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Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells

(experimental metastatic efficiency/gene amplification)

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ABSTRACT **Cultured cells subjected to oxygen deprivation have been shown to undergo anomalous DNA synthesis, which can result in DNA overreplication and the generation of cellular variants [Rice, G. C., Hoy, C. & Schimke, R. T. (1986) *Proc. Natl. Acad. Sci. USA* 83, 5978–5982]. In the present study, murine tumor cells were exposed to severe hypoxia and then tested for their ability to form experimental metastases. Upon reoxygenation, cells transiently, yet dramatically, increased their metastatic potential. Flow cytometric analysis confirmed that hypoxia and reoxygenation induced cell cycle perturbations and DNA overreplication in these tumor cell lines. Fibrosarcoma cells with overreplicated DNA isolated by fluorescence-activated cell sorting proved to be highly metastatic, although cells with 2–4 times the haploid DNA content in populations treated with hypoxia were also markedly more metastatic than oxic populations. These results support the hypothesis that hypoxic conditions existing in regions of solid tumors promote cellular heterogeneity and tumor progression.**

Gene amplification is believed to be an important mechanism of tumor cell diversification (1, 2). Our previous investigations have used quantitative genetic analysis to study the stochastic generation of metastatic variants in populations of murine tumor cells. The rates measured for the generation and reversion of metastatic variants (3) are similar to rates reported for the generation and reversion of drug-resistant variants which arise as a result of low levels of gene amplification (4–7), as opposed to those associated with genetic changes arising from point mutations or deletions. Our group has published data which demonstrate that the ability of murine melanoma sublines to generate drug-resistant variants correlates with their ability to generate metastatic variants (8). The cytotoxic drugs used in these studies (methotrexate, *N*-phosphonacetyl-L-aspartate) were chosen because resistance is most often conferred by amplification of a defined genetic locus. Furthermore, recent investigations have established that the presence of a specific homogeneously staining region, a cytogenetic marker of stable and extensive gene amplification, correlates with increased tumorigenicity and metastatic potential of human melanoma cells in a xenograft model (9, 10). Invasion of amniotic membranes by human melanoma cells has been shown to select for cells with an increased incidence of double-minute chromosomes (11), which are also markers of gene amplification. Collectively, these studies provide indirect evidence for a role of gene amplification in cancer metastasis.

One model of gene amplification postulates that additional copies of genomic sequences are produced when an unscheduled round of DNA synthesis is initiated in the wake of a primary round of synthesis which has been temporarily interrupted (12). Recent investigations have reported that large-scale DNA overreplication (13–15) and gene amplification (14, 16) can occur in cells when DNA synthesis is inhibited by oxygen starvation. These observations suggest a means by which large portions of the genome may be amplified in a nonspecific manner. The present investigation was conducted to determine whether hypoxia-induced DNA overreplication affects the metastatic behavior (lung colonization potential) of cancer cells.

MATERIALS AND METHODS

Tumor Cell Lines and Culture Conditions. The origins and propagation of the KHT-C2-LP1 fibrosarcoma and B16F10-A1 melanoma cell lines used in this investigation have been previously reported (17). For these experiments, monolayers of cells were grown in 8-oz. (240-ml) glass Brockway bottles containing 30 ml of α-minimal essential medium and 10% fetal bovine serum. Bottles were sealed with silicon stoppers and hypoxic conditions were produced by equilibrating logarithmic-phase subconfluent cultures (at a density $<2.5 \times 10^4$ cells per cm^2) with humidified gas containing 5% carbon dioxide and 95% nitrogen (<10 ppm oxygen). This mixture was delivered at a rate of 5–7 liters per bottle per hr via short lengths of Tygon tubing which fed from a glass manifold.

The oxygen concentrations in both gas and aqueous phases of cultures continuously purged with this oxygen-deficient mixture were monitored by using an electronic sensor (18) to determine the degree of hypoxia that was established. The gas phase quickly developed an oxygen tension comparable to that of the delivered mixture, while the depletion of oxygen from the culture medium was more gradual. These measurements indicate that sufficient amounts of oxygen were removed from the medium to achieve radiobiological hypoxia (0.1% oxygen) by 3 hr after the onset of gassing, and levels approaching anoxia were obtained within 16 hr. The actual concentration of oxygen to which the cells were exposed will be lower than that measured in the medium since oxygen in the unstirred layers surrounding the monolayer is rapidly consumed by cellular respiration.

