Fluctuating and Diffusion-Limited Hypoxia in Hypoxia-Induced Metastasis

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Abstract

Purpose: Most tumors develop regions with hypoxic cells during growth, owing to permanent limitations in oxygen diffusion (chronic or diffusion-limited hypoxia) and/or transient limitations in blood perfusion (acute or fluctuating hypoxia). The aim of this study was to investigate the relative significance of chronic and acute hypoxia in the development of metastatic disease.

Experimental Design: D-12 and R-18 human melanoma xenografts were used as models of human cancer. D-12 tumors metastasize to the lungs, whereas R-18 tumors develop lymph node metastases. Fraction of radiobiologically hypoxic cells (HF_rad) was measured in individual primary tumors by using a radiobiological assay based on the paired survival curve method. Fraction of immunohistochemically hypoxic cells (HF_imm) was assessed in the same tumors by using a pimonidazole-based immunohistochemical assay optimized with respect to achieving selective staining of chronically hypoxic cells. HF_imm and the difference between HF_rad and HF_imm were verified to be adequate variables for fraction of chronically hypoxic cells and fraction of acutely hypoxic cells, respectively.

Results: Chronic as well as acute hypoxia were found to promote spontaneous metastasis of D-12 and R-18 tumors. Acute hypoxia influenced metastasis to a greater extent than chronic hypoxia, partly because the fraction of acutely hypoxic cells was larger than the fraction of chronically hypoxic cells in most tumors and partly because acutely hypoxic cells showed a higher metastatic potential than chronically hypoxic cells.

Conclusions: It may be beneficial to focus on fluctuating hypoxia rather than diffusion-limited hypoxia when searching for hypoxia-related prognostic variables and predictive assays.

Most experimental and human tumors are heterogeneous in oxygen tension (pO_2) and develop regions with hypoxic (pO_2 <10 mmHg) cells during growth (1). Two main causes of tumor hypoxia have been recognized: permanent limitations in oxygen diffusion and transient limitations in blood perfusion (2). Permanent limitations in oxygen diffusion result in development of hypoxic cells in regions far from blood vessels, typically adjacent to necrotic regions. These cells are termed chronically hypoxic and are in untreated tumors believed to remain hypoxic until they die because of lack of oxygen or nutrients. Chronically hypoxic cells have a lifetime within the range of 4 to 10 days in most experimental tumors (3). Transient limitations in blood perfusion (i.e., transient cessations in microregional blood flow or temporal variations in microvessel RBC flux) result in development of hypoxic cells downstream of perfusion-impairing vessel abnormalities. These cells are termed acutely hypoxic and are believed to experience several short-term periods of hypoxia during their lifetime. The duration of these periods has been shown to range from less than a minute to several hours in experimental tumors (4). This type of tumor hypoxia, often referred to as fluctuating hypoxia, should be distinguished from diffusion-limited hypoxia because cells in regions with fluctuating hypoxia may differ biologically from those in regions with diffusion-limited hypoxia (2–4).

A large number of methods have been introduced for assessing the fraction of hypoxic cells in tumors (5, 6). Differing cell subpopulations are detected in different assays, implying that data derived from different assays may supplement one another (7). Radiobiological assays measure the fraction of the clonogenic cells in tumors that are hypoxic (5–7). This hypoxic fraction is termed fraction of radiobiologically hypoxic cells and involves both chronically and acutely hypoxic cells. The fraction of radiobiologically hypoxic cells is considered to be of greater clinical significance than hypoxic fractions derived from non-radiobiological assays, as only clonogenic cells are of relevance for tumor growth and response to treatment (7, 8). Immunohistochemical assays have been suggested to represent a useful supplement to radiobiological assays (9). In these assays, antibodies to nitroimidazole-protein adducts are used to identify hypoxic cells in histologic preparations from tumors. Immunohistochemical assays preferentially detect tumor
regions with diffusion-limited hypoxia as opposed to fluctuating hypoxia, particularly in paraformaldehyde-fixed tissue (10) but cannot be used to distinguish between clonogenic and nonclonogenic cells (5, 6, 9).

