Acute Hypoxia Enhances Spontaneous Lymph Node Metastasis in an Orthotopic Murine Model of Human Cervical Carcinoma

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ABSTRACT

An orthotopic mouse model of cervical carcinoma has been used to investigate the relationship between acute (cyclic) hypoxia and spontaneous lymph node metastasis in vivo. The human cervical carcinoma cell line ME-180 was stably transfected to express the fluorescent protein DsRed2, which allowed the in vivo optical monitoring of tumor growth and metastasis by fluorescent microscopy. The surgically implanted primary tumors metastasize initially to local lymph nodes and later to lung, a pattern consistent with the clinical course of the disease. The effect of acute hypoxia on the growth and spread of these tumors was examined by exposing tumor-bearing mice to treatment consisting of exposure to 12 cycles of 10 min 7% O2 followed by 10 min air (total 4 h) daily during tumor growth. After 21 days, the tumors were excised, lymph node and lung metastases were quantified, and the hypoxic fraction and relative vascular area of the primary tumors were assessed by immunohistochemical staining for the hypoxic marker drug EF5 [2-(2-nitro-1H-imidazole-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide] and the vascular marker CD31, respectively. In untreated mice, the primary tumor size was directly correlated with lymph node metastatic burden. The acute hypoxia treatment resulted in a significant decrease in the size of the primary tumors at the time of excision. However, the mice in the acute hypoxia group had an increased number of positive lymph nodes (2–4) as compared with control mice (1–3). Lung metastasis was not affected. The acute hypoxia treatment also decreased the relative vascular area in the primary tumors but did not affect the hypoxic fraction. These results suggest that fluctuating oxygenation in cervical carcinoma tumors may reduce tumor growth rate, but it may also enhance the ability of tumor cells to metastasize to local lymph nodes.

INTRODUCTION

The microenvironment of solid tumors is characterized by regions of nutrient deprivation, low extracellular pH, high interstitial fluid pressure, and hypoxia. Specifically, although the oxygen tension (pO2) in normal tissue ranges, in general, between 10 and 80 mm Hg, depending on the tissue type, tumors often contain significant regions where the pO2 is <5 mm Hg (1–7). 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).

Hypoxia in solid tumors is thought to be primarily attributable to the abnormal vasculature that develops during tumor angiogenesis. The blood vessels in solid tumors 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 contribute to a situation in which a significant proportion of tumor cells lie in hypoxic regions beyond the diffusion distance of oxygen (100–150 μm) where they are exposed to chronically low oxygen tensions.

However, tumor hypoxia can also occur transiently. Substantial instability in microregional blood flow and tissue oxygenation has been demonstrated to occur in animal and human tumors, using a number of techniques (11–17). These fluctuations are thought to be attributable to transient occlusion, narrowing of vessels, and arteriolar vasomotion. Also, the abnormal architecture of the vascular system itself may produce variation in red cell traffic patterns (13, 14). The high interstitial fluid pressure, characteristic of solid tumors, may further exacerbate the situation, as suggested by recent attempts to model these temporal changes (18). The available data suggest that this blood flow instability, in the context of an already poorly organized and regulated vascular system, can produce temporal (acute) changes in oxygenation in substantial volumes of solid tumors (12, 16, 17).

Clinically, tumor hypoxia, as measured using needle electrodes, has been shown to correlate with poor disease-free and overall survival in carcinoma of the uterine cervix, soft-tissue sarcoma, and squamous cell carcinoma of the head and neck (1–5, 7). In cervical carcinoma it has been shown that this correlation exists in groups of patients treated with radiation or surgery, with or without chemotherapy, demonstrating that this relationship is not due solely to the radioprotection afforded by low oxygen concentrations (5). Furthermore, hypoxia has been found to be associated with the presence of lymph node metastases at presentation and with higher rates of distant failure, suggesting that it may be a marker of progression in cervix cancer (4, 7, 19, 20).