KHT cells could be maintained under hypoxic conditions for up to 48 hr before any significant number of cells (10–20%) detached from the glass surface. In contrast, B16F10 cells could tolerate only 18 hr of hypoxia before a similar number of cells became detached. Counts of cells recovered at various times after the initiation of gassing indicated that cellular proliferation was completely inhibited after 12–18 hr. The average pH of the medium remained between 7.3 and 7.5 throughout chronic hypoxic exposures. Reoxygenation of cultures was accomplished by replacing the medium with

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Abbreviations: C, haploid amount of DNA; EME, experimental metastatic efficiency; FACS, fluorescence-activated cell sorting; FALS, forward-angle light scatter; PE, plating efficiency.

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fresh (air-equilibrated) medium and returning the bottles to a standard incubator.

Suspensions of cultured cells were prepared by washing monolayers with calcium- and magnesium-free phosphate-buffered saline, followed by a 5-min exposure to 0.25% trypsin solution (GIBCO, no. 610-5050). Cells harvested from chronically treated cultures were >95% viable as assessed by trypan blue dye exclusion and >95% were single cells. Cell numbers were determined with an electronic particle counter and cells were then pelleted and resuspended to 10^5 per ml. The cell concentration was verified with the use of a hemocytometer. Plating efficiency (PE) was determined by transferring a known number of cells onto 100-mm plastic plates containing growth medium and counting the number of colonies (containing >50 cells) which arose 10 days later.

Experimental Metastasis Assay. Cells were harvested by trypsin treatment, counted, pelleted, and resuspended in growth medium to 10^5 per ml. The lung colonization assay used in this investigation involves the intravenous injection of cells into groups of six or seven mice as previously described (17). Inbred strains of age-matched male C3H/HeJ and female C57BL/6J mice (syngeneic to KHT sarcoma and B16 melanoma cells, respectively) were obtained from The Jackson Laboratory. Animals were housed in the specific pathogen-free colony of the Ontario Cancer Institute and sustained on acidified water and mouse/rat chow pellets ad lib. Each mouse received 2×10^4 cells via the tail vein. Groups of mice were sacrificed 20 days later, at which time their lungs were removed and fixed in Bouin's solution. The number of pulmonary tumors which had developed was determined with the aid of a dissecting microscope.

Flow Cytometric Analysis of Tumor Cell Size and DNA Content. Suspensions of KHT cells were analyzed for fluorescence and forward-angle light scatter (FALS) properties by using an EPICS V flow cytometer (Coulter) after cells were stained for 45 min in 5 μM Hoechst 33342 dye, using a previously described protocol (19). Fluorochrome incorporated by cells was excited by 30 mW of 340-nm light emitted by an argon laser. The data were processed and displayed by a MDADS computer and related software. For sorting experiments, cells were discriminated according to both blue fluorescence (DNA content) and FALS (cell size) values. Isolated populations were pelleted and resuspended at 10^5 cells per ml in growth medium and tested for plating and metastatic efficiency by using the standard assays.

RESULTS

The effects of a single hypoxic exposure on the colonization ability of KHT and B16F10 cells are presented in Fig. 1. Tumor cells assayed immediately after hypoxic culture exhibited a slight decrease in their ability to form metastases; however, this is attributable to a loss of viability caused by oxygen starvation. Cells which were allowed to recover under oxic conditions displayed progressively larger increases in metastatic potential. For both lines studied, a maximum effect was observed approximately 18 hr after reoxygenation. The experimental metastatic efficiency (EME) for these treatment groups was calculated by taking into consideration the number of viable cells (as assessed by *in vitro* clonogenicity) injected into animals. KHT cells demonstrated, at maximum, a 14-fold increase, and B16F10 cells a 6-fold increase, in EME compared to oxic controls. The reduced effect in B16F10 populations may be due to the shorter hypoxic exposure (18 hr) used for treatment of this cell line. The enhancement of colonization ability induced by hypoxia was not a stable alteration, since the EME of treated populations consistently returned to values comparable to those for untreated controls by about 48 hr after reoxygenation.