Recent studies have suggested that hypoxia promotes malignant progression and metastatic spread of tumor tissue by selecting for aggressive cell phenotypes and by up-regulating the expression of genes involved in the metastatic process (11–13). Thus, clinical investigations involving several histologic types of cancer have shown that invasive growth, development of metastatic disease, or poor disease-free or overall survival rate is associated with the expression of hypoxia-inducible gene products or low pO2 in the primary tumor (14–17). Studies of experimental tumors have indicated that tumors with high fractions of hypoxic cells metastasize more frequently than genetically equivalent control tumors with low fractions of hypoxic cells, whether high hypoxic fractions are occurring naturally (18, 19) or are imposed by irradiating the tumor bed (20, 21) or by keeping the host mice in a low-oxygen atmosphere (22, 23). These experimental and clinical studies, however, do not provide information on the nature of the hypoxia that promotes the development of metastatic cell phenotypes, as hypoxia was detected by the use of pO2 electrodes or intrinsic and/or extrinsic hypoxia markers, methods that do not distinguish between fluctuating and diffusion-limited hypoxia (11–23).

Molecular mechanisms of hypoxia-induced metastasis have been studied in our laboratory by using D-12 and R-18 human melanoma xenografts as preclinical models of human cancer and pimonidazole as extrinsic hypoxia marker (18–21). D-12 tumors form spontaneous pulmonary metastases in athymic nude mice, and hypoxia was found to promote metastasis primarily by up-regulating the proangiogenic factor interleukin-8 (II-8). R-18 xenografts develop spontaneous lymph node metastases, and hypoxia was found to promote metastasis primarily by up-regulating the invasive growth-promoting receptor urokinase-type plasminogen activator receptor (uPAR). In the work reported here, we used these tumor models to study the nature of the hypoxia that promotes metastasis. To do so, fraction of radiobiologically hypoxic cells (HFRad) and fraction of immunohistochemically hypoxic cells (HFImm) were measured in individual D-12 and R-18 primary tumors and related to the metastatic status of the host. HFRad was measured by using an assay based on the paired survival curve method. This assay is particularly useful for accurate assessment of HFr atd in individual D-12 and R-18 tumors (24). HFImm was assessed by using a pimonidazole-based assay optimized with the purpose of achieving selective staining of chronically hypoxic cells (24). HFRad, HFImm, and the difference between HFr atd and HFImm (HFRad - HFImm) were assumed to represent total fraction of hypoxic cells, fraction of chronically hypoxic cells, and fraction of acutely hypoxic cells, respectively. These assumptions have been verified to be valid for D-12 and R-18 tumors (24). Therefore, fraction of chronically hypoxic cells is equated with HFImm and fraction of acutely hypoxic cells is equated with HFRad - HFImm in the present communication. The experiments gave results suggesting that metastasis of D-12 and R-18 tumors is promoted by diffusion-limited as well as by fluctuating hypoxia, with fluctuating hypoxia being more important than diffusion-limited hypoxia in both tumor lines.

Materials and Methods

**Mice and tumors.** Adult (8–10 weeks of age) female BALB/c nu/nu mice, maintained as described elsewhere (25), were used as host animals for xenografted tumors. Primary tumors were initiated from D-12 or R-18 monolayer cell cultures (25). Approximately 3.5 × 10^5 cells suspended in 10 μL Ca^{2+}- and Mg^{2+}-free HBSS were inoculated i.d. into the left mouse flank (26). Tumor volume (V) was calculated as V = π / 6 × ab^2, where a is the longer and b is the shorter of two orthogonal diameters (26). The animal experiments were approved by the Institutional Committee on Research Animal Care and carried out according to the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing, and Education (New York Academy of Sciences, New York, NY).

**Tumor irradiation.** A Siemens (Erlangen, Germany) Stabilipan X-ray unit, operated at 220 kV, 19 to 20 mA, and with 0.5-mm copper filtration, was used for irradiation. The tumors were irradiated at a dose rate of 5.1 Gy/min, using a radiation field of 15 × 15 mm (24). The mice were anesthetized with ketamine (33 mg/kg) and azaperone (25 mg/kg), and the body core temperature of the mice was kept at 37°C to 38°C during irradiation by using a heating lamp. The anesthesia did not alter the total blood perfusion or the extent of transient blood perfusion in the tumors significantly, as verified in separate experiments by using the 444Rb uptake method and the double-fluorescent staining technique, respectively. Hypoxic tumors were obtained by occluding the blood-supply with a clamp 5 min before irradiation (24).

