Acute (Cyclic) Hypoxia Enhances Spontaneous Metastasis of KHT Murine Tumors

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ABSTRACT

Hypoxia exists in most human and rodent solid tumors and has been shown to correlate with poor survival in carcinoma of the cervix, carcinoma of the head and neck, and soft tissue sarcoma. It exists both chronically, due to the poorly organized vasculature of solid tumors, and acutely, due to fluctuations in blood flow. It has been found that tumors that are more hypoxic are more likely to metastasize in humans and in rodent models, and it has been demonstrated that exposure of tumor cells to hypoxia in vitro can transiently enhance their metastatic potential when they are reinjected i.v. into mice. The purpose of the present study was to determine whether experimentally imposed hypoxic stress in vivo, either chronic or acute, affects the process of spontaneous metastasis in tumor-bearing mice. We exposed mice bearing KHT tumors to low oxygen conditions (5–7% O2 breathing) daily during tumor growth in an attempt to induce additional chronic (2 h/day) and acute (12 × 10 min/day) hypoxia in their tumors. By monitoring tumor pO2 levels over the course of treatment, we demonstrated that these treatments produce acute and chronic hypoxia within the tumor tissue. The acute but not the chronic hypoxia treatment significantly increased the number of spontaneous microscopic lung metastases in the mice by a factor of about 2, and the results suggest that this effect was due to the changes induced in the primary tumor. This study describes a novel method for studying the effects of hypoxia in solid tumors and demonstrates that acute and chronic hypoxia can have different effects on tumor cell behavior in vivo.

INTRODUCTION

Most human and rodent solid tumors contain a substantial fraction of cells that are hypoxic. Normal tissue pO2 ranges, in general, between 10 and 80 mm Hg, depending on the tissue type, whereas tumors often contain significant regions in which the pO2 is <5 mm Hg (1–7).2 Tumor hypoxia has been found to be heterogeneous among tumors of identical histopathological type, and it does not correlate with standard prognostic factors such as tumor size, stage, and grade (1–7).2

Tumor hypoxia seems to arise because of the stochastic development of the vasculature during tumor growth. Due to the often rapid and poorly regulated angiogenesis that occurs during tumor development, the vascular network of tumors has characteristic structural and functional abnormalities. Tumor blood vessels have highly irregular architecture, which includes such features as blind ends, arteriovenous shunts, and high angle branching patterns (8, 9). They also lack smooth muscle and enervation and may have an incomplete endothelial lining and basement membrane, which makes them more “leaky” than vessels in normal tissues (8, 9). Furthermore, the vessels supplying tumors often develop from post capillary venules, creating severe longitudinal pO2 gradients within the vessels themselves (10).

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suggest that it is the former physiological situation that is responsible for accelerating metastasis formation, and they provide a model for studying the underlying molecular mechanisms in vivo.

**MATERIALS AND METHODS**

Mice and Tumor Cell Lines. Experiments were performed using the KHT-C murine fibrosarcoma cell line described previously (35). Cells were maintained by alternate in vitro and in vivo passage. In vitro, cells were grown as monolayers in plastic flasks using α-MEM (Life Technologies, Inc., Burlington, Canada) supplemented with 10% fetal bovine serum (Wisent, Quebec, Canada). Cells between the second and fourth in vitro passage were removed from the flasks during exponential growth using 0.05% trypsin for 5 min at 37°C and transferred into syngeneic 8–12-week-old C3H/HeJ male mice (Jackson Laboratory, Bar Harbor, ME). Each tumor was initiated by injecting 2 × 10^4 cells in 50 μl of media into the left gastrocnemius muscle. Tumor growth was monitored by an external measurement of leg diameter. Animals were sacrificed when the leg diameter reached a predetermined size as described below. For i.v. injection of tumor cells, 2.5 × 10^4 to 2 × 10^6 cells, depending on the experiment, were injected via the tail vein, and animals were sacrificed at various time points. Animals were housed at the Ontario Cancer Institute animal colony and had access to food and water ad libitum. All experiments were performed according to the regulations of the Canadian Council on Animal Care.