The vasculature of cervical tumors, as assessed primarily by immunohistochemical staining, is another physiological parameter that has been shown to have predictive value in terms of treatment outcome and may be related to tumor hypoxia. This relationship was first observed by Kolstad (21) in the 1960s, where large intercapillary distances on the tumor surface were found to predict for poor outcome after radiotherapy in cervical carcinoma. However, these data are diverse, because several different techniques and parameters have been used to describe the vasculature in cervical carcinoma. The techniques can be broadly grouped into two categories, those that quantify the microvessel density in so called “hot spots” of tumor angiogenesis and those that measure global parameters of vascularity over the whole of the tumor. In general, a high hot spot microvessel density, which is thought to be a marker of angiogenesis, has been shown to be predictive of poor outcome (20, 22, 23), whereas a high global vascularity has been shown to be predictive of a more favorable outcome (24–27). It has been proposed that this latter relationship may be attributable to high levels of hypoxia in tumors with low global vascular densities. In support of this idea, a recent study in a single group of cervix cancer patients has shown that hot spot microvessel density does not correlate with hypoxia as measured using the Eppendorf microelectrode, whereas the global intercapillary dis-
tance is proportional to the hypoxic fraction (26). However, data from other tumor sites have shown no correlation between measures of global vascularity and hypoxia (28). Interestingly, in cervical carcinoma, no study has shown a link between any measure of the vasculature and the presence of lymph node metastasis at diagnosis.

A link between hypoxia and metastasis has been demonstrated in a number of experimental systems, and the available data have been reviewed by Rosfjord (29) and Subarsky and Hill (30). Exposure to hypoxia in vitro has been found to transiently enhance the experimental metastatic potential of murine KHT fibrosarcoma, B16 melanoma, and SCC-VII squamous cell carcinoma, as well as human melanoma cell lines (31–34). In human melanoma xenografts, hypoxia in the primary tumor has been shown to correlate with the incidence of lymph node metastasis (35). Similarly, in the KHT murine fibrosarcoma model, we have reported that, at small tumor sizes, the pH2 level in the primary tumor correlates with the probability of spontaneous metastatic spread (36). Furthermore, exposing tumor-bearing animals to periods of fluctuating hypoxia during tumor growth can enhance spontaneous metastasis to the lungs (37). These findings are consistent with the clinical data and suggest that fluctuations in oxygen concentration can directly enhance the metastatic efficiency of solid tumors.

The direct effects of acute hypoxic exposure on lymph node metastasis have not been examined in an animal model. Because the molecular mechanisms underlying the spread of tumor cells to lymph nodes may be significantly different from those involved in hematogenous spread, these types of experiments may be useful in determining the consequences of hypoxia in malignancies such as cervical carcinoma, where lymph node metastasis is the predominant route of dissemination. To this end, we have recently developed an orthotopic mouse model of cervical cancer that allows for the study of the late stages of this disease. The human cervical carcinoma cell line ME-180 was stably transfected to express high levels of the fluorescent protein DsRed2, which allows for in vivo optical monitoring of tumor growth and metastasis using a fluorescent stereomicroscope. Tumor fragments are surgically implanted into the cervix of severe combined immunodeficient mice and grow to involve the entire reproductive tract, incorporating the normal tissues of the uterus, cervix, and portions of the vagina. These tumors metastasize initially to local lymph nodes and later to lung, a pattern consistent with the clinical course of the disease.

The purpose of the present study was to determine whether hypoxia and/or vascularity correlates with metastasis in this model system and to examine whether imposing additional acute hypoxia on the developing tumors could enhance the spread of disease to the lymph nodes and the lungs.

MATERIALS AND METHODS

Tumor Cell Lines and Mice. ME-180 human cervical carcinoma cells were obtained from the American Type Culture Collection. The construction and characterization of the fluorescent DsRed expressing cells have been described previously. Briefly, the cells were transfected using Clontexin (Clontech, Palo Alto, CA), with a plasmid containing the DsRed2 gene under the control of the cytomegalovirus promoter and the neomycin resistance gene under the control of the SV40 early promoter (Clontech). The cells were selected, cloned, and maintained by alternate growth in vitro and in vivo to minimize the selection of cells adapted to grow in either situation. In vitro, cells were grown as monolayers in α-MEM (Life Technologies, Inc., Burlington, Ontario, Canada) containing 800 μg/ml G418 (Life Technologies, Inc., Burlington, Ontario, Canada), and supplemented with 10% fetal bovine serum (Wisent, St. Bruno, Quebec, Canada) at 37°C in 5% CO2. For in vivo growth, cells between the 2nd to 4th in vitro passage were removed from the flasks during exponential growth using 0.05% trypsin for 10 min at 37°C and transferred into 8–12-week-old female severe combined immunodeficient mice obtained from an in-house breeding program. Each tumor was initiated by injecting 2.5 × 106 cells in 50 μl of media into the left gastrocnemius muscle. Tumor growth was monitored by an external measurement of leg diameter. 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.