**Cell survival assay.** Tumor cell survival was measured in vitro, using a plastic surface colony assay (27). A standardized mechanical and enzymatic procedure was used to prepare single-cell suspensions (28). Briefly, the tumors were minced in cold HBSS before enzymatic treatment at 37°C for 2 h. The enzyme solution consisted of 0.2% collagenase, 0.05% Pronase, and 0.02% DNase in HBSS. Trypan blue-negative tumor cells were plated in 5 mL medium [RPMI 1640 (25 mmol/L HEPES and t-glutamine) supplemented with 13% bovine calf serum, 250 mg/L penicillin, and 50 mg/L streptomycin] in 25-cm^2 tissue culture flasks and incubated at 37°C for 14 days. Cells causing colonies with >50 cells were scored as clonogenic. The cell surviving fraction of an irradiated tumor was calculated from the plating efficiency of the cells of the tumor and the mean plating efficiency of the cells of six untreated control tumors.

**Radiobiological hypoxia.** Fraction of radiobiologically hypoxic cells was determined for individual tumors by using a procedure based on the paired survival curve method (24). The tumors were irradiated with 10 Gy under unclamped conditions, and fraction of surviving cells [SF_{10} (unclamped)] was determined for each tumor as described above. Fraction of radiobiologically hypoxic cells was then calculated as SF_{10} (unclamped) / SF_{10} (clamped), where SF_{10} (clamped) represents the mean cell surviving fraction of 10 tumors irradiated with 10 Gy under clamped conditions. The determination of the fraction of radiobiologically hypoxic cells of individual D-12 and R-18 tumors from measurements of SF_{10} has been justified previously (24).

**Immunohistochemical hypoxia.** Fraction of chronically hypoxic cells was determined for individual tumors by using a peroxidase-based immunohistochemical assay, optimized with the purpose of staining chronically hypoxic cells adequately without staining normoxic or acutely hypoxic cells significantly. The assay and a critical experimental evaluation of the assay have been reported in detail elsewhere (24). Briefly, pimonidazole [1-[(2-hydroxy-3-piperidinyl)-propyl]-2-nitroimidazole], a well-characterized hypoxia marker (29), was administered i.p. in doses of 30 mg/kg body weight 4 h before tumor resection. Histologic preparations, produced from tumor tissue fixed in phosphate-buffered 4% paraformaldehyde, were incubated with polyclonal rabbit antiserum to pimonidazole-protein adducts (dilution of 1:1,600), a kind gift from Prof. J.A. Raleigh (Department of Radiation Oncology, University of North Carolina School of Medicine, Chapel Hill, NC). Diaminobenzidine was used as chromogen, and hematoxylin was used for counterstaining. Quantitative studies of hypoxia were...
based on six cross-sections of each tumor. Fraction of chronically hypoxic cells (i.e., the area fraction of the nonnecrotic tissue showing positive pimonidazole staining) was determined by image analysis.

Metastasis. The primary tumors were resected at predetermined times after initiation. The host mice were then examined daily for clinical signs of metastases (listlessness, weight loss, or hunched posture). They were euthanized 3 months after the primary tumor resection or when moribund. Mice having borne D-12 primary tumors were examined for pulmonary metastases (i.e., the lungs were fixed in Bouin’s solution for 24 h and inspected for macroscopic metastases by stereomicroscopy; ref. 18). Mice having borne R-18 primary tumors were examined for external lymph node metastases in the interscapular, submandibular, axillary, and inguinal regions and internal lymph node metastases in the abdomen and mediastinum (19). Metastases were always found in moribund mice. The presence of metastases was confirmed by histologic examinations. Mice were scored to be metastasis negative if pulmonary (D-12) or lymph node (R-18) metastases could not be detected by autopsy 3 months after the primary tumor was resected. Further details of the assays have been reported elsewhere (18–21, 26).