**Hypoxia Treatment.** Unanesthetized mice were placed in 6-liter incubator chambers (Billups-Rothenberg Inc., Del Mar, California) and exposed to a continuous flow of a humidified gas mixture to induce in vivo hypoxia. The chronic hypoxia treatment consisted of a single 2-h exposure to 5–7% O_2; balance N_2, given once per day, 7 days per week. The acute hypoxia treatment consisted of 12 cycles of 10-min exposure to 5–7% O_2; balance N_2/10 min air, given once per day, 7 days per week. Control animals were placed in identical chambers and exposed to a continuous flow of humidified laboratory air. The flow rate was monitored and kept between 3 and 6 liters/min throughout the treatments, and the temperature was held constant at 25°C.

**Tumor Oxygenation Measurements.** Measurements of tumor pO_2 were made using the Oxylite 4000 system (Oxford Optronix Ltd., Oxford, United Kingdom). This system uses a fiberoptic probe that contains a fluorescent dye immobilized at the tip. The fluorescent lifetime of the dye is inversely proportional to oxygen concentration, and measurements are made by interrogating the probe with a light pulse and converting the fluorescent lifetime to a pO_2 value. The probe was introduced through a 20-gauge needle into tumors or leg muscle of mice anesthetized using Ketalean (2.5 mg/kg ketamine hydrochloride i.m.; MTC Pharmaceuticals, Cambridge, Canada) and Innovar-Vet (0.08 mg/kg fentanyl and 4 mg/kg droperidol, i.p.; MTC Pharmaceuticals, Mississauga, Canada). Measurements were recorded in real time by exporting the data to a portable computer running Chart v3.4 (AD Instruments).

**Histological Assessment of Micrometastases.** Microscopic metastases were examined histologically as described previously (29). Briefly, animals were sacrificed when the tumor-bearing leg reached 9 mm (0.3–0.4 g), and the lungs were removed and fixed in Bouin’s solution (BDH Inc., Toronto, Canada). The lungs were embedded in paraffin, and four histological sections 200 μm apart were cut through each lobe and stained with H&E. The presence of microscopic metastases was evaluated by two independent blinded observers using a transmitted light microscope. Groups of two or more clearly identifiable tumor cells were scored as a metastasis. Agreement between observers was generally very good, but in the event of discrepancies, the section was reviewed by both observers, and a consensus was reached. The size of the lesions was measured using a graticule at ×400 magnification. Area was calculated by multiplying the longest diameter and the perpendicular diameter because lesions were generally elliptical in shape, and the geometric mean was calculated.

**Colony-forming Assay for Micrometastases.** Individual lungs were enzymatically and mechanically digested to obtain a single cell suspension as described previously (36).Suspensions were counted on a hemocytometer, and 5 × 10^4 total cells were plated on 10-cm culture dishes in 10 ml of α-MEM + 10% fetal bovine serum. Twelve days later, the easily identifiable KHT-C tumor cell colonies were counted.

**Assessment of Macroscopic Metastases.** For macroscopic metastases, lungs were removed at the indicated times and fixed in Bouin’s solution as described above. The number of pulmonary metastases visible on the lung surface was counted with the aid of a dissecting microscope as described previously (21, 29).

**Statistical Analysis.** Because the distribution of lung metastases in a group of mice is not normally distributed, nonparametric methods were used (37). The Kruskal-Wallis test was used to analyze the difference in the number of metastases between multiple groups, and Dunn’s test was used to perform multiple comparisons against the control group. For comparisons of two groups, the Mann-Whitney rank-sum test was used.

**RESULTS**

In a series of initial experiments, we examined different treatment strategies designed to induce additional chronic and acute hypoxia within KHT mouse tumors. Based on those initial studies, we chose two treatments for the current study: (a) a chronic hypoxia treatment that involved exposing animals to 5–7% O_2 for 120 min each day; and (b) an acute hypoxia treatment that involved exposing animals to 12 cycles of 10 min 5–7% O_2/10 min air each day. This level of oxygen concentration is close to the limit that can be tolerated by the mice over this time period. The total time of exposure was chosen to exert a significant toxic stress in the tumors yet minimize adaptation to the stress in the whole animal. These treatments were continued daily until the animals were sacrificed.

