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Hypoxia in Microscopic Tumors

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Abstract

Tumor hypoxia has been commonly observed in a broad spectrum of primary solid malignancies. Hypoxia is associated with tumor progression, increased aggressiveness, enhanced metastatic potential and poor prognosis. Hypoxic tumor cells are resistant to radiotherapy and some forms of chemotherapy. Using an animal model, we recently showed that microscopic tumors less than 1 mm diameter were severely hypoxic. In this review, models and techniques for the study of hypoxia in microscopic tumors are discussed.

Keywords

hypoxia; micrometastases; microscopic tumors; angiogenesis; hypoxic resistance

1. Introduction

Most cancer-related deaths are due to the development of metastatic disease rather than the growth of primary tumors. The prevention of this development or the elimination of metastases before they become clinically detectable would be expected to result in improvements in cancer mortality rates. Adjuvant treatment in the form of chemotherapy and/or radiotherapy is generally given after surgical removal of the primary cancer. However, although many patients with cancer respond to therapy, this often does not translate into substantial improvements in overall survival [1]. This may be because, at least some, sub-clinical micrometastases are resistant to cytotoxic therapy. Micrometastases can exist in an apparently dormant state characterized by zero or indolent net cellular proliferation and absence of vascularity. Such micrometastases may be hypoxic depending on their size and the oxygen tension of the milieu in which they exist. Hypoxia coupled with restricted cellular proliferation has the potential to confer a significant degree of resistance to cytotoxic agents. Subsequently, dormant micrometastases may become activated by an “angiogenic switch” triggered by changes in the balance between pro- and anti-angiogenic factors. Such an event could be caused by local ischemia/reperfusion [2] or removal of the primary tumor. Alternatively, it has been suggested that the achievement of dormancy through the use of anti-angiogenic agents is a legitimate therapeutic goal possibly leading to a situation of “cancer without disease” [3–5]. The issues of resistance, activation and dormancy are important and it is necessary to understand the micrometastatic environment including hypoxia and its interaction with angiogenesis and cell proliferation and how this affects the development of metastases.

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Hypoxia is a common feature of primary solid malignancies and is recognized as an important determinant of clinical outcome [6–10]. We have recently shown, in an animal model of sub-clinical colorectal cancer micrometastases in the peritoneal cavity, that microscopic tumors of less than 1 mm diameter were extremely hypoxic while those of greater size (~1–4 mm diameter) were not significantly hypoxic [11]. Here we briefly review the literature concerning hypoxia in microscopic tumors, including models of microscopic disease, techniques for the detection of hypoxia in sub-millimeter deposits, and the effects of hypoxia on angiogenesis, cell proliferation and initial tumor development.

2. Animal models of microscopic disease

Models of disseminated microscopic malignant disease may be generated in the lung, bone, liver and peritoneum by intravenous [12–14], left ventricular [12,15], intrasplenic [2,16] and intraperitoneal [11,17,18] injection of tumor cells, respectively. So far the use of these types of model systems to study tumor hypoxia has been very limited.

Additionally, subcutaneous inoculation of tumors cells frequently gives rise to microscopic tumors of sub-millimeter dimensions [19–21]. However these apparent “non-takes” have generally been studied in the context of tumor dormancy with few reports of the hypoxic status of the microscopic tumors. The time course of angiogenesis and the development of hypoxia (as assessed by hypoxia-induced fluorescence) in sub-millimeter tumors in rodent dorsal skin-fold window chambers has been reported [22]. This elegant assay system provides intriguing information but is restricted to a tight anatomical location and cannot be used to examine the typical sites of metastatic dispersion.

We have shown recently that a model of disseminated microscopic peritoneal and ascites tumors was suitable for studying hypoxia [11]. In this model disseminated peritoneal microscopic tumors were induced by injecting suspensions of HT29 or HCT-8 tumor cells into the peritoneal cavity of nude mice. Mice sacrificed 6–7 weeks (HT29) or 3–4 weeks (HCT-8) after tumor initiation displayed a distribution of tumors of sizes ranging from a few hundred micrometers up to several millimeters in diameter on or in the intestinal serosa. Ascites fluid containing a distribution of free-floating tumor cell aggregates of up to 1 mm in diameter was also present in mice inoculated with HT29 cells. The data also suggested that intradermal injection of similar tumor cell suspensions could give rise to unitary microscopic tumors useful for studying hypoxia. An intradermal system has the potential advantage that growth curves (and hence dose-response relationships) for microscopic tumors could be more easily generated via ultrasound or optical imaging.

