these experiments, the clone A and D lines were freshly established from stock cultures frozen in liquid nitrogen. Cell lines were maintained in RPMI-1640 medium with 10% fetal bovine serum, 1% anti-pleuropneumonia-like organism agent, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 1% $NaHCO_3$, and 0.04% Gentamicin (all cell culture reagents were purchased from the Grand Island Biological Co., Grand Island, NY).

Production of Xenografted Tumors. Male nude mice (7 wk of age) were obtained from the Charles River Breeding Laboratories, Wilmington, MA. Mice were quarantined for 1 wk and housed (5/large cage, with dust covers) in a laminar flow hood (Thoren Industries, King of Prussia, PA) in a dedicated animal room in the Animal Care Facility of Brown University. To produce solid tumors, 1×10^7 human colon cancer cells taken from exponentially growing cultures were suspended in Hank's basic salt solution and injected into the right flank regions of mice in a total volume of 0.25 ml (one tumor/mouse) (22). Mice receiving injections were then ear tagged and randomly assigned to various treatment groups.

Measurement of Tumor Volumes. Tumor volumes were determined using the formula for a prolate ellipsoid, as we have previously described (22). All tumor volume measurements were done by a single individual. Growth curves were used to determine equivalent tumor volumes for the assay of intratumor hypoxia and to determine tumor doubling times.

Hypoxic Fraction Bioassay Using the Paired Survival Curve Technique. For determination of hypoxic fractions, the parallel line excision bioassay technique was used. The assumptions and limitations of this approach have been described (7–9, 12), and we have used this technique in a number of previous studies with xenografted tumors (*e.g.*, Ref. 22). Complete survival curves were generated, and the hypoxic fractions were determined from the relative survival of cells irradiated with high doses of either air-breathing or nitrogen gas-asphyxiated (10 min) tumor-bearing mice. Irradiations of solid tumors were performed with a Philips (Einthoven, the Netherlands) 250-kVp therapeutic X-ray machine operated at 250 kVp and 15 mA. For irradiation of tumors in air-breathing animals, mice were briefly anesthetized with Metafane (Pitman-Moore, Philadelphia, PA), restrained on a Lucite irradiation platform, and allowed to fully recover from anesthesia before irradiation. Two mice were exposed at a time at ambient temperature, and the trunks of the animals were shielded with 2 mm of lead. The target-to-skin distance was 39 cm. Exposure doses were measured with an exposure meter (Victoreen Co., Cleveland, OH) and converted to

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Table 1 *Parameters of disaggregation, in vivo growth, and hypoxia of xenografted human colon tumors*

	Cell yield (cells/mg × 10^4)	Colony-forming efficiency (%)	Doubling time (days)[a]	Latency (days)[b]	Hypoxia (%)
Clone D					
Oxic	2.11 (0.10)[c]	37.6 (9.1)[c]	2.9	5.9	0.12 (0.02–0.72)[c]
Hypoxic	2.07 (0.14)	34.2 (6.2)			
Clone A					
Oxic	1.83 (0.11)	34.2 (9.7)	2.5	6.3	3.0 (1.8–5.0)
Hypoxic	1.65 (0.09)	32.1 (8.3)			
LOVO					
Oxic	2.57 (0.73)	4.45 (1.8)	5.0	6.6	7.60 (4.9–11.8)
Hypoxic	1.87 (0.84)	5.32 (3.2)			
HCT-15					
Oxic	3.40 (0.97)	11.2 (3.7)	6.0	2.0	8.90 (4.6–17.4)
Hypoxic	2.32 (1.16)	14.1 (4.4)			
COLO 320HSR					
Oxic	5.96 (2.03)	3.52 (1.5)	3.3	17.4	9.80 (5.9–16.3)
Hypoxic	7.11 (2.68)	4.11 (2.9)			
SW620					
Oxic	3.28 (0.89)	28.3 (7.0)	3.8	12.1	13.9 (5.3–36.4)
Hypoxic	3.80 (0.61)	23.1 (5.2)			
WiDR					
Oxic	1.51 (0.43)	16.0 (5.4)	5.1	3.5	14.0 (8.3–23.7)
Hypoxic	1.79 (0.22)	14.1 (8.0)			
SW480					
Oxic	2.85 (0.59)	11.0 (4.5)	9.2	8.6	15.3 (8.1–28.8)
Hypoxic	3.19 (0.60)	10.7 (3.2)			
HT-29					
Oxic	2.39 (0.92)	7.77 (3.9)	6.1	11.2	16.8 (9.2–30.6)
Hypoxic	2.52 (1.54)	6.98 (4.1)			
LS174T					
Oxic	2.68 (0.76)	16.8 (8.4)	3.3	5.1	18.5 (10.0–34.1)
Hypoxic	2.24 (0.67)	18.8 (7.6)			
HCT-8					
Oxic	3.66 (1.43)	13.6 (4.3)	5.5	6.8	82.1 (46.8–144.2)
Hypoxic	4.84 (2.34)	15.9 (4.1)			