The effects of varying the duration of hypoxia on the ability of tumor cells to form experimental metastases were investigated with the KHT sarcoma subline. Cell viability declined as the duration of hypoxic treatment increased, such that after 48 hr cells harvested from monolayers expressed a PE one-quarter that of oxic controls. This loss of viability is consistent with previous reports on the toxicity of hypoxia in other monolayer systems (20, 21). Due to the loss of *in vitro* clonogenicity encountered with chronic hypoxic exposures, the EME was calculated and used as an index of metastatic potential. The data, presented in Fig. 2, demonstrate that, as hypoxic treatment was prolonged, greater increases in metastatic potential were observed. This relationship was evident in cells injected immediately after treatment but was much more prominent when the populations were reoxygenated. The EME of cells exposed to hypoxia for 48 hr followed by 18 hr of oxic recovery was 50-fold greater than that of the

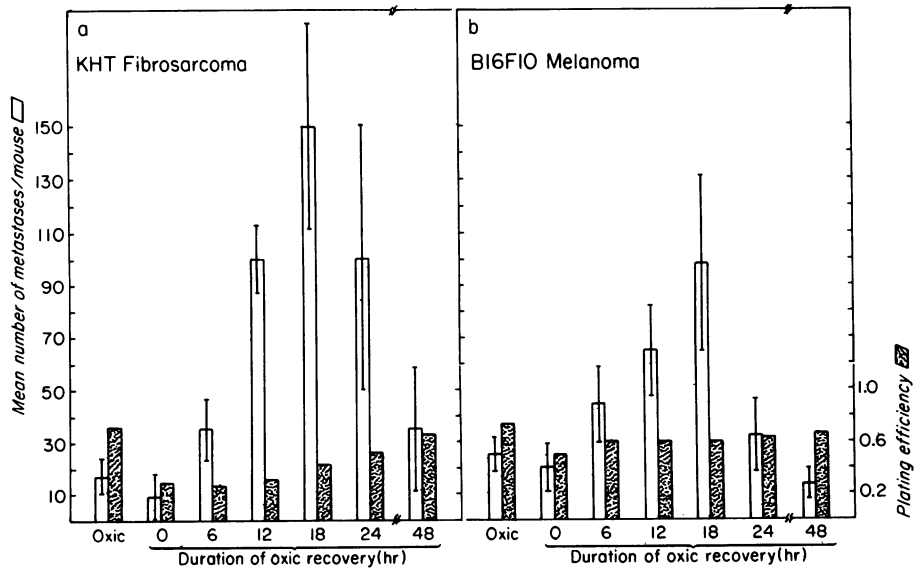

FIG. 1. Effects of hypoxic culture on the lung colonization ability of tumor cells. Monolayers of KHT-C2-LP1 cells (*a*) and B16F10-A1 cells (*b*) were subjected to hypoxic culture conditions and then tested for their ability to form experimental metastases at various times after reaeration. KHT cells were exposed to hypoxia for 24 hr, while B16F10 cells were treated for 18 hr. Open bars (left ordinate) represent the mean number of lung metastases (±1 SD) for the treatment group. Stippled bars (right ordinate) indicate the corresponding plating efficiency of the sample.