3. Methodologies for the detection of hypoxia in microscopic tumors

In studies of macroscopic tumors, hypoxia can be assessed in ways that are either problematic or not feasible for microscopic tumors such as pO_2 probe measurements and non-invasive imaging with positron emitting hypoxic tracers. In general, for microscopic disease, invasive methods of assessing hypoxia are required. These rely on the detection of either (1) an exogenous substance (tracer) that preferentially localizes in hypoxic tissue or (2) the expression of endogenous markers of hypoxia.

The most commonly used exogenous hypoxia markers, - pimonidazole, EF5 and CCI-103F - are 2-nitroimidazole compounds that are selectively reduced in hypoxic regions (generally $pO_2 < 10$ mmHg) of tumors, subsequently binding to intracellular macromolecules [23]. The spatial pattern of binding of these markers can be visualized on tumor sections by immunohistochemical methods. Exogenous hypoxia markers have been widely used in clinical and experimental studies of tumor hypoxia [24–28]. In an analogous approach, the spatial pattern of binding of radiolabeled hypoxia tracers in tumor sections may be visualized by

autoradiographic means. Suitable radiolabeled tracers for this purpose include ^{18}F -fluoromisonidazole (FMISO) [29,30], ^{125}I -iodoazomycinogalactopyranoside (IAZGP) [31] and in some circumstances ^{18}F -fluoro-deoxyglucose (FDG) [32]. Modern digital autoradiographic detection systems can visualize the spatial distribution of activity with a pixel resolution of 25–50 μm . The advantage of immunohistochemical detection of hypoxia markers is high spatial resolution, that of autoradiographic detection is quantification. Typically exogenous hypoxia tracers are administered 1–3 hours prior to tumor retrieval and their binding reflects the level of tumor hypoxia over the relatively short time period over which the marker is available in the circulation.

A large number of cellular proteins are regulated by hypoxia through the HIF pathway [33]. The patterns of expression of these proteins in tumor sections, detected by immunohistochemical methods, can be used to assess the spatial distribution of hypoxia. Potential endogenous markers of hypoxia include hypoxia inducible factor 1 α (HIF1 α), carbonic anhydrase 9 (CA9) [34–37] and glucose transporters 1 and 3 (GLUT1 and GLUT3) [23,38,39,40]. However, in principle, any protein with enhanced expression in hypoxic regions could be used for this purpose. The choice of an appropriate protein marker of hypoxia is model-dependent; for example we found CA9 to be useful in xenograft tumors derived from HT29 cells but not HCT-8 cells [11]. At a practical level, candidate proteins are best identified in unperturbed macroscopic tumors in which they should show suitably enhanced expression in perinecrotic regions and spatial co-localization with exogenous hypoxia tracers. The information provided by endogenous markers of hypoxia is influenced by the rates of protein accumulation and loss. For example HIF1 α is degraded very rapidly when oxygen becomes available with a half-time of the order of a few minutes [41,42], whereas the degradation of CA9 is a relatively slow process with estimated half-times of the order of a few days [43,44]. These kinetic differences mean that the spatial distributions of enhanced protein expression will not necessarily coincide. A rapidly-degrading protein would be expected to indicate instantaneous hypoxia at the time of assay while a slowly-degrading protein would more appropriately indicate “historical” hypoxia. Thus combinations of endogenous protein markers used in conjunction with exogenous tracers can be used to detect changes in tumor hypoxia. Such an approach may be used to identify regions of transient hypoxia [45] or monitor the effect of interventions that perturb the intratumoral distribution of hypoxia [11].

4. Hypoxia in macroscopic and microscopic tumors

In the clinic, the existence of tumor hypoxia is a common feature of primary solid malignancies [6,7,8,10,44]. In animal model studies on syngeneic or xenografted tumors the use of pO_2 probes or magnetic resonance methods have indicated that smaller tumors tend to be better oxygenated than larger ones [46–50]. Milross et al investigated oxygen status as a function of tumor size in three syngeneic murine carcinomas grown in C3Hf/Kam mice. As tumor volume increased from 200 to 1400 mm^3 , the percentage of measured pO_2 values ≤ 5 mm Hg in MCa-4, OCa-I, and SCC-VII tumors increased from 55 to 96, 70 to 81 and 57 to 98 respectively [46]. Zhao et al reported that the hypoxic fraction was 14% in small ($<1\text{ cm}^3$) Dunning prostate R3327 HI rat tumors, increasing to 55% in tumors larger than 3 cm^3 , with a statistically significant positive correlation between hypoxia fraction and tumor size [47]. However, the smallest tumors examined in these studies were ~ 8 mm in diameter, a practical limit set by the methodologies used.