Orthotopic Implantation. The development of the orthotopic implantation technique has been described previously. Briefly, i.m. ME-180 tumors 0.6–0.8 g in size were excised and dissected under sterile conditions. The tumors were cut into 2–3 mm3 fragments in α-MEM and placed on ice. Female severe combined immunodeficient mice were anesthetized by isofluorane inhalation, and the uterus was exposed by an abdominal midline incision. A small incision was made in the uterus at the level of the cervix, and a 2–3 mm3 tumor fragment was sutured in place using a single 0–0 silk suture. The abdomen was closed in two layers using 4–0 silk sutures and stainless steel wound clips.

In the present study, each of four i.m. tumors was used to initiate 8–12 cervical tumors, and the mice from each donor tumor were divided equally into the treatment and control groups (total 40 animals). The acute hypoxia treatment had no noticeable effect on healing of the surgical incision, and all mice had the wound clips removed after 7 days.

Acute Hypoxia Treatment. Beginning on the day after tumor implantation, unanesthetized mice were placed in 6-liter incubator chambers (Billups-Rothenberg Inc., Del Mar, CA) and exposed to a continuous flow of a humidified gas mixture to induce in vivo hypoxia. The acute hypoxia treatment consisted of 12 cycles of 10 min 7% O2, balance N2/10 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 was kept between 3 and 6 liter/min throughout the treatments, and the temperature was held constant at 25°C. This protocol had been shown previously to induce periods of hypoxia in the tumor tissue over the entire 4 h course of treatment, as measured using the Oxylite 4000 PO2 monitoring system (Oxford Optronix, Oxford, United Kingdom; Ref. 37). Hematocrit was determined after 3 weeks of treatment by a standard microhematocrit method.

EFS Administration. To identify hypoxic regions of the primary tumor, mice received an injection of the hypoxia marker drug EFS (2-[2-(nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamidene]) (obtained from Dr. Cameron Koch, University of Pennsylvania), 3 h before tumor excision (38). To achieve optimal drug distribution and tumor staining, each mouse received 5 μg EFS i.p. of a 10 mM EFS stock solution in 0.9% saline, giving a total body dose of 100 μmol/kg. Two mice were left unanesthetized as negative controls.

Immunohistochemistry. Immunohistochemical quantification of hypoxia and the amount of vasculature was achieved by staining for EFS and CD31, respectively. Immediately after excision, primary tumors were embedded in optimal cutting temperature embedding medium (Tissue-Tek, Sakura) and snap frozen in liquid nitrogen. Samples were stored at −70°C until processing. For each marker, 5 sections, 5-μm thick, were cut at 200-μm intervals, to address the issue of intratumor heterogeneity. A separate serial section was used for each marker. A subset of 10 tumors was also stained for the hypoxia inducible protein carbonic anhydrase 9 (CA9) as an intrinsic marker of hypoxia (39).

Before staining, slides were fixed in 2% paraformaldehyde for 20 min at 4°C and rinsed three times in PBS. All incubations during the staining procedures were carried out at room temperature. Endogenous peroxidase was blocked using a 3% H2O2, and endogenous biotin was blocked using the Vector Lab biotin blocking kit (Vector Laboratories, Burlington, Ontario, Canada). Slides were then blocked with 5% normal serum in PBS for 10 min. Slides were then incubated with primary antibody for 60 min and rinsed three times in PBS. The primary antibodies and their dilutions were as follows: for EFS, the biotinylated antibody ELK3-51 (1:500; a gift of Dr. Cameron Koch, University of Pennsylvania); for CD31, the rat antimouse CD31, clone MEC 13.3 (1:500; Pharmingen International); and for CA9, the monoclonal antibody M75 (1:50; a gift from Dr. Adrian L. Harris and Dr. Nigel J. Beasley, John Radcliffe Hospital, Oxford). For CD31 and CA9, slides were then incubated for 30 min with biotinylated antirat IgG, mouse adsorbed (1:200) for CD31, and with biotinylated antimouse IgG (1:200) for CA9 (Vector Laboratories).

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Slides were then rinsed three times in PBS and incubated with streptavidin-horseradish peroxidase from the Signet UltraStreptavidin kit (Signet Laboratories, Dedham, MA) for 20 min. After rinsing three times in PBS, slides were incubated with freshly prepared NovaRed substrate (Vector Laboratories) for 5 min and rinsed in distilled water. All slides were counterstained with Mayer’s hematoxylin.