Statistical analysis. Experimental data are presented as arithmetic mean ± SE unless otherwise stated. The Pearson product moment correlation test was used to search for correlations between variables. Statistical comparisons of data sets were carried out by using the Student’s t test (single comparisons) or by one-way ANOVA (multiple comparisons) when the data sets complied with the conditions of normality and equal variance. Under other conditions, comparisons were carried out by nonparametric analysis using the Mann-Whitney rank-sum test (single comparisons) or the Kruskal-Wallis one-way ANOVA on ranks (multiple comparisons). The Bonferroni method (parametric tests) or the Dunett method (nonparametric tests) was used to identify data sets that differed from the control data in multiple comparisons. The Kolmogorov-Smirnov method was used to test for normality. Errors inherent in intermediate analyses were propagated through to the final analyses. Probability values of P < 0.05, determined from two-sided tests, were considered significant. The statistical analysis was carried out by using SigmaStat statistical software (Jandel Scientific GmbH, Erkrath, Germany).

Results

Fifty mice with D-12 tumors and 50 mice with R-18 tumors were included in the metastasis experiments. When having reached a volume of 400 to 500 mm³, the tumors were irradiated with 10 Gy, resected, and cut into ~1-mm-thick slices. One half of each tumor (every second slice) was processed for measurement of SF₁₀ (unclamped) and fraction of radiobiologically hypoxic cells, whereas the other half was processed for assessment of fraction of chronically hypoxic cells. The host mice were examined for development of macroscopic metastases for the next 3 months.

SF₁₀ (unclamped) differed by a factor of ~7.5, from 1.57 × 10⁻³ to 1.17 × 10⁻², among the 50 D-12 tumors (Fig. 1A), and by a factor of ~4.3, from 2.46 × 10⁻² to 1.05 × 10⁻¹, among the 50 R-18 tumors (Fig. 1B). In comparison, SF₁₀ (clamped) of 10 D-12 control tumors and SF₁₅ (clamped) of 10 D-12 control tumors differed by factors of ~1.7 and ~2.0, respectively (Fig. 1A), and SF₁₀ (clamped) of 10 R-18 control tumors and SF₁₅ (clamped) of 10 R-18 control tumors differed by factors of ~1.8 and ~1.9, respectively (Fig. 1B). The geometric means of SF₁₀ (unclamped), SF₁₀ (clamped), and SF₁₅ (clamped), indicated at Fig. 1 (horizontal lines), were 4.47 × 10⁻³, 1.74 × 10⁻², and 1.86 × 10⁻³, respectively, for the D-12 tumors and 5.84 × 10⁻², 1.17 × 10⁻¹, and 2.08 × 10⁻², respectively, for the R-18 tumors. The coefficients of variation were 48% [SF₁₀ (unclamped)], 17% [SF₁₀ (clamped)], and 21% [SF₁₅ (clamped)] for the D-12 tumors and 34% [SF₁₀ (unclamped)], 21% [SF₁₀ (clamped)], and 20% [SF₁₅ (clamped)] for the R-18 tumors. The differences in SF₁₀ (unclamped) among the 50 D-12 tumors and the differences in SF₁₀ (unclamped) among the 50 R-18 tumors thus primarily reflected differences in fraction of radiobiologically hypoxic cells and not experimental uncertainty. The fraction of radiobiologically hypoxic cells of the 50 D-12 tumors differed from 9% to 67%, with a geometric mean of 26% and an arithmetic mean of 29%, and the fraction of radiobiologically hypoxic cells of the 50 R-18 tumors differed from 21% to 90%, with a geometric mean of 50% and an arithmetic mean of 53%.