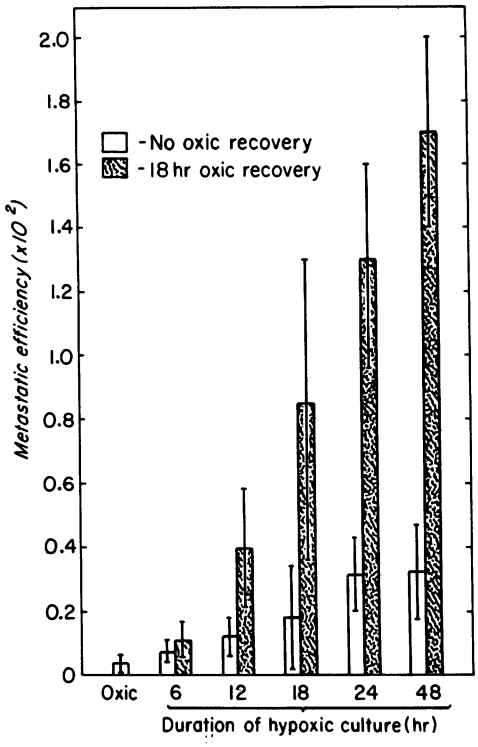

FIG. 2. Effect of the duration of hypoxic culture on the lung colonization ability of KHT-C2-LP1 cells. Values for the experimental metastatic efficiency (EME) of samples represent the number of lung metastases per viable tumor cell injected. Cell viability was assessed by *in vitro* clonogenicity. Error bars represent ±1 SD.

oxic control. Nearly 2% of the viable cells in these populations were capable of forming metastases.

Changes in the DNA content and size of cells caused by hypoxic treatment were evaluated by flow cytometric analysis of cells stained with the fluorescent DNA-binding dye Hoechst 33342. Fig. 3 illustrates fluorescence and FALS histograms for populations of KHT cells grown under oxic and hypoxic conditions. Oxic cultures in logarithmic-phase growth typically had 50% of cells in G_1 phase, 35% in S phase, and 15% in G_2/M phase. Exposure of these cells to chronic hypoxia resulted in an accumulation of cells in G_1 phase, which is consistent with an inhibition of DNA synthesis. After 48 hr of hypoxic culture, 75% of the cells exhibited a G_1 phase DNA content, while only 15% had S phase DNA content. Partial G_1 phase synchronization has been previously reported in studies which have examined the effects of hypoxia on progression of cells through the cell cycle (14, 15, 21).

Highly reproducible sequential changes in DNA profiles were observed upon reoxygenation of cultures. An increase in the number of cells with S phase DNA content was apparent within 12 hr of the restoration of normal oxygen levels, and at this point the DNA histogram resembles that of logarithmic-phase populations. This apparent return to normality was completely altered 18 hr after reoxygenation, when the partial G_1 synchronization led to a disproportionate number of cells entering S phase. In addition, the first evidence of DNA overreplication became apparent with the generation of a subset of cells exhibiting >4C DNA content (C is normal haploid DNA content). By 24 hr the DNA overreplicated subpopulation (possessing a modal DNA content ≈ 8C) represented 10% of the total cells. The number of cells in this subpopulation began to diminish by 48 hr, when 5–7% of the cells possessed a >4C DNA content, and the histogram appears more characteristic of oxic controls. These DNA histograms were reproduced (data not shown) by using a flow cytometric technique which involves analysis of