**Quantifying Hypoxia and Vascularity.** Images of entire tumor sections were digitised using a Polaroid SprintScan 4000 slide scanner at a resolution of 6 μm/pixel using standardized acquisition settings throughout. The tumor area was delineated manually, and all artifacts and large areas of necrosis were removed using Adobe Photoshop version 5.5 (Adobe Systems Inc., San Jose, CA). The images were then analyzed using Northern Eclipse image analysis software (Empix Imaging, Mississauga, Ontario, Canada). The positively stained areas (EF5 for hypoxia or CD31 for vasculature) were segmented using a single threshold level for all slides, and all areas >2 pixels (72 μm²) in size were counted as positive regions. The stained fraction (%) for each marker was then calculated by dividing the stained area by the total tumor area to give the hypoxic fraction and the relative vascular area. For CD31, the number of vascular elements per unit area was also calculated to give the global vascular density. The images presented have been adjusted for brightness, contrast, and color balance for ease of viewing, but quantification was performed on unmanipulated images.

**Quantifying Metastasis.** A Leica MZ FLIII fluorescent stereomicroscope with a 100 W mercury lamp was used to observe fluorescent tumors and metastases. DsRed-labeled cells were observed using a 560/40 excitation filter and a 610 long-pass emission filter. Images were acquired using a Leica DC350 digital camera and analyzed using Northern Eclipse software (Empix Imaging). Immediately after dissection, images of each side of each lung lobe were acquired using a Leica Fluorescence stereomicroscope and a 610 long-pass emission filter. Images were acquired using a Leica MZ FLIII fluorescent stereomicroscope, after dissection of animals bearing orthotopic ME-180 DsRed expressing primary tumors. Local lymph nodes and the lungs on a fluorescent stereomicroscope were digitised. Images of entire tumor sections were acquired using standardized acquisition settings. For lymph node metastases, the images were segmented into fluorescent and nonfluorescent areas, using the same threshold level throughout, to identify and measure metastases. This protocol is able to identify small numbers of tumor cells in the lymph nodes, with the smallest lesion identified in this series of animals corresponding to approximately 125 cells. For lung metastases, a relative thresholding function was applied that identifies objects relative to their local background (40 × 40 pixel area), to highlight small metastatic lesions. A single common threshold was then applied to identify and measure the larger metastases. The same protocol and settings were used for all lungs. Where reported, the volume of the metastases was calculated by measuring two perpendicular diameters and assuming an ellipsoid geometry. The images presented have been adjusted for contrast, brightness, and color balance for ease of viewing, but quantification was performed on unmanipulated images.

**Statistical Analysis.** Relationships between tumor physiological variables were examined using linear regression. Continuous variables were also dichotomised (above and below the median value), and pairwise comparisons were made using the Student’s t test and the Bonferroni correction for multiple comparisons. ANOVA was used to compare the inter- and intratumor variation in EF5 and CD31 staining. Two-way ANOVA was used to test for differences between the four sets of animals and between the two treatment groups. Nonparametric methods were used to test for differences in the number of metastases. For comparisons of two groups, the Mann-Whitney rank-sum test was used. Differences in the incidence of lung metastasis were tested using a 2 × 2 contingency table.

**RESULTS**

**Relationship between Tumor Physiological Parameters and Metastasis.** We examined initially the relationship between several tumor physiological parameters and metastasis to lymph nodes and to lungs in the group of 20 untreated mice bearing fluorescent orthotopic cervical tumors (four sets of four to six animals, each derived from a different i.m. donor tumor). Three weeks after implantation of the tumor fragments, the primary tumors were excised and weighed, and metastasis was quantified by dissecting the animal and imaging the local lymph nodes and the lungs on a fluorescent stereomicroscope (Fig. 1). The expression of the DsRed fluorescent protein by the tumor cells allowed the incidence, number, and size of the metastatic lesions to be scored. Tumor hypoxia, relative vascular area, and global vascular density were assessed by immunohistochemical staining for the hypoxia marker drug EF5 and vascular marker CD31, respectively, on serial sections of a ME-180 orthotopic cervical tumor. Scale bars indicate 500 μm. C and F are brightfield images, and D and E are the corresponding fluorescent images, acquired on a Leica MZ FLIII stereomicroscope, after dissection of animals bearing orthotopic ME-180 DsRed expressing primary tumors. C and D show local lymph node metastases (primary tumor removed). Scale bar indicates 4 mm. E and F show an individual lung lobe in which metastatic lesions can be observed in the fluorescent image. Scale bar indicates 1 mm.
relationship between tumor size and metastasis to the lungs (Table 1). There was no linear relationship between tumor size and either the hypoxic fraction or the vascular parameters (data not shown).

We next assessed whether the hypoxic fraction and vasculature in the primary tumor correlated with metastasis. The hypoxic fraction was assessed by immunohistochemical staining for the hypoxia marker drug EF5 (hypoxic fraction). The vasculature was assessed by measuring the fraction of the entire tumor area staining positive for the vascular marker CD31 (relative vascular area), and by measuring the number of vascular elements per unit area (global vascular density). Both of these vascular parameters give a measure of the amount of vasculature over the whole of the tumor, and there was a weak but significant positive correlation between the two (data not shown). The results reported in this study were not different depending on which parameter was used, and therefore, these data have been presented using relative vascular area.