We recently reported on the hypoxic status of microscopic tumors established intraperitoneally and intradermally using the HT29 and HCT8 colorectal cancer lines [11]. In general, the smallest tumor deposits (less than ~ 1 mm diameter) showed intense hypoxia (hypoxic fraction as high as 90% based on pimonidazole staining) with little or no blood perfusion (as visualized by Hoechst 33342 fluorescence) or CD31-positive vasculature. Tumors in the range 1–4 mm

diameter seemed relatively well vascularized and well perfused and generally displayed little or no hypoxia. In tumors larger than 4 mm diameter, hypoxia reappeared in the characteristically perinecrotic distribution pattern seen in macroscopic tumors. We have also studied hypoxia in experimental lung metastases derived from non-small cell lung cancer A549 cells and observed similar patterns of tumor hypoxia in this model to those exhibited by the peritoneal and intradermal tumors (Li et al unpublished observation).

It will be important to investigate to what extent is severe hypoxia a general feature of microscopic tumors (micrometastases) that is independent of cell type and location. For instance, what are the patterns of hypoxia in experimental micrometastases to lung, liver and bone using cells derived from breast, prostate and lung cancers? Also important is whether the differing patterns of hypoxia seen in tumors of differing sizes reflect a true temporal progression. For example, have well-vascularized non-hypoxic tumors 1–4mm in size at some point passed through a stage where they were intensely hypoxic with no vasculature - or do the small hypoxic tumors represent a form of dormancy? Future studies are needed to assess hypoxia in patient samples of micrometastases, which may be obtained by careful histologic examination of resected tissue from patients diagnosed with cancers [51].

5. Hypoxia and cellular proliferation in dormant microscopic tumors

It has been observed that human microscopic tumors (<1 mm) may be avascular and in a state of dormancy, i.e. non-expanding in mass [5,52]. Some studies have reported that in dormant tumors, cells were either dividing very slowly or were in G₀ phase [51,53,54,55]. Others have found proliferation in dormant tumors to be as high as in macroscopic vascularized tumors but that the dormant tumors did not grow beyond a threshold size due to a kinetic balance between proliferation and cell death through apoptosis [20,21]. We observed cellular proliferation in the non-hypoxic rim but not the interior hypoxic core of sub-millimeter avascular HT29 tumors, whereas proliferating cells were found throughout larger tumors 1–4 mm in dimension with little to no hypoxia [11]. Several studies have found cellular proliferation and hypoxia to be mutually exclusive in both microscopic [11] and macroscopic tumors [32,56,57].

Although the extent of hypoxia has not been reported in most studies of tumor dormancy, our results support the contention that dormant tumors may be hypoxic and that hypoxia may be causally related to low proliferation rates and thus tumor dormancy. Obviously, further studies will be required to examine these issues.

6. Hypoxia and angiogenesis in microscopic tumor models

Hypoxia has been recognized as a primary physiological regulator of angiogenesis [58,59]. The key regulators of hypoxia-induced angiogenesis are the HIF family of protein transcription factors [60]. HIF regulates multiple factors such as VEGF, and nitric oxide synthases to promote tumor growth and angiogenesis [61]. In addition, HIF-independent pathways that are capable for inducing angiogenesis in response to hypoxia have also been identified [62].

Our results using HT29 and HCT-8 colorectal tumor cell lines in both peritoneal and intradermal sites suggest the possibility that the existence of severe hypoxia in microscopic tumors is common irrespective of cell line and tumor location and reflects the pre-angiogenic stage of tumor development. As peritoneal and intradermal tumors increased in size to the diameter range ~1 to 4 mm, there was a drastic reduction in tumor hypoxia coupled with the appearance of functional tumor vascularization [11]. This suggests the sequence of events are that cells become hypoxic when tumors reach several hundred micrometers, hypoxia is a key factor which drives angiogenesis, previously hypoxic cells become oxygenated and the neovascularized tumors grow beyond the size threshold (approximately 1mm).