The changes in tumor oxygenation induced by the 5–7% O_2 exposure were measured using the Oxylite 4000 system. Fig. 1 presents results from representative experiments, showing the patterns of pO_2 change that were observed during the chronic and acute treatments. As expected, the absolute pretreatment pO_2 values varied quite widely from 40 to 0 mm Hg, depending on where the probe was positioned within the tumor. However, we consistently observed a drop in pO_2 down to hypoxic values (<5 mm Hg) when 5–7% O_2 was breathed and a rise in pO_2 when air was reintroduced. A similar pattern was observed when pO_2 was measured in normal muscle. However, absolute pO_2 values were much higher and rarely dropped below 5 mm Hg during the low oxygen exposures (Fig. 1A). We also performed these measurements on tumors in animals that had been exposed daily to chronic and acute hypoxia treatments for 7 days. We observed no change in the oxygen profiles of the tumors in these animals, which suggests that the treatments are short enough to avoid adaptation. These observations confirm that we are able to modify tumor pO_2 acutely or chronically by exposing animals to low O_2 atmospheres.

We next examined the effect of these treatments on spontaneous metastasis of KHT tumors. Mice were exposed to either chronic or acute hypoxia treatment or to normal laboratory air (control) each day after i.m. injection of 2 × 10^5 cells. Neither of the treatments affected the rate of tumor growth compared with the control animals. When the diameter of the tumor-bearing leg reached 9 mm (~8 days), corresponding to a tumor mass of approximately 0.4 g, the lungs were excised, and four histological sections separated by 200 μm were examined for the presence of microscopic metastases (Fig. 2A). We observed a highly significant increase (P < 0.01) in the number of lung micrometastases in the group receiving the acute hypoxia treatment relative to the control group. However, there was no difference between the group receiving the chronic hypoxia treatment and the control group. This finding is the first demonstration that exposure to acute hypoxia in vivo can directly stimulate spontaneous metastasis.

This method of quantifying microscopic metastases involves counting the lesions in four histological sections, which is only a sample of the total lung tissue. If we assume that the metastases are distributed and oriented randomly with respect to the plane of the section, then the number of metastases that are counted using this method will be proportional not only to the actual number of metastases present but also to the size of the metastases (specifically, the diameter of the...
lesion perpendicular to the plane of the sections). To determine whether the acute hypoxia treatment increased the growth rate of the lung metastases, we measured all of the lesions in both the control and acute hypoxia-treated groups. There was no significant difference in lesion size between the two groups (Fig. 2B), suggesting that the acute hypoxia treatment did not affect the growth rate of metastases in the mouse lungs. To confirm these results using an alternate method and to assess the viability of the microscopic lung colonies, we used a colony-forming assay to measure the tumor burden in the mouse lungs. Control and acute hypoxia-treated mice, as described above, had their lungs removed and digested to obtain single cell suspensions when their tumors reached 0.4 g. For the lung colony experiments, this occurred at approximately 10 days after tumor implantation. A total of $5 \times 10^4$ lung cells were plated, and the number of KHT-C colonies that developed was counted. This assay gives a measure of the relative number of viable tumor cells in the lungs. As with the histological assessment, the acute hypoxia treatment caused a significant increase in the number of clonogenic tumor cells in the lungs of the mice (Fig. 2C). Because the tumor cells make up an extremely small proportion of the lung at this stage, the data are presented as the number of viable tumor cells relative to the number of lung cells plated. This eliminates any variation introduced by the digestion procedure. However, analysis of the data using the total number of viable tumor cells recovered as an end point yielded similar results (data not shown).