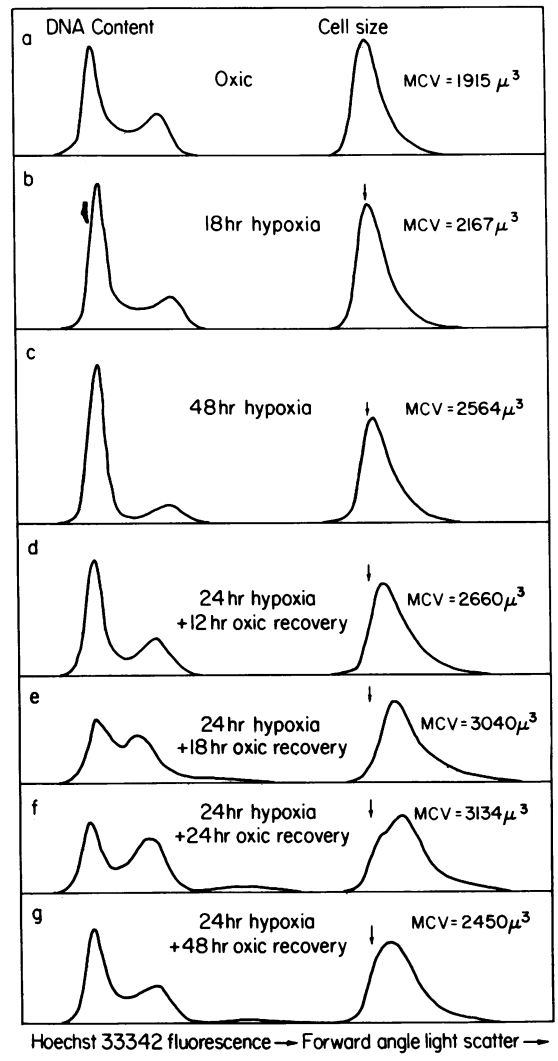

FIG. 3. Effects of hypoxia and reoxygenation on the DNA content and size of KHT-C2-LP1 cells. Cell fluorescence (after staining with Hoechst 33342) and FALS values were obtained by flow cytometric analysis of cell populations cultured under the indicated conditions. Data are presented as paired histograms. FALS is a function of cell size (22), and arrows indicate the position of the mode observed for the oxic population. Increases in size indicated by changes in FALS signals corresponded with increases in cell impedance measured on an electronic particle counter coupled to a multichannel pulse-height analyzer. The median cell volume (MCV) was determined by comparing cell impedances with a standard curve produced by analysis of plastic beads of defined sizes ($\mu^3 = \mu m^3$).

individual nuclei stained with propidium iodide (23). The fact that cells with >4C DNA content were detected by using this procedure indicates that this subpopulation does not consist of binucleate cells and that the overreplicated DNA is contained in a single nuclear envelope. Evidence of DNA overreplication has also been observed in B16F10 melanoma cells exposed to hypoxic culture conditions (data not shown).

Hypoxic culture also produced an increase in the FALS signal of these cells, which is indicative of an increase in cell volume. Reoxygenation was accompanied by additional increases in the modal and median FALS signal, which reached a maximum after 24 hr. FALS values measured at 48 hr indicate that cells undergo a reduction in size with continued culture. The changes in cell size were confirmed by using an electronic particle counter coupled to a multichannel pulse-height analyzer. Use of this instrument permitted the median cell volume (MCV) to be estimated for each population (see Fig. 3).

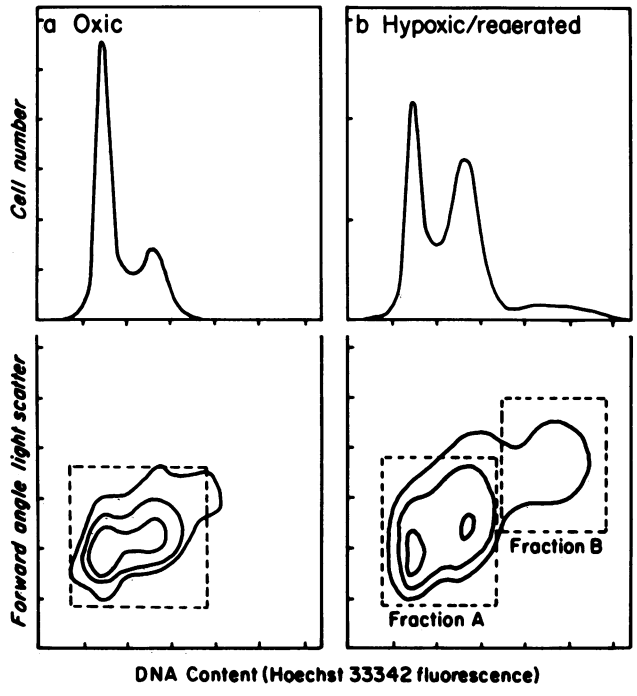

FIG. 4. Isolation of DNA-overreplicated KHT-C2-LP1 cells by using FACS. KHT cells cultured under oxic conditions (*a*) or exposed to 24 hr of hypoxia followed by 24 hr of oxic recovery (*b*) were vitally stained with Hoechst 33342 dye and analyzed by flow cytometry (40,000 counts for oxic; 30,000 for hypoxic/reaerated). Upper panels are DNA histograms for each sample (ordinate scale for left histogram is twice that of the right). Lower panels are two-parameter plots (FALS vs. fluorescence) with isometric contours representing 30 (outer), 143 (middle), and 1147 (inner) counts. Cells were then sorted according to the delineated fractions. Samples were washed once in growth medium and tested for plating efficiency and metastatic ability by using the standard assays (see Table 1 for results).