For both the hypoxic fraction and the relative vascular area, we observed some intratumoral variation between the different sections of the individual tumors, but the variation between tumors was significantly larger based on an ANOVA, with mean values ranging from 1–47% (mean 16.5%) in the case of EF5, and from 3.0–13.1% (mean 6.6%) in the case of CD31 (Fig. 3). As discussed previously, in some studies, the extent of hypoxia has been found to correlate with global measures of vascularity, whereas in others, no relationship has been observed (26, 28). In this group of animals, although there was wide variation in both parameters, there was no correlation between the two (Fig. 3), irrespective of whether they were treated as continuous variables or whether the data were dichotomised about the median value. In addition, there was no relationship between hypoxia or relative vascular area and either lymph node or lung metastasis (Table 1). These findings were true whether the mice were dichotomised about the median value as presented in Table 1 or whether the parameters were treated as continuous variables and linear regressions were performed.

In a subset of 10 animals, we also assessed the fraction of the tumor staining positive for CA9, a gene that is induced under hypoxic

Table 1  The relationship between pathophysiological parameters and metastasis in orthotopic ME-180 xenograft tumors

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Tumor Size</th>
<th>Hypoxic Fraction</th>
<th>Relative Vascular Area</th>
<th>Acute hypoxia Tumor size</th>
<th>Hypoxic fraction</th>
<th>Relative Vascular Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node metastases*</td>
<td>2 (2–3)</td>
<td>1 (1–3)</td>
<td>2 (1–3)</td>
<td>3 (2–4)</td>
<td>2 (2–4)</td>
<td>2 (1–3)</td>
</tr>
<tr>
<td>Lymph node burden**</td>
<td>4.6 (±3.1)*</td>
<td>3.1 (±2.4)</td>
<td>3.9 (±3.4)</td>
<td>3.0 (±2.0)</td>
<td>4.0 (±2.0)*</td>
<td>1.9 (±1.2)</td>
</tr>
<tr>
<td>Lung metastasis*</td>
<td>0 (0–72)</td>
<td>0 (0–18)</td>
<td>0 (0–72)</td>
<td>0 (0–29)</td>
<td>0 (0–29)</td>
<td>0 (0–72)</td>
</tr>
<tr>
<td>Lung metastasis**</td>
<td>0 (0–72)</td>
<td>0 (0–18)</td>
<td>0 (0–72)</td>
<td>0 (0–29)</td>
<td>0 (0–29)</td>
<td>0 (0–72)</td>
</tr>
</tbody>
</table>

*Median number of involved lymph nodes per animal (range of values).
**Mean lymph node burden (sum of volumes of all involved nodes) (± 1 SD).

Fig. 2. The relationship between tumor size and lymph node metastasis. Fluorescent orthotopic ME-180 tumors derived from four i.m. donor tumors were excised after 3 weeks of growth and weighed. After dissection, local lymph node metastases were quantified by fluorescent microscopy. Lymph node burden is the sum of the volumes of all involved nodes. Data are shown in solid symbols for untreated animals (control) and in open symbols for animals exposed to the acute hypoxia treatment daily during tumor development (acute hypoxia). Symbols represent tumors derived from the same i.m. donor tumor. Each point represents an individual animal. The correlation between tumor size and lymph node burden is statistically significant for animals in the control group (P < 0.03).

Fig. 3. The relationship between hypoxic fraction and relative vascular area. After 21 days of tumor growth, the hypoxic fraction and the relative vascular area were calculated by quantifying the proportion of the tumor area staining positive for EF5 and CD31, respectively, on five histological sections per tumor. Data are shown for untreated animals (control) and animals exposed daily to the acute hypoxia treatment (acute hypoxia). Each point represents an individual animal, and different symbols represent orthotopic tumors derived from the four i.m. donor tumors. Error bars represent 1 SD. EF5, 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide.
The obvious difference we observed between the two groups was that the difference in the extent of local invasion was greater in control animals (Fig. 3A). When the size of the tumors was measured, the difference was found to be attributable to an increase in the number of small lesions, because the number of larger lesions was similar between the two groups (Fig. 3B). These small nodes were also more distant from the primary tumor, being located further up the aortic lymph node chain (Fig. 3C). Interestingly, at the time of analysis, the distribution of lesion size appears to be bimodal, and the nodes involved are primarily the first two pairs of nodes in the aortic lymph node chain. This is consistent with a model in which the lymph nodes are sequentially colonized from an initial pair of draining nodes.