It has been documented that lung injury can cause an increase in metastatic efficiency (37, 38). Therefore, we performed experiments to determine whether the increased number of metastases observed in the acute hypoxia-treated group was due to the effect of the treatment on the primary tumor or to the effect of the treatment on the mouse as a whole. We have determined that in this model, tumors growing in the leg begin to seed cells to the lung at approximately 3–4 days after implantation, or at a leg diameter of 7.5 mm. To mimic this situation, we exposed animals without tumors to the acute hypoxia treatment daily and injected $2 \times 10^7$ tumor cells i.v. via the tail vein on day 3. These mice were treated for an additional 4 days, which is equivalent to the treatment that the initial group of tumor-bearing animals received at a later stage of tumor growth. Mice were injected with $2 \times 10^5$ KHT-C cells i.m. and treated daily with air or the acute hypoxia treatment during the growth of their tumors to a leg diameter of 15 mm (2.0 g and ~15 days). At this time, the lungs were removed, and the number of macroscopic metastases was counted. There was a nonsignificant difference ($P > 0.1$) between the two groups, showing a trend toward the acute hypoxia-treated group having more metastases (Fig. 4).

### DISCUSSION

Previously, we have shown that exposure of murine KHT cells to hypoxia and reoxygenation in vitro dramatically enhances their met-
astatic potential when reinjected into mice (21). In vivo, measurements using the Eppendorf \( \text{PO}_2 \) histogram have revealed that there is inter- and intratumor heterogeneity in oxygenation of KHT tumors (30). Furthermore, it was found that animals bearing the more poorly oxygenated tumors had a significantly higher incidence of micrometastasis (29). These results are consistent with studies in humans and suggest that the KHT model may be useful for studies of the mechanisms underlying these observations. The use of orthotopic tumor implantation is also advantageous in that orthotopic systems tend to model the clinical situation more closely (39–41).

The current study sought to integrate the findings of both of these prior studies. We used a direct experimental technique to modify tumor oxygenation rather than relying on naturally occurring variation, so that causal relationships could be investigated. Our strategy was to develop treatments that allowed us to modify tumor \( \text{PO}_2 \) in vivo and induce additional acute or chronic hypoxia in tumors. This approach allowed direct assessment of the effects of chronic and acute hypoxia and potentially provides a model for investigating the underlying molecular mechanisms.

As expected, we were able to modulate the \( \text{PO}_2 \) in KHT tumors by...
subjecting tumor-bearing mice to low oxygen atmospheres. Using the Oxylite system, we found that there was heterogeneity in pretreatment oxygenation, which is consistent with the spatial heterogeneity observed in our previous studies (30). We observed a consistent pattern of change in tumor pO2 levels, with kinetics on the scale of minutes, which allowed us to induce acute hypoxia over a relatively short period of time. Over the course of the 4-h exposures, we also observed pO2 fluctuations that were independent of the treatment (see Fig. 1D). We believe that this is a reflection of the naturally occurring instability in pO2 levels within these tumors. The kinetics of these fluctuations are consistent with the previously reported temperature fluctuations that occur in these tumors, which were attributed to blood flow instability (42). However, because the probe diameter is 220 μm, the Oxylite system tends to average out some of the microregional heterogeneity in tumor oxygenation, as compared with true microelectrodes, and therefore should be considered a more regional measure of pO2 (43). In normal muscle, we saw a similar pattern of change in pO2 during the treatments (Fig. 1A). However, pretreatment oxygen tension was much higher than that in the tumors, and the absolute pO2 did not drop below 5 mm Hg, suggesting that we were not inducing substantial hypoxia in normal tissue (Fig. 1A). We did not observe any naturally occurring fluctuations in normal muscle pO2, although another group, using microelectrodes and Fourier analysis, has observed low amplitude, low frequency fluctuations in pO2 in rat skeletal muscle (44). This difference may be due to the different measurement devices and types of analysis used. Overall, the assessment of our treatments is consistent with the concept of temporal and spatial heterogeneity within these tumors and with our strategy of inducing acute and chronic hypoxia in vivo in tumors. It is possible that the treatments could also alter the tumor pH, but we have been unable to detect any significant changes in tumor pH as a result of these treatments (data not shown).