The tumors of both lines showed highly heterogeneous staining for pimonidazole. The staining pattern was consistent with staining of chronically hypoxic cells without staining of normoxic or acutely hypoxic cells (Fig. 2). The D-12 tumors had developed regions with large necroses, regions composed of tumor chords and small necroses, and large regions without necrosis (Fig. 2A). The necrotic regions were always encompassed by a rim of pimonidazole-positive cells, two to four cell layers thick. The periphery of the tumor chords also stained

![Fig. 1. Cell surviving fraction versus radiation dose for D-12 (A) and R-18 (B) tumors irradiated under clamped (hypoxic; ○) or unclamped (●) conditions. Points, individual tumors. Horizontal lines, geometric means.](image-url)
positive for pimonidazole. Moreover, foci of pimonidazole-positive cells scattered throughout the tissue were observed in tumor regions without necrosis. The R-18 tumors, on the other hand, had not developed macroscopic necrotic regions at resection (Fig. 2B). Foci of tumor cells staining positive for pimonidazole were seen throughout the tissue. These pimonidazole-positive foci differed substantially in size and shape, and a few necrotic or necrotizing cells were seen in the middle of some of the largest foci. In tumors of both lines, the boundary line between stained and unstained cells was sharp (i.e., the cells showed either intense brown staining or no staining). Positive staining of cells adjacent to identifiable blood vessels or cells in the center of tumor chords could not be detected in any tumor.

The fraction of chronically hypoxic cells of the 50 D-12 tumors ranged from 5% to 28% with a mean value of 16.1%, and the fraction of chronically hypoxic cells of the 50 R-18 tumors ranged from 4% to 19% with a mean value of 9.5%. There was a weak positive correlation between fraction of radiobiologically hypoxic cells and fraction of chronically hypoxic cells, for both the D-12 tumors (correlation coefficient, 0.41; \( P = 0.0030 \); Fig. 3A) and the R-18 tumors (correlation coefficient, 0.34; \( P = 0.016 \); Fig. 3B). However, fraction of radiobiologically hypoxic cells was higher than fraction of chronically hypoxic cells in all individual tumors (Fig. 3A and B). Fraction of acutely hypoxic cells differed from 1% to 53% with a mean value of 12.6% for the D-12 tumors and from 10% to 82% with a mean value of 43.8% for the R-18 tumors. There was no correlation between fraction of acutely hypoxic cells and fraction of chronically hypoxic cells (\( P > 0.05 \) for both D-12 and R-18 tumors; Fig. 4). Mean fraction of acutely hypoxic cells was lower than mean fraction of chronically hypoxic cells for the D-12 tumors (\( P = 0.0012 \)) and higher than mean fraction of chronically hypoxic cells for the R-18 tumors (\( P < 0.000001 \)). Only 13 of the 50 D-12 tumors showed a fraction of acutely hypoxic cells that was higher than the fraction of chronically hypoxic cells (Fig. 4A), whereas the fraction of acutely hypoxic cells was higher than the fraction of chronically hypoxic cells in 49 of the 50 R-18 tumors (Fig. 4B).

Twenty-seven of the 50 mice bearing D-12 tumors developed pulmonary metastases, whereas 23 mice did not. Extralymph- nary metastases were not observed in any mouse. The tumors that caused metastases showed ~1.7-fold higher fraction of radiobiologically hypoxic cells than those that did not metastasize (\( P < 0.000001 \); Fig. 5A). Fraction of chronically hypoxic cells was ~1.3-fold higher in the tumors that metastasized than in those that did not (\( P = 0.015 \); Fig. 5B), and fraction of acutely hypoxic cells was ~2.5-fold higher in the metastatic than in the nonmetastatic tumors (\( P = 0.0024 \); Fig. 5C). Moreover, 26 of the 50 mice bearing R-18 tumors developed lymph node metastases, whereas 24 mice did not. Extralymphatic metastases were not detected in any mouse. The tumors that metastasized showed ~1.5-fold higher fraction of radiobiologically hypoxic cells than those that did not metastasize (\( P < 0.000001 \); Fig. 6A). Fraction of chronically hypoxic cells was ~1.4-fold higher in the metastatic than in the nonmetastatic tumors (\( P = 0.0078 \); Fig. 6B), and the metastatic tumors showed ~1.6-fold higher fraction of acutely hypoxic cells than the nonmetastatic tumors (\( P < 0.000001 \); Fig. 6C). Thus, metastatic and nonmetastatic tumors of both lines differed significantly in fraction of radiobiologically hypoxic cells, fraction of chronically hypoxic cells, and fraction of acutely hypoxic cells (Figs. 5 and 6).