However this schema may be overly simplistic. Recently, Cao and colleagues using a rodent dorsal skin-fold window chamber system observed the initial phase of angiogenesis in microscopic tumors to occur before the development of hypoxia. Subsequently tumors did become hypoxic and this coincided with an intensification of angiogenesis [22]. This study featured HCT116 human colon carcinoma cells and 4T1 mouse mammary carcinoma cells engineered to express green fluorescence protein (GFP) under HIF transcriptional control as a reporter for hypoxia. To counter the possibility that tumor hypoxia was present before the levels of GFP became detectable, the investigators used the hypoxic toxin tirapazamine to eliminate or reduce the number of hypoxic cells. This delayed the detection of the GFP signal but did not significantly affect the onset of incipient angiogenesis.

7. Hypoxia, glucose metabolism and acidosis in microscopic tumors

Hypoxic cells undergo a switch from aerobic to anaerobic glucose metabolism. This adaptive response to hypoxia involves the coordinated expression of many genes regulated by HIF, such as those that encode GLUT1, GLUT3, and various glycolytic enzymes [63]. Overexpression of GLUT1 and GLUT3 in perinecrotic regions of macroscopic tumors has been reported, suggesting a relationship with diffusion-limited hypoxia [39,40]. In addition, a correlation has been observed between GLUT1 overexpression and pimonidazole binding in carcinomas of the uterine cervix [38].

Glycolysis may be assessed functionally by examining the uptake of ^{18}F -FDG. FDG is an analog of glucose, and like glucose, is phosphorylated by hexokinase, but, unlike glucose, is not metabolized further. Since the phosphorylated metabolite is unable to leave the cell, the intracellular accumulation of FDG may be assayed. In vitro experiments show that incubation in hypoxic conditions induces an increase in cellular FDG uptake [64–66]. However, there were differences between these studies in terms of the kinetics of hypoxia-induced increases in FDG uptake and the likely underlying mechanisms. In the study of Clavo et al the increase in uptake was correlated with increased membrane expression of GLUT-1 [64,65], while in the study of Burgman et al enhanced FDG uptake appeared to result from a hypoxia-induced increase in glucose transporter activity rather than transcriptional upregulation [66]. It was recently shown that the intratumoral distribution of ^{18}F -FDG in R3327-AT rat prostatic carcinoma xenografts positively correlated with that of the hypoxic marker pimonidazole [32]. Currently, there is no data available on how micrometastases adaptively respond to hypoxia by switching to anaerobic glucose metabolism. However we have observed intense FDG uptake in hypoxic microscopic tumors in the peritoneum (Li et al, unpublished).

Increased glycolysis and lactate production under hypoxic conditions results in acidosis, which has been implicated in hypoxic cell death [67,68]. CA9 is a transmembrane isoform of carbonic anhydrase characterized by high H^+ transfer that functions to relieve intracellular acidosis. CA9 has been identified as a HIF target gene, and is considered an endogenous hypoxic marker. Its expression under hypoxia is cell line dependent [69,70]. The role of CA9 in the regulation of intracellular pH of hypoxic cells and its ability to promote cell survival in conditions of acidosis requires further investigation, particularly in regard to microscopic tumors.

8. Perturbing hypoxia in microscopic tumors

Changes in tumor hypoxia can be detected using double hypoxic markers. The principle of this method is that one marker represents previous and one current hypoxic status. With exogenous hypoxic markers such as the 2-nitroimidazole compounds pimonidazole, EF5 and CCI-103F two markers are administered separated by a time gap. Subsequently the differing patterns of binding may be visualized by immunofluorescence microscopy. Radiolabeled hypoxia tracers may also be used; in which case a tracer labeled with a long half-life radionuclide (e.g. ^{131}I -IAZGP) is administered first followed, after a suitable time, by one with a short half-life

(e.g. ^{18}F -FMISO). A digital autoradiogram acquired immediately after tumor removal will show the combined distribution of both tracers while one acquired after sufficient time for the short half-life radionuclide to decay to negligible levels shows only the distribution of the long half-life tracer. Subsequently, if necessary the contribution of the long half-life tracer to the early autoradiogram may be removed by digital image subtraction. Assays based on endogenous hypoxia-regulated protein such as HIF1 α , CA9 and GLUT1 can also be used. These rely on differences in the protein lifetime so that current hypoxia (e.g. HIF1 α distribution) can be compared with “historic” hypoxia (e.g. CA9 distribution). In addition exogenous and endogenous tracers can be combined in an appropriate manner so that current and previous hypoxia can be visualized.