[a] Doubling times were determined for tumors at the time of excision for determination of intratumor hypoxia levels.
[b] Latent period defined as the time for implanted tumor cells to growth to an average volume of 100 mm^3.
[c] Numbers in parentheses and the ranges on the hypoxic percentages, 95% confidence interval (31).

absorbed doses using appropriate temperature, pressure, and Roentgen-Gy factors (29). The dose-rate was about 1 Gy/min.

After irradiation, tumors were immediately excised and placed into ice-cold Hanks' basic salt solution, weighed, chopped into 1-mm^3 fragments, and disaggregated at 37°C for 40 min using an enzyme cocktail of 0.2% RNase-free DNase (Sigma Chemical Co., St. Louis, MO), 0.25% collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 0.25% nuclease-free neutral Pronase (Calbiochem Corp., San Diego, CA) in complete RPMI-1640 medium without fetal calf serum. The digestate was then filtered through an 80 μm rectangular stainless steel mesh and pelleted (1000 rpm, 10 min, 4°C). After resuspension, cells were counted using phase-contrast microscopy, and appropriate numbers of cells were seeded into 60- or 100-mm plastic dishes (all plasticware from B-D Labware, Trenton, NJ) at several dilutions for enumeration of survival by clongenicity. Typically, there were 8 to 10 individual tumors assayed per dose point per condition (*i.e.*, oxic or hypoxic) for each tumor line.

As we have recently shown (30) that some of these human colon tumor cell lines require heavily irradiated (30 Gy, 10 Gy/min) "feeder cells" (Model 68A ^{137}Cs source; J. L. Shepard & Co., Glendale, CA) for optimal CFE,[3] we added FCs when needed to bring the minimal number of cells/60-mm dish to 10^5. Colonies were allowed to develop at 37°C in a humidified incubator under an atmosphere of 5% CO_2 and 95% air for 10 to 14 days, after which time colonies were fixed and stained with 0.5% crystal violet in absolute methanol. Colonies were inspected visually to ensure that no counting bias was incurred by the presence of giant cells.

RESULTS

In Table 1, we list the *in vitro* (cell yields and CFE) and *in vivo* (latencies, doubling times and calculated hypoxic percentages) values found for the various disaggregated tumors.

Although absolute cell yields vary among different tumor lines, there were no differences in yield for oxic *versus* hypoxic tumors. The mean cell yield was 2.98 × 10^4 cells/mg (95% confidence interval, 2.02 to 3.94 × 10^4 cells/mg).

There was significant variation in the CFEs among colon tumor lines. Again, the condition of imposed hypoxia had no effect upon the CFE. The mean (log) CFE from the 11 tumors was 13.2%, with 95% confidence interval from about 8.0 to 21.7% (31). As noted in "Materials and Methods," CFE values were obtained using heavily irradiated FCs for all tumor lines because, of the 11 colon tumor lines studied, 4 (WiDR, LS174T, LoVo, and HT-29) show significant increases in CFE when FCs are used (30).

[3] The abbreviations used are: CFE, colony-forming efficiency; FCs, feeder cells; n, extrapolation number (back extrapolation of the linear portion of an X-ray survival curve to the y-axis); D_q (Gy) or quasithreshold dose, intersection of the back extrapolation of the linear portion of an X-ray survival curve with a line parallel to the x-axis at the 100% survival level; D_0 (Gy) or mean lethal dose, the dose required to reduce survival on the linear portion of the X-ray survival curve by a factor of $1/e$ (36.8%).