We found that the acute but not the chronic hypoxia treatment increased the number of spontaneous micrometastases in mouse lungs. Importantly, we have also demonstrated that this observation represents an increase in the number of clonogenically viable tumor cells present in the lungs. Because we were unable to observe any effect on the metastatic efficiency of cells introduced i.v., we conclude that the enhancement is due to the effect of the acute hypoxia treatment on the primary tumor and is not due to other potential effects of the treatment such as damage to the lung endothelium. In addition, because we did not observe a change in the timing of the initial metastatic spread or in the growth rate of the primary tumor or the metastases, we propose that the enhancement of metastasis is due to an increase in the rate of accumulation of tumor cells in the lung. This could be achieved by an increase in the efficiency of seeding or by an increase in the number of cells released from the primary tumor. These experiments demonstrate, for the first time, that hypoxia has a direct effect on metastasis in vivo and suggest that acute fluctuating hypoxia is of primary importance. Clinically, this finding suggests that it may be important to develop diagnostic tools that address not only total hypoxia but also acute and chronic hypoxia independently because these two situations may have different effects on patient outcome.

The fact that we did not observe a significant effect of the acute hypoxia treatment when metastases were scored macroscopically is consistent with other results obtained using the KHT system. Previously, we found that the proportion of animals with micrometastases was significantly correlated with the extent of hypoxia in the primary tumor but that the number of macroscopic metastases observed when tumors reached a size of 2 g showed only a nonsignificant trend toward a correlation with hypoxia in the primary tumor (29). We suggested that the large number of metastases observed when the KHT tumors are allowed to grow to a large size makes it difficult to observe the effect of hypoxia at these larger sizes. In addition, at larger sizes, all KHT tumors become very hypoxic, which could confound the interpretation of these results (29). However, it is also possible that the effect of the acute hypoxia treatment is transient, as observed in the experiments involving cells exposed to hypoxia in vitro, and is only apparent at an early stage of metastatic growth (21). Additional studies with a less aggressive tumor model may help to resolve these issues.

The mechanism responsible for the effect of hypoxia on metastasis is currently unknown, but two possibilities arise from work published in the last few years. Hypoxia has been shown to increase genetic instability and select for cells that are resistant to apoptosis (31, 34). It may therefore accelerate tumor progression. Whereas our acute hypoxia treatment does appear to cause DNA strand breaks, possibly by increasing oxidative stress (data not shown), we have no evidence that the rate of tumor progression is altered during the short time course of these experiments. However, hypoxia and/or oxidative stress may also be causing changes in gene expression that enhance the ability of cells to metastasize.

The expression of many genes is regulated by hypoxia and oxidative stress, and several of these genes have also been implicated in the metastatic process. Specifically, vascular endothelial growth factor has been shown to be primarily responsible for the increase in metastatic potential of human melanoma cells on exposure to hypoxia in vitro (20). Furthermore, both interleukin 8 and tissue factor have been shown to be up-regulated by hypoxia and to have the ability to increase metastatic potential in pancreatic and melanoma cells, respectively (45–48). In addition, several groups have found that hypoxia can increase the invasive capacity of tumor cells using a variety of in vitro assays, and several genes encoding components of various protease systems have been found to be up-regulated under low oxygen conditions (24, 49). However, due to the complex, multistep process of metastasis, there are many other genes that may be involved, and many of these may exert their effects in a cell type-specific manner.

This study describes a novel model for studying the effects of hypoxia in solid tumors and demonstrates that acute and chronic hypoxia can have different effects on tumor behavior. Specifically, acute but not chronic hypoxia was found to increase metastasis of
KHT murine tumors. Further work to extend these studies to other models including a human xenograft system is in progress to determine if the observations can be generalized to other cell types. This model will allow investigation of the mechanisms underlying the effects of hypoxia on metastasis and may point to new ways that hypoxia in solid tumors needs to be addressed in clinical practice.

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