At the 21-day time point investigated in this study, the process of lymph metastasis in the animals was in its early stages, with fewer than 50% of animals showing observable lesions. Of those animals that were positive for lung metastasis, the mean diameter of the lesions was approximately 100 μm based on analysis of the fluorescent images (Table 2). There was no difference in lung metastasis observed between mice in the acute and control groups. This was true for the incidence, number of lesions per mouse, and size distribution of the lesions (Table 2).

We also measured the hypoxic fraction and the relative vascular area in the tumors exposed to acute hypoxia. As in the control tumors, there was considerable heterogeneity in these parameters between tumors but the two parameters were independent of one another (Fig. 3). We observed that the tumors treated with acute hypoxia had a statistically significantly lower vascular density than control tumors (Fig. 3A). The acute hypoxia treatment caused a small but statistically

**Effects of Acute Hypoxia Treatment.** The second part of this study examined the effects of imposing additional acute hypoxia on tumors during their development. This was accomplished by exposing the tumor-bearing animals to cycles of 10 min 7% O2/10 min air, for 4 h each day during tumor growth. This gassing regimen has been shown previously to enhance spontaneous lung metastasis of KHT murine sarcoma tumors growing i.m. (37). As was observed previously for the KHT tumors (37), this treatment predictably modified the oxygen concentration in the ME-180 orthotopic cervix tumors, as measured using the Oxylite tissue pO2 sensor (data not shown).

After 3 weeks of treatment, the tumors were processed as described above for the matched control animals. The relationships discussed above, between the various tumor physiological parameters and the extent of metastasis, were generally the same in the acute hypoxia group as they were in the untreated animals, although the correlation between tumor size and lymph node burden was not statistically significant in the treated group (Table 1; Figs. 2 and 3). The first obvious difference we observed between the two groups was that the tumors in the animals treated with acute hypoxia were smaller than those in control animals (Fig. 4). A two-way ANOVA indicated that this difference was statistically significant \((P < 0.05)\) and was consistent across the four sets of animals. Histologically, there were no obvious differences between the two groups, because both gave rise to well-differentiated tumors with very few areas of necrosis. There were also no gross differences in the extent of local invasion of the proximal abdominal structures such as the bladder and large intestine.

When lymph node metastasis was quantified, we observed that the animals in the acute hypoxia group had significantly more involved nodes than control animals (Fig. 5A). When the size of the nodes was measured, the difference was found to be attributable to an increase in the number of small lesions, because the number of larger lesions was similar between the two groups (Fig. 5B). These small nodes were also more distant from the primary tumor, being located further up the aortic lymph node chain (Fig. 5C). Interestingly, at the time of analysis, the distribution of lesion size appears to be bimodal, and the nodes involved are primarily the first two pairs of nodes in the aortic lymph node chain. This is consistent with a model in which the lymph nodes are sequentially colonized from an initial pair of draining nodes.

At the 21-day time point investigated in this study, the process of lung metastasis in the animals was in its early stages, with fewer than 50% of animals showing observable lesions. Of those animals that were positive for lung metastasis, the mean diameter of the lesions was approximately 100 μm based on analysis of the fluorescent images (Table 2). There was no difference in lung metastasis observed between mice in the acute and control groups. This was true for the incidence, number of lesions per mouse, and size distribution of the lesions (Table 2).

We also measured the hypoxic fraction and the relative vascular area in the tumors exposed to acute hypoxia. As in the control tumors, there was considerable heterogeneity in these parameters between tumors but the two parameters were independent of one another (Fig. 3). We observed that the tumors treated with acute hypoxia had a statistically significantly lower vascular density than control tumors (Fig. 6A). The acute hypoxia treatment caused a small but statistically
animals exposed to either acute hypoxia during tumor growth (acute) or to normal air (control).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Incidence</th>
<th>Number(^a) (median (range))</th>
<th>Size(^b) (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9/20</td>
<td>14 (3–72)</td>
<td>102 (±95)</td>
</tr>
<tr>
<td>Acute</td>
<td>7/20</td>
<td>14 (3–67)</td>
<td>100 (±102)</td>
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</table>

\(^a\) Median number of lesions in mice positive for lung metastasis followed by the range of values.
\(^b\) Mean lesion diameter of all lung metastases in each group ±1 SD.

significant increase in hematocrit (mean = 50.3 for controls versus 52.9 for acute hypoxia-treated animals). There was no difference in the hypoxic fraction between the treated and untreated tumors (Fig. 6B). This parameter was not affected directly by the treatment, because mice were not exposed to acute hypoxia for 24 h leading up to EF5 administration and tumor excision. As in the control animals, there was no relationship between the relative vascular area and metastasis; however, in the treated group, animals with a hypoxic fraction greater than the median value of 12.7% had higher tumor burden in the regional lymph nodes (Table 1).