The latent periods (days to reach 100 mm^3 in volume; Table 1) vary from a minimum of 2.0 days (HCT-5) to 17.4 days (COLO 320HSR). The mean latency was 7.8 days (95% confidence interval, 4.8 to 10.8 days) (31). Doubling time values range from 2.5 days (clone A) to 9.2 days (SW480). The mean doubling time was 4.8 days (95% confidence interval, 3.5 to 6.1 days) (31). These doubling times are defined for the tumor volumes at the time of excision for determination of hypoxic fractions. This volume was held constant for all 11 tumor lines and was 750 mm^3.

In Fig. 1, we present 3 typical excision assay survival curves for SW620, SW480, and WiDR xenografted colon tumors to illustrate shapes of the dose-response curves. Also schematically included in Fig. 1 for purposes of comparison are survival curves representing the survival of exponentially growing cells *in vitro*. The radiation survival parameters describing the exponential portions of the survival curves derived from tumors in air-breathing or N_2-asphyxiated hosts for all tumor lines are listed in Table 2. We have used the single-hit, multitarget formalism to parameterize (n, D_0, D_q) responses.

The hypoxic percentages were quite variable, ranging from less than 1% (clone D) to over 80% (HCT-8) (Table 1; results are arranged in order of increasing percentage of hypoxia). Averaging the (log) hypoxic percentages yields a mean hypoxic fraction of 10.4% (95% confidence interval, 4.90 to 22.1%) (31). Statistical analyses indicated that the percentage of hypoxia of the xenografted colon tumors did not significantly correlate with any of the disaggregation or *in vivo* growth parameters.

In Fig. 2, we have plotted a cumulative frequency distribution for the extent of hypoxia within the two classes of xenografted human tumors for which a reasonable amount of data exist, colorectal cancers and melanoma [data for the melanomas and for one additional human colon tumor, HRT-8, were taken from Ref. 12]. For all determinations (n = 20), the median value of hypoxia is roughly about 15%. However, if the hypoxic fractions for colorectal cancers and melanomas are ranked, and if the nonparametric Mann-Whitney U test (32) is used to analyze the data, there is a significant difference between the extent of hypoxia between the two tumor types ($P < 0.025$, one-tailed test); *i.e.*, hypoxic percentages are greater in melanoma than in colorectal cancer. A linear least-squares regression analysis of the unweighted hypoxic percentages for all tumor lines with the exception of clone D and NA11 indicates that the data can well be described by a straight line with an equation of (probit) $Y = 2.326 + 2.252$ (log) X (n = 18, correlation coefficient = 0.982, $t = 20.6$, $P < 0.001$) (31).

Table 2 *Survival curve parameters of the exponential regions of cells from xenografted human colorectal tumors irradiated in vivo*

Tumor	D_0 (Gy)	n	D_q (Gy)
Clone D			
Air breathing	2.52 ± 0.27[a]	0.04	
N_2 asphyxiated	2.37 ± 0.23	3.02	2.62 ± 0.95
Clone A			
Air breathing	2.27 ± 0.32	0.12	
N_2 asphyxiated	2.11 ± 0.35	3.92	2.88 ± 0.85
LOVO			
Air breathing	2.55 ± 0.24	0.20	
N_2 asphyxiated	2.64 ± 0.39	2.35	2.26 ± 0.67
COLO 320HSR			
Air breathing	2.64 ± 0.27	0.32	
N_2 asphyxiated	2.54 ± 0.38	2.99	2.78 ± 0.75
SW620			
Air breathing	2.45 ± 0.35	1.92	1.60 ± 0.89
N_2 asphyxiated	2.40 ± 0.14	8.30	5.08 ± 1.12
WiDR			
Air breathing	3.60 ± 0.35	0.32	
N_2 asphyxiated	3.50 ± 0.21	1.59	1.62 ± 0.89
SW480			
Air breathing	3.10 ± 0.17	0.38	
N_2 asphyxiated	2.79 ± 0.27	6.19	4.92 ± 0.75
HT-29			
Air breathing	3.04 ± 0.13	0.42	
N_2 asphyxiated	3.14 ± 0.27	1.96	2.12 ± 0.70
LS174T			
Air breathing	2.89 ± 0.29	0.44	
N_2 asphyxiated	2.72 ± 0.41	2.38	2.36 ± 0.87
HCT-8			
Air breathing	2.65 ± 0.32	2.11	1.98 ± 0.89
N_2 asphyxiated	2.41 ± 0.16	2.60	2.31 ± 1.04

[a] Mean ± SE (31).