KHT tumor cells which had overreplicated their DNA were isolated by using fluorescence-activated cell sorting (FACS) from cultures which had been exposed to 24 hr of hypoxia followed by 24 hr of oxic recovery. Sorting criteria were based on both DNA content and FALS, since this information could be used to clearly discriminate between cells of the two subpopulations (Fig. 4*b*). Cells collected from both 2–4C (fraction A) and DNA-overreplicated (fraction B) subpopulations were assessed for viability (PE) and lung colonization ability. Cells with >4C DNA content were also sorted directly onto glass slides for microscopic examination, which revealed that this subpopulation was indeed composed of single cells and not cell aggregates. Cells grown under oxic conditions were also stained and sorted (see Fig. 4*a*) to provide control populations.

The results of a representative sorting experiment are summarized in Table 1, and similar results were obtained in three other experiments. FACS using this Hoechst 33342 procedure leads to a small (15–20%), but consistent, reduction in the viability of both oxic and hypoxic tumor cells. This loss of viability appears to be due to damage suffered during sorting, since cells which are stained, but not sorted, exhibit values of PE and EME similar to unstained controls (data not shown). For this experiment, the EME of the hypoxic/reaerated population was 10-fold greater than that of the oxic control. Comparisons of the EME between fractions A and B indicate that the DNA-overreplicated cells were on average 2.5-fold more efficient at forming experimental metastases than the 2–4C subpopulation. Analysis of these data by a nonparametric test (Mann–Whitney *U*) indicates that this difference is highly significant ($P < 0.01$). The fact that no consistent differences were observed in the PE values of fractions A and B supports the argument that a real difference exists between the colonization abilities of these two subpopulations. Assessment of fraction A for metastatic potential indicated that cells in the 2–4C subpopulation exhibit an EME which is 6-fold greater than that of oxic cells, which is also a statistically significant difference ($P < 0.01$).

DISCUSSION

The results of this investigation have demonstrated that anaerobically cultured tumor cells have an enhanced ability to form experimental metastases. This effect is dependent on the duration of exposure to hypoxia and becomes much more dramatic when a period of oxic recovery is permitted before the cells are assayed. Potentiation of lung colonization ability is a transient phenomenon and the induction is essentially lost 48 hr after treatment (see Fig. 1). The observation that cells injected immediately after harvesting from oxygen-depleted cultures exhibit only modest increases in EME implies either that the conditions in the microcirculation are nonpermissive for development of the phenotype or that changes in cellular characteristics are not expressed soon enough to confer resistance to metastatic selection.

Anaerobiosis is associated with widespread changes in cell physiology, and flow cytometric analysis of hypoxically cultured cells indicates that oxygen starvation can dramatically alter cell cycle distribution. A cell cycle effect of lung colonization ability has been reported by Suzuki *et al.* (24), who used centrifugal elutriation to fractionate fibrosarcoma cells according to their position in the cell cycle. This study found that cells in S and G_2/M phase were 10- to 20-fold more efficient at forming experimental metastases than cells in G_1 phase. However, our own studies, which used FACS to obtain KHT cells in different phases of the cell cycle, indicate that G_2/M cells in this tumor model have a EME which is only 3-fold greater than G_1 cells (unpublished results). It is unlikely that redistribution of cells in the cell cycle is responsible for the

Table 1. Lung colonization ability of KHT cell populations isolated from oxic and hypoxic/reaerated cultures by FACS

Culture conditions	Sample	PE	No. of lung nodules	EME × 10^2
Oxic	Total (unstained)	0.72	7, 4, 12, 11, 10, 11, 16	0.070
	Total (sorted)	0.57	1, 7, 6, 2, 5, 8, 10	0.049
Hypoxic/ reaerated	Total (unstained)	0.54	93, 54, 83, 80, 31, 106, 101	0.72
	Total (sorted)	0.49	37, 29, 50, 44, 35, 46, 45	0.42
	2–4C (fraction A)	0.49	44, 39, 3, 37, 28, 35, 23	0.30
	>4C (fraction B)	0.52	55, 46, 102, 47, 136, 83, 60	0.73