DISCUSSION

The orthotopic model of cervical carcinoma used for this study mirrors the late stages of the clinical disease in several respects, including the pattern of spread, where early metastasis to the local lymph nodes is followed by distant spread to the lungs (5, 7). Also, as in the clinic, tumor size was found to correlate with the extent of lymph node metastasis (19). Interestingly, there was no relationship between lung metastasis and either tumor size or lymph node metastasis, even though the fluorescent tumor cells allowed for a detailed quantification of the incidence, number, and size of the metastatic lesions. Although this finding is consistent with the idea that lymph node and hematogenous metastasis are distinct processes that may be influenced by independent factors, the data in this study do not rule out the possibility that tumor cells may also spread from the lymph nodes to other distant sites such as the lungs. In the future, this model system should allow for clarification of this issue.

Both the hypoxic fraction and the relative vascular area were found to show greater intertumoral than intratumoral variation, as assessed by examining multiple sections of individual tumors. This is also consistent with clinical data, where hypoxic fractions of cervical carcinomas can range from 0 to 99% (7) and suggests that there are unique patterns of vascular development that occur in individual tumors, which result in a wide variation in the extent of hypoxia (24, 42). Also, as in patient tumors, the hypoxic fraction and relative vascular area were not related to tumor size, further supporting the concept that these microenvironmental parameters are unique to individual tumors. However, in contrast to several clinical reports, there was no correlation between the hypoxic fraction and the extent of lymph node metastasis (19, 20). There could be several explanations for this finding. The clinical reports relating hypoxia to metastasis used the Eppendorf microelectrode rather than a hypoxia marker drug such as EF5, and these techniques do not always correlate with one another (43–45). Oxygen electrode measurements provide information about oxygen concentration at a single time point and therefore reflect both chronic and acute hypoxia. The binding of hypoxia marker drugs, on the other hand, depends on drug concentration, oxygen concentration, and time, so that regions of severe, chronic hypoxia will be stained to a greater degree than regions that are more moderately hypoxic for shorter periods during the drug exposure. Consistent with this interpretation, EF5 staining in this group of animals correlated very well with staining for CA9, a hypoxia-responsive gene that is thought to be a marker of chronic hypoxia because of its requirement for extended periods of hypoxia for induction (39, 41). Therefore, it may be that in our model system, chronic, diffusion-
limited hypoxia predominates in the primary tumors, and is not related to metastatic efficiency, as opposed to the observed effect of acute hypoxia treatment.

The lack of a correlation between CD31 staining and metastasis is consistent with the clinical data, where there is no relationship between vascular density and the presence of lymph node metastasis before treatment, whether the hot spot method is used or whether global parameters of vascularity, such as those used in this study, are used (20, 22, 23, 25–27). However, it was reported recently that, in human cervical carcinoma, global intercapillary distance correlates with hypoxia, whereas in the present study, there was no relationship between the EF5-positive fraction and the relative vascular area (26). Again, this may reflect the different techniques used to assess hypoxia, because the clinical data relied on Eppendorf microelectrode measurements whereas the present study used the hypoxia marker drug EF5. It also illustrates the concept that morphometric vascular parameters based on staining for vascular markers do not necessarily quantify functional vasculature and tissue oxygenation.

Previously, we have shown that exposing mice bearing KHT murine sarcoma tumors to acute hypoxia enhances the ability of these tumors to form lung metastases (37). Using the OxyLite probe, we demonstrated that by changing the concentration of inspired gas from 21% to 7% O₂, the oxygen tension of the tumor tissue could be reduced to a new equilibrium level within 2 min (37). We observed similar responses in tumor pO₂ in the ME-180 orthotopic tumors used in this study. It is possible that this treatment may also alter the tumor pH, but we have been unable to detect any significant changes in tissue pH as a result of these treatments. Although the hematocrit of treated animals was slightly elevated, the hypoxic fraction in treated animals, 24 h after the last treatment cycle, was not significantly different from control animals (Fig. 6B), suggesting that tumor oxygenation was not substantially altered during the 20 h/day the animals were breathing normal air.