DISCUSSION

The primary purpose of this work was to gather additional data about the hypoxic status of xenografted human colon tumors. There were previously only three published reports on the extent of hypoxia in unperturbed xenografted human colon

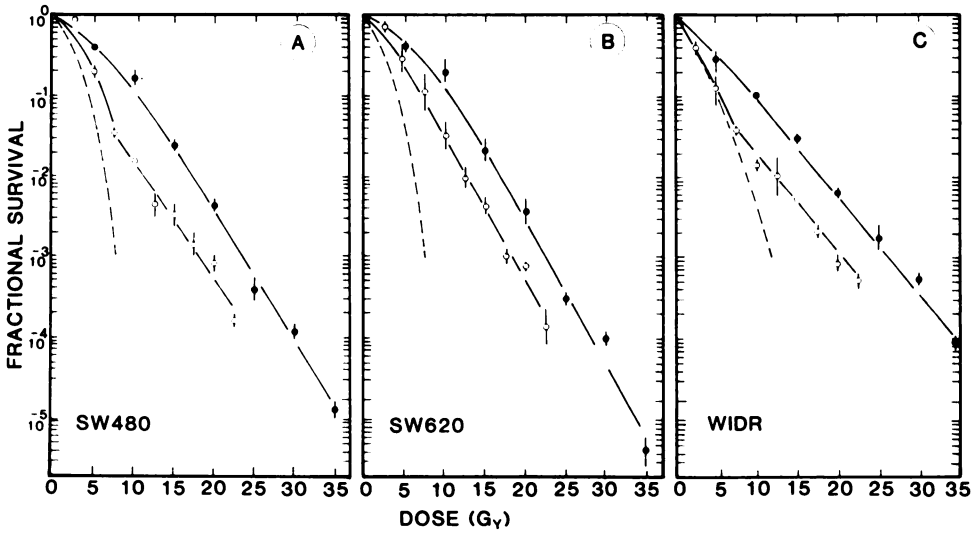

Fig. 1. Paired survival curve bioassay dose-response *curves* for typical human colorectal xenografted tumours. In *A*, *B*, *C*, respectively, oxic (O) and hypoxic (●) cell responses for SW480, SW620, and WiDR tumors are shown. *Points*, mean; *bars*, SEM. The *dashed line* represents the graded dose X-ray survival response of the respective exponentially growing cell lines *in vitro*.

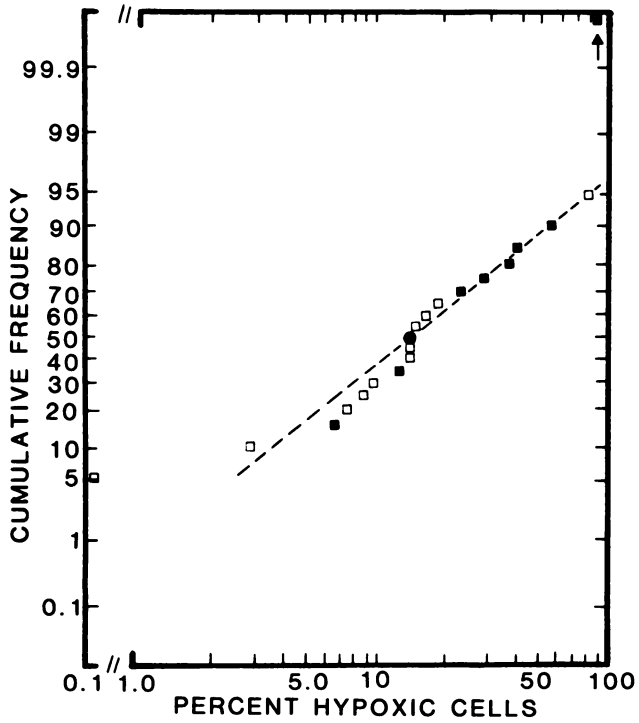

Fig. 2. Cumulative frequency plot of the percentage of hypoxic cells in xenografted human colon (□, this paper; ●, from Ref. 12) and melanoma (■) tumors. Cumulative frequency data between 10 and 95% have been fit to a linear least-squares regression equation (31).