The hypoxic/reaerated cells were subjected to hypoxia for 24 hr and then reoxygenated for 24 hr. After culture, samples were tested before any manipulation [Total (unstained)] and after staining and sorting [Total (sorted)]. The number of lung nodules in each mouse is recorded in the fourth column.

hypoxia-induced enhancement of lung colonization potential observed in this study, since the maximum effect was obtained at a time (18 hr after reaeration) when populations were quite asynchronous (see Fig. 3*e*).

Cells subjected to hypoxia demonstrated definite signs of enlargement, and increased cell volume has been postulated to promote metastasis formation by facilitating the arrest of circulating tumor cells. However, it is clear that the majority (70–90%) of aerobically cultured cells injected intravenously are arrested, at least temporarily, in the microcirculation of the first organ encountered without the benefit of an induced increase in cell volume (25, 26). Furthermore, models describing the interaction of tumor cells with the microcirculation formulated by Weiss and co-workers (27, 28) predict that an increase in cell volume would actually be detrimental to metastasis formation, since increased stress on the cell membrane increases the probability of rupture and cell death. The present finding of an enhanced EME for cells with 2–4C DNA content isolated from hypoxic/reaerated cultures is not likely explained by more efficient retention in the microcirculation as a result of increased cell volume, since this fraction has a size distribution which is similar (although not identical) to that of oxic controls (compare FALS windows for sorting and contours described in Fig. 4).

Flow cytometric analyses performed on populations of cells arrested by hypoxia indicate that, upon reaeration, a significant number of cells may overreplicate their DNA (13–15). This has been shown to result in the induction of low levels of gene amplification at two genetic loci (dihydrofolate reductase and P-glycoprotein) which are known to be located on different chromosomes (14, 16). Preliminary studies (data not shown) which indicate that hypoxic culture can also increase the resistance of KHT cells to the drugs methotrexate and adriamycin are consistent with these results. DNA-overreplicated cells are believed to arise from cells in S phase, where transient inhibition of DNA synthesis results in segments of the genome being replicated more than once in a single cell cycle (29–31). It is clear that large amounts of DNA can be overreplicated after the resumption of synthesis, and it is therefore likely that amplification occurs at many loci, including genes which may enhance metastatic potential.

The hypoxia-induced increase in EME correlated with the generation of cells with overreplicated DNA, and the effect was shown to diminish as the proportion of cells with >4C DNA declined. The subpopulation with >4C DNA content proved to be 15-fold more efficient at forming experimental metastases than oxic controls. However, these cells at most represented only ≈10% of the total population, and it was demonstrated that cells with normal DNA content (2–4C) in hypoxic/reaerated populations also exhibited an increased EME (6-fold greater than oxic controls). One explanation for this observation is that extensive DNA overreplication may not be required for the enhancement of metastatic potential. The degree of DNA overreplication occurring in treated cells appears to be variable (see Fig. 3*f*), and cells in the 2–4C range may overreplicate DNA on a scale which is not detectable by using the described flow cytometric technique. The hypothesis that gene amplification is an important mechanism involved in the enhancement of colonization potential by hypoxia is supported by the observation that other agents which inhibit DNA synthesis and induce DNA overreplication and gene amplification (UV radiation, hydroxyurea, methotrexate, aphidicolin) (32–35) can also increase the metastatic potential of tumor cell populations (36–38).

This investigation has demonstrated that exposure to hypoxia can enhance the ability of tumor cells to form experimental metastases. We have argued that the induction of gene amplification may be important for this effect; however, the participation of other factors cannot currently be ruled out. Regardless of mechanism, these results raise the possibility that circulating tumor cells released from reoxygenated regions of solid tumors may have increased colonization potential. This influence may be particularly important for cancer metastasis formation if the detachment of hypoxic tumor cells from the primary site is facilitated by degradative enzymes released from adjacent necrotic regions (39, 40).

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