In the D-12 line, the difference between metastatic and nonmetastatic tumors was 14.3% for mean fraction of radiobiologically hypoxic cells, 3.8% for mean fraction of chronically hypoxic cells, and 10.5% for mean fraction of acutely hypoxic cells [i.e., the difference was ~3.8-fold larger for fraction of radiobiologically hypoxic cells than for fraction of chronically hypoxic cells (\( P = 0.0042 \)) and ~2.8-fold larger for fraction of acutely hypoxic cells than for fraction of chronically hypoxic cells (\( P = 0.047 \); Fig. 5)]. In the R-18 line, the difference between the tumors that metastasized and those that did not was 22.3% for mean fraction of radiobiologically hypoxic cells, 3.0% for mean fraction of chronically hypoxic cells, and 19.3% for mean fraction of acutely hypoxic cells [i.e., the difference was ~7.4-fold larger for fraction of radiobiologically hypoxic cells than for fraction of chronically hypoxic cells (\( P = 0.00014 \)) and ~6.4-fold larger for fraction of acutely hypoxic cells than for fraction of chronically hypoxic cells (\( P = 0.0020 \); Fig. 6)]. Thus, fraction of radiobiologically hypoxic cells and fraction of acutely hypoxic cells discriminated significantly better between metastatic and nonmetastatic tumors than did fraction of chronically hypoxic cells.

Fig. 2. Immunohistochemical preparations of a D-12 (A) and an R-18 (B) tumor stained with anti-pimonidazole antibody to visualize regions with chronically hypoxic cells. Hypoxic regions appear dark brown. Nec, necrotic regions.
Discussion

Previous studies in our laboratory have shown that hypoxia promotes spontaneous pulmonary metastasis of D-12 tumors primarily by up-regulating IL-8 and spontaneous lymph node metastasis of R-18 tumors primarily by up-regulating uPAR (18–21). Tumors may develop hypoxia by different mechanisms and may thus contain distinctly different subpopulations of hypoxic cells (1, 4). In the work reported here, the potential of different subtypes of hypoxia in promoting metastasis was studied. Similar results were obtained for D-12 and R-18 tumors. Metastasis was found to be positively correlated to fraction of radiobiologically hypoxic cells, fraction of chronically hypoxic cells, and fraction of acutely hypoxic cells, and fraction of radiobiologically hypoxic cells and fraction of acutely hypoxic cells differentiated more clearly between metastatic and nonmetastatic tumors than did fraction of chronically hypoxic cells.

Three conditions indicate that the extent of chronic tumor hypoxia was overestimated and the extent of acute tumor hypoxia was underestimated in our study. First, the clonogenicity of chronically hypoxic cells may be lower than the clonogenicity of normoxic and acutely hypoxic cells (7, 30). Second, pimonidazole staining occurs at pO2 < 7.5 to 10.0 mmHg (24), whereas 50% radiosensitization occurs at pO2 = 3.0 to 7.5 mmHg (5, 31). Third, chronically hypoxic cells may be more sensitive to radiation than acutely hypoxic cells (32). Consequently, fraction of chronically hypoxic cells, defined here as being equal to HFImm, represents the upper limit of the fraction of the clonogenic tumor cells that are chronically hypoxic, and fraction of acutely hypoxic cells, defined here as being equal to HFRad/C0 HFImm, represents the lower limit of the fraction of the clonogenic tumor cells that are acutely hypoxic.