cancers (16, 22, 23), describing 6 different tumors. We therefore examined 11 human colon cancer cell lines [including some of these previously studied by Guichard *et al.* (16) and West and Sutherland (23)] and have determined that hypoxic fractions ranged from less than 1% (clone D) to over 80% (HCT-8) (Table 1). Our data compare well with previously reported data on HCT-8 (16) and WiDR (23). We repeated our previous studies on the hypoxic fractions of tumors of clones A and D (22), because the work used 0.5% trypsin as a disaggregating agent as contrasted to the enzyme cocktail used in the present work. Other investigators (33) have shown that different methods of tumor disaggregation can, in some instances, produce variable results. Our current results agree well with previous work for disaggregation and clonogenic responses of tumors of clones A and D.

There are several aspects of this work that require further discussion. (*a*) Unperturbed xenografted colorectal cancers as a class do not seem to contain large percentages of hypoxic cells (with the exception of HCT-8) (Table 1), when assayed at average volumes of about 750 mm^3. It must be remembered, however, that hypoxic fractions may vary, depending on the volume at which they are assayed (8, 12). With regard to the relevance of this finding for therapy, ionizing radiation is used in several different formats in the overall treatment of colorectal cancer (34), and the data herein infer that the presence of intratumor hypoxia with, consequently, radioresistance tumor cells may not generally be a primary cause of failure. This would support the use of radiation treatment in the treatment of colorectal cancer. However, in regard to clinical response, *in vitro* studies of the distribution of comparative radiosensitivities of various classes of oxic human cancer cells have shown that colon cancer was intrinsically radioresistant (30).

(*b*) Comparison of the xenograft colon cancer results to published data on xenografted melanomas indicates that these neoplasms contain significantly more hypoxic cells than do colorectal cancers (Fig. 2). This finding infers that intratumor hypoxia may be a major factor in curability of melanoma, indicating that interventions related to the state of tumor oxygenation may be particularly relevant for this disease. In support of this argument, Gatenby *et al.* (4) have correlated intratumor oxygen levels (assayed by microelectrodes) with patient outcome in squamous cell carcinoma. Their results indicated that a critical level may exist at which intratumor hypoxia becomes a clearly limiting factor in curability, and Gatenby *et al.* (4) placed this at about 26% (*i.e.*, 26% of tumor cells existed in an environment where the pO_2 was 8 mm of Hg or less). With regard to studies of hypoxia in xenografted tumors, this level (26%) was exceeded in 5 of 8 melanomas (10) (Fig. 2), but in only one of 11 colorectal cancers (Table 1), suggesting that melanomas might normally contain sufficient numbers of hypoxic cells to affect clinical outcome. Additional studies on hypoxic percentages in xenografted human tumors are needed to enlarge the data base from which inferences such as the above for colorectal cancer and melanomas can be drawn.

Irrespective of documentation of hypoxia within neoplasms, however, a more fundamental question must be considered. The question is, what biological mechanisms underlie the observation that hypoxic percentages vary by approximately two orders of magnitude, even within neoplasms of the same histological class. One factor of importance may be an inverse relationship between expression of hypoxia and the levels of angiogenic polypeptides, such as transforming growth factor α and fibroblast growth factor β produced by individual cancer cell lines.[4] For example, the levels of fibroblast growth factor β in line HCT-8 cells were the lowest of all of the 11 human colon lines studied in this report. Therefore, the degree of hypoxia may lie in the paracrine relationships between the tumor and the host. Unraveling such relationships is of importance, but the use of xenografted tumors must be viewed with caution in this regard, as it must not be forgotten that results are obtained in the situation where human parenchymal tumor cells are juxtaposed with murine stroma and vasculature (35, 36).

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