These methods can be used to study the effects of interventions that perturb tumor hypoxia. Ljungkvist et al observed that carbogen (95% O₂, 5% CO₂) breathing led to significantly decreased hypoxia in SCCNij3 xenografts using CCI-103F and pimonidazole double staining [27]. Our group has studied the effect of carbogen breathing on hypoxia in HT29 tumors using a CA9-pimonidazole double marker technique. In air-breathing conditions the distributions of pimonidazole binding and CA9 expression were similar in HT29 macroscopic xenografts [11,71]. Following 2 h of carbogen breathing (1 h before and 1 h after pimonidazole administration), there was a significant reduction in pimonidazole immunofluorescence relative to that of CA9 [71]. In contrast, administration of hydralazine caused a significant increase in pimonidazole binding in comparison with CA9 expression in macroscopic HT29 tumors.

In air-breathing conditions, microscopic peritoneal and ascites HT29 tumors <1 mm diameter were strongly positive for both pimonidazole and CA9 [11]. Following carbogen breathing we found a significant reduction in pimonidazole binding in some but not all peritoneal tumors and in all ascites tumors. This led us to speculate that carbogen breathing, and possibly other hypoxia-modulating agents, may have a role in the treatment of micrometastatic disease.

9. Conclusions and Perspectives

There is little published information on measurements of the hypoxic status of microscopic tumors and micrometastases. In preclinical animal models, avascular microscopic tumors (<1mm) can be intensely hypoxic. Future studies should examine the hypoxic status of microscopic tumors for a range of cancer cell lines of different origins in a range of anatomical sites and, wherever feasible, in patient derived material.

As the efficacy of cytotoxic treatment for micrometastatic disease may be compromised by hypoxic resistance it will be important to examine the impact of therapeutic interventions on the hypoxic status of microscopic disease in addition to more conventional measures of response.

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Figure 1.

Hypoxia in microscopic HT29. Disseminated peritoneal metastases were induced by i.p. injection of colon cancer HT29 cells suspensions (10^7 cells in 0.2ml PBS). Experiments were performed 6 weeks post inoculation. Animals were intravenously injected pimonidazole (2mg) one hour before sacrifice and with Hoechst 33342 (0.5 mg) 1 min before sacrifice. Peritoneal tumors (adhering to the intestinal serosa) were collected and washed with cold PBS to remove any attached ascitic tumor cells before freezing and embedding in OCT. Sets of contiguous 8 μ m thick tissue sections were cut. **A.** Fluorescence overlay image (green = pimonidazole, blue = Hoechst 33342, red = CA9) contains a variety of large and small peritoneal tumors. The series of smaller tumors of sub-millimeter diameter as arrows indicated show intense pimonidazole and CA9 fluorescence, suggesting significant hypoxia, with little to no Hoechst 33342 fluorescence. This contrasts with the larger tumor (circled) which shows extensive Hoechst 33342 but lesser pimonidazole or CA9 fluorescence. **B.** Contiguous section stained for CD31. It can be seen that the small tumors that appear hypoxic in Fig. 1A are essentially avascular whereas the large tumors have extensive vasculature. **C.** Same section as shown in Fig. 1A stained with hematoxylin and eosin provided for reference. All scale bars = 400 μ m.

Figure 2.

The relationship between tumor size and pimonidazole positive fraction (PPF) for microscopic HT29 tumors growing in either the peritoneal cavity or intradermally. PPF was defined as the ratio of tumor area with positive pimonidazole staining to total tumor area. Tumors less than 1 mm possessed little to no vasculature or blood perfusion, tumors larger than 1 mm were highly perfused and had widespread vasculature. The x-axis represents effective tumor diameter (D) derived from the tumor cross-sectional area (A) according to $D=2\sqrt{A/\pi}$. Circle peritoneal, triangle: intradermal.

Figure 3.

Effect of carbogen (95% O₂, 5% CO₂) breathing on hypoxia in peritoneal microscopic metastases of HT29 cells. The effect of carbogen breathing on hypoxia was studied using a CA9-pimonidazole double marker technique in same frozen tumor section. **A.** One section containing a collection of HT29 peritoneal tumors from an air-breathing host. Fluorescence image shows distributions of pimonidazole (green) binding and CA9 (red) expression are similar in microscopic tumor deposits as arrows indicated, which exclude from Hoechst 33342 (blue) staining. Same section also stained with hematoxylin and eosin (H&E) provided for reference. **B.** One section containing a collection of HT29 peritoneal tumors from a carbogen-breathing host. Following 2 h of carbogen breathing (1 h before and 1 h after pimonidazole administration), fluorescence image shows intense CA9 staining but low levels of pimonidazole (green) staining in microscopic deposits as arrows indicated, suggesting oxygenation. Same section stained with hematoxylin and eosin provided for reference. All scale bars = 400 μ m.