We found that the acute hypoxia treatment significantly reduced the growth rate of the primary tumors, and reduced the relative vascular area, but did not alter the hypoxic fraction as measured by EF5 staining. Although it is possible that the acute hypoxia treatment reduced the expression of the vascular marker CD31, rather than the number of vessels, we saw no reduction in staining intensity, and did not observe any obviously un stained vascular elements, suggesting that the reduction in the relative area of CD31 staining was attributable to a reduction in the amount of vasculature. These are novel findings, and the mechanisms involved remain to be elucidated. It is known that there is a decrease in both mRNA and protein synthesis in response to hypoxia (46, 47). If the acute hypoxia treatment also produced a decrease in macromolecular synthesis, then a reduction in proliferation could result. It is also possible that decreased proliferation or increased cell death could be induced in response to oxidative stress caused by the acute hypoxia treatment.

In the ischemia-reperfusion injury associated with myocardial infarction, stroke, and transplant surgery, reactive oxygen species are produced during the reoxygenation of hypoxic tissues (48–50). It is likely that the same effect is occurring during cycles of hypoxia and reoxygenation in solid tumors. In animal experiments, oxygen radicals have been shown to be involved in the antitumor effects observed after experimentally imposing ischemia and reperfusion on experimental tumors, although in these experiments, the tumors were made ischemic for 3 h (51). Consistent with this data, we have observed an increase in the number of DNA single-strand breaks in tumor cells obtained by fine needle aspiration biopsies of KHT fibrosarcomas after the acute hypoxia treatment (data not shown). Therefore, oxidative stress, or its downstream effects, may be inducing cell cycle arrest and/or cell death leading to a reduction in tumor growth rate. Similarly, oxidative stress may be responsible for the decrease in vascular density in the orthotopic ME-180 tumors. Oxidative stress can induce apoptosis in vascular endothelial cells, and the antiangiogenic effects of some chemotherapeutic drugs are partially mediated by reactive oxygen species (51–53). It is possible that these two effects may be linked and that the decrease in tumor growth rate is tied to the reduction in vascular density. However, additional experiments are required to determine whether reactive oxygen species are involved in either of the effects observed in this study.

We found that although the acute hypoxia treatment reduced the tumor growth rate, it enhanced lymph node metastasis. This increase in lymph node metastases in the acute hypoxia group was attributable to an increase in the number of small lesions, more distant from the primary tumor. These findings, combined with the fact that the more distant lymph nodes were always smaller than the more proximal nodes, suggests that tumor cells are colonizing the lymph nodes sequentially from a symmetrical pair of initial draining nodes and that this process is accelerated in the mice exposed to acute hypoxia. We believe that the observed increase in metastasis is attributable to the effects of the treatment on the primary tumor; nevertheless, because the whole mouse is being exposed to the acute hypoxia treatment, we cannot completely rule out the possibility that the enhancement is attributable to changes induced in the host tissues. However, our previous experiments have shown that exposing nontumor-bearing mice to this treatment does not affect the ability of cells to form lung metastases after i.v. injection, suggesting that any changes in metastatic efficiency in response to such treatments are dependent on effects on the primary tumor (37). Although the process of lymph node metastasis was accelerated in response to the treatment, lung metastasis was not affected. This suggests that the effects of the acute hypoxia treatment are cell-type and/or tumor-site specific, because lung metastasis was enhanced in the previous KHT experiments (37).

The mechanisms responsible for the effect on metastasis are unknown and may be multifactorial. There are at least the following two possibilities: (a) that the treatment is accelerating mutation and exerting a selective pressure that encourages the outgrowth of more aggressive cellular variants (54, 55), or (b) that the stress associated with the treatment is inducing epigenetic changes such as alterations in gene expression that enhance the ability of cells to form viable metastases (reviewed in Ref. 30). 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, interleukin-8, c-met, chemokine receptor 4, and mdm-2 have been shown to be up-regulated by hypoxia and have been implicated in various stages of metastasis (Refs. 31, 56–58, and reviewed in 30). It will be of particular interest to examine the effect of acute hypoxia on the vascular endothelial growth factor-C/vascular endothelial growth factor receptor 3 signaling pathway, because this pathway has been shown experimentally to enhance lymph node metastasis and is associated with lymph node metastasis in patients with cervical carcinoma (59–60). However, metastasis is a multistep process, and many other genes may be involved, including some that may exert their effects in a cell type-specific manner. Combined with our previous data on the KHT sarcoma (37), these findings suggest that it will be important to develop diagnostic tools that address not only total hypoxia but also temporal changes in oxygenation, because these two situations may have different effects on patient outcome.

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Acute Hypoxia Enhances Spontaneous Lymph Node Metastasis in an Orthotopic Murine Model of Human Cervical Carcinoma

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