The present experiments suggest that the ratio between fraction of acutely hypoxic cells and fraction of chronically hypoxic cells may differ substantially among tumor lines and among individual tumors of the same line. Thus, mean fraction of acutely hypoxic cells and mean fraction of chronically hypoxic cells were 12.6% and 16.1%, respectively, for the D-12
tumors and 43.8% and 9.5%, respectively, for the R-18 tumors, and fraction of acutely hypoxic cells was larger than fraction of chronically hypoxic cells in 13 of the 50 individual D-12 tumors and in 49 of the 50 individual R-18 tumors. We have measured fraction of acutely hypoxic cells and fraction of chronically hypoxic cells for other human melanoma xenograft lines previously, and fraction of acutely hypoxic cells was found to be larger than fraction of chronically hypoxic cells in 21 of 25 individual A-07 tumors and in 13 of 16 individual U-25 tumors (24). Taken together, these data suggest that the fraction of acutely hypoxic cells often is larger than the fraction of chronically hypoxic cells in human melanoma xenografts. Apparently, tumors of other histologies may show hypoxia patterns diverging from those of melanomas. However, Durand and LePard (33) estimated the extent of acute hypoxia in SCCVII murine tumors by using a dual-staining technique, and they concluded that the majority of the 15% to 20% radiobiologically hypoxic cells were acutely hypoxic. A similar conclusion was reached by Kimura et al. (34) who estimated the extent of acute hypoxia in R3230Ac rat tumors grown in dorsal flap window chambers from measurements of fluctuations in RBC flux and pO2. Recently, oxygen electrodes have been used to study fluctuations in pO2 in a large number of tumors, and it has been shown that tissue pO2 may fluctuate around threshold values in the range 3 to 10 mmHg in large regions of rodent tumors (34–37), human tumor xenografts (38–40), and spontaneous canine tumors (41).

Experimental studies of correlations between tumor hypoxia and metastasis require tumor models that make it possible to distinguish clearly between metastatic and nonmetastatic primary tumors, as is the case for the D-12 and R-18 melanomas. Separate experiments with the D-12 melanoma have shown that (a) macroscopic metastases and/or micrometastatic disease are present in the lungs of some mice at the time of primary tumor resection and (b) mice appearing healthy 3 months after the resection show lungs without histologic signs of metastatic disease, whereas mice showing clinical signs of metastatic disease at that time have lungs with macroscopic metastases only. Moreover, studies involving D-12 and R-18 tumors have shown that mice appearing healthy 3 months after the primary tumor is resected also are free from metastases 3 months later, as revealed by detailed histologic examinations of lungs and lymph nodes (18–21). Consequently, it is highly unlikely that micrometastatic disease was present in mice scored as metastasis-negative in our study.

The work reported here suggests that diffusion-limited as well as fluctuating hypoxia in the primary tumor promote metastasis of D-12 and R-18 tumors. This suggestion is based on the observations that there was no correlation between fraction of chronically hypoxic cells and fraction of acutely hypoxic cells and that metastasis was positively correlated to fraction of chronically hypoxic cells as well as fraction of acutely hypoxic cells. Furthermore, because fraction of acutely hypoxic cells discriminated better between metastatic and nonmetastatic tumors than did fraction of chronically hypoxic cells, fluctuating hypoxia probably influenced metastasis to a greater extent than diffusion-limited hypoxia. In R-18 tumors, this conclusion is consistent with the observation that the fraction of acutely hypoxic cells generally was substantially larger than the fraction of chronically hypoxic cells. However, this conclusion is valid also for the D-12 line where the fraction of acutely hypoxic cells was smaller than the fraction of chronically hypoxic cells in the majority of the tumors, suggesting that acutely hypoxic cells have a higher metastatic potential than chronically hypoxic cells.

Differences in metastatic potential between acutely and chronically hypoxic cells may be related to their localization within tumors. Chronically hypoxic cells are in most tumors located at some distance from functional blood vessels and lymphatics and often adjacent to necrotic regions (42). The pimonidazole staining pattern observed here suggests that this is the case also for D-12 and R-18 tumors. Acutely hypoxic cells, on the other hand, can be located adjacent to functional blood

![Fig. 5.](https://www.aacrjournals.org/clinicscancerresearch/article-pdf/13/7/1976/202356/clinicscancerresearch1371976.pdf)
vessels and in the tumor periphery close to functional lymphatics (42). In addition to having easier access to functional vessels, acutely hypoxic cells may have higher energy status than chronically hypoxic cells (3, 7, 30, 32) and thus be in better condition to accomplish the different steps of the metastatic process.

Moreover, it is an open question whether metastatic dissemination of acutely and chronically hypoxic cells involves the same or different molecular mechanisms. Previous studies have shown that hypoxia-induced up-regulation of IL-8 promotes metastasis of D-12 tumors and hypoxia-induced up-regulation of uPAR promotes metastasis of R-18 tumors (18–21). These conclusions were based on the findings that (a) metastatic tumors showed higher hypoxic fractions and higher expression of IL-8 (D-12) or uPAR (R-18) than nonmetastatic tumors, (b) metastasis was inhibited by treatment with neutralizing antibody against IL-8 (D-12) or uPAR (R-18), and (c) tumor regions staining positive for IL-8 (D-12) or uPAR (R-18) colocalized with regions with chronically hypoxic cells (i.e., regions staining positive for pimonidazole). Interestingly, the IL-8–positive foci were 1.4- to 1.8-fold larger than the pimonidazole-positive foci in D-12 tumors (18, 21) and the uPAR-positive foci were 1.3- to 1.5-fold larger than the pimonidazole-positive foci in R-18 tumors (19, 21). Tumor regions showing acute hypoxia caused by temporal variations in microvessel RBC flux are expected to be located adjacent to chronically hypoxic tumor regions (2, 4). Consequently, the staining patterns of IL-8 (D-12) and uPAR (R-18) were consistent with up-regulation of IL-8 and uPAR in acutely as well as chronically hypoxic tumor regions, suggesting that acute as well as chronic hypoxia promoted pulmonary metastasis of D-12 tumors by up-regulating IL-8 and lymph node metastasis of R-18 tumors by up-regulating uPAR. Our data do of course not exclude the possibility that other molecular mechanisms, similar or different for acutely and chronically hypoxic cells, also might have been involved.

It has been suggested that tumor hypoxia may promote metastatic dissemination also by selecting for hypoxia-resistant, highly metastatic cell variants (43). This is not a likely mechanism for hypoxia-promoted metastasis in D-12 and R-18 tumors. Studies in our laboratory have shown that long-term culturing of D-12 and R-18 cells under chronic or cyclic (acute) hypoxia in vitro does not induce or select for cell variants with enhanced metastatic potential.

Cairns et al. (22) have also carried out experimental studies, suggesting that acutely hypoxic cells have a higher metastatic potential than chronically hypoxic cells. They induced chronic or acute hypoxia in KHT tumors by exposing tumor-bearing mice to a low-oxygen atmosphere of 5% to 7% O2 during tumor growth and observed that mice exposed to acute hypoxia developed a larger number of microscopic lung metastases than mice exposed to chronic hypoxia. However, there is a fundamental difference between this study and the study reported here. For the KHT tumors, all tumor cells were exposed to chronic or acute hypoxia independent of their nutritional status and localization within the tumors (22). In the present work with D-12 and R-18 tumors, effects of naturally occurring acute and chronic hypoxia on metastatic dissemination and growth were studied.

The observation that metastatic spread may be promoted by chronic as well as acute tumor hypoxia has significant clinical implications. It has been suggested that induction of tumor hypoxia may be a useful strategy for enhancing the efficacy of some therapeutic agents [e.g., hyperthermia (44) and hypoxic cell cytotoxins (45)]. One consequence of our study is that treatment strategies involving deliberate tumor hypoxification should be avoided. Moreover, some treatment strategies may increase the fraction of hypoxic cells in tumors by impairing tumor blood supply [e.g., antiangiogenic treatment (46), treatment with vascular disrupting agents (47), and some forms of photodynamic therapy (48)]. The possibility that such treatments may cause tumor hypoxia and hence promote metastasis should be studied in detail before large-scale clinical investigations are initiated.

In summary, tumors may contain chronically hypoxic cells caused by permanent limitations in oxygen diffusion and
acutely hypoxic cells caused by fluctuations in blood perfusion. Hypoxia may promote metastasis by up-regulating the expression of metastasis-promoting gene products. The preclinical study reported here suggests that fluctuating hypoxia may represent a more serious problem to cancer patients than diffusion-limited hypoxia, partly because the fraction of acutely hypoxic cells may be larger than the fraction of chronically hypoxic cells in most tumors and partly because acutely hypoxic cells may have a higher metastatic potential than chronically hypoxic cells. Consequently, it may be beneficial to focus on fluctuating hypoxia rather than diffusion-limited hypoxia when searching for hypoxia-related prognostic variables and predictive assays of treatment outcome.

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