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Mechanisms of blood flow and hypoxia production in rat 9L-epigastric tumors

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Abstract

Classical descriptions of tumor physiology suggest two origins for tumor hypoxia; steady-state (diffusion-limited) hypoxia and cycling (perfusion-modulated) hypoxia. Both origins, primarily studied and characterized in murine models, predict relatively small, isolated foci or thin shells of hypoxic tissue interspersed with contrasting oxidic tissue. These foci or shells would not be expected to scale with overall tumor size since the oxygen diffusion distance (determined by oxygen permeability and tissue oxygen consumption rate) is not known to vary dramatically from tumor to tumor. We have identified much larger (macroscopic) regions of hypoxia in rat gliosarcoma tumors and in larger human tumors (notably sarcomas and high-grade glial tumors), as indicated by biochemical binding of the hypoxia marker, EF5. Thus, we considered an alternative cause of tumor hypoxia related to a phenomenon first observed in window-chamber tumor models: namely longitudinal arteriole gradients. Although longitudinal arteriole gradients, as originally described, are also microscopic in nature, it is possible for them to scale with tumor size if tumor blood flow is organized in an appropriate manner. In this organization, inflowing blood would arise from relatively well-oxygenated sources and would branch and then coalesce to poorly-oxygenated outflowing blood over distances much larger than the length of conventional arterioles (multi-millimeter scale). This novel concept differs from the common characterization of tumor blood flow as disorganized and/or chaotic. The organization of blood flow to produce extended longitudinal gradients and macroscopic regional hypoxia has many important implications for the imaging, therapy and biological properties of tumors. Herein, we report the first experimental evidence for such blood flow, using rat 9L gliosarcoma tumors grown on the epigastric artery/vein pair.

Keywords

Bloodflow; Hypoxia; EF5; Imaging; Static Hypoxia; Acute Hypoxia; Macroscopic Regional hypoxia; 9L tumors; DNA-damage

Introduction

Hypoxic tumor regions (reduced oxygen content compared to normal tissue) are thought to arise by two principal mechanisms. Thomlinson and Gray first predicted static, diffusion-limited tumor hypoxia based on their findings that necrosis was found at the center of tumor tissue cords in human bronchial carcinomas exceeding 300–400 μ diameter [1]. The width of the annulus of viable tissue in these cords was consistent with the predicted oxygen diffusion distance [2]. A second mechanism for the production of hypoxia, described as cycling or perfusion-modulated, was predicted by J. Martin Brown in 1979 [3]. His hypothesis was confirmed a decade later by Trotter and colleagues using dye-mismatch experiments [4]. In these experiments, two blood perfusion markers, Hoechst 33342 and DiOC7, were injected into tumor-bearing mice. Both markers bound to the DNA of exposed cells but could be differentiated by their fluorescence wavelength. Both dyes were found to label the walls of the same tumor vessels when injected simultaneously. However, when the dyes were injected at times separated by about 20 minutes, occasional individual vessels were found that showed binding of only one of the two dyes [4,5]. This was interpreted to mean that blood flow was cycling on-and-off in the mismatched vessels. Subsequently, Dewhirst and colleagues demonstrated several subtleties to this scenario in tumor ‘window chamber’ models, including the possibilities of blood flow reversal and large local variations in hematocrit for individually monitored vessels [6–8]. Currently, there are no alternative methods to the dye-mismatch technique to distinguish diffusion-limited *versus* perfusion-modulated hypoxia, and to our knowledge this has not been investigated in large tumors. Additionally, DNA-binding dyes cannot be used in humans and the dyes themselves have been shown to be vasoactive [4]. Thus, new methods are constantly being sought that could identify perfusion-modulated hypoxia, preferably with application to humans. For example, Krishna and colleagues have suggested the use of EPR-based assays to measure cycling hypoxia directly - however, these methods are not yet scaleable to use in large human tumors – [9] see [10] for review. Thus, while widely assumed to be of prominent importance, there is no direct evidence for perfusion-modulated hypoxia in human tumors [8,11,12].

EF5 is a 2-nitroimidazole that is differentially metabolized by hypoxic compared with aerobic cells, forming macromolecular adducts that can be detected by monoclonal antibodies. This enables the use of EF5 as a ‘hypoxia marker’ and we have developed quantitative immunohistochemical (IHC) techniques to allow EF5 adducts to be interpreted as absolute tissue oxygen partial pressure – for review see [13]. Using these methods, we made an initial characterization of EF5 binding in 9L gliosarcoma tumors grown as epigastric pedicles [14]. This site of tumor growth has the property that the tumor vasculature arises almost entirely from a single source, the epigastric artery-vein pair (see description in methods). In contrast to their relatively oxic characteristic (uniformly low EF5 binding) when grown intracranially, 9L epigastric pedicle tumors were found to have highly heterogeneous hypoxia, both within and between tumors, as measured by EF5 binding [14]. 9L subcutaneous tumors have similar variability in extent and degree of hypoxia and we showed, for the first time in a tumor model, that intertumoral heterogeneity in radiation response could be predicted by EF5 binding [15]. This has been recently expanded to

include an ^{18}F -labelled, non-invasive EF5 binding assay [16]. In some of these tumors, we found regions of high EF5 binding that extended over dimensions of much greater than 1 mm [17]. At the time, this was considered to be unexpectedly large, since the oxygen diffusion distance is typically of the order of 125–175 microns [1]. Thus viable cells (essential for EF5 metabolism) should not exist beyond approximately 150 micrometers from the nearest blood vessel, and both current models for hypoxia predict relatively small zones of hypoxia (see Figure 1a). At the time, we interpreted the macroscopic regions of hypoxia (MRH) as arising from some combination of diffusion-limited and perfusion-modulated hypoxia - for example, it was possible that cycling blood flow was occurring in large groups of vessels, though this has not been demonstrated experimentally and would be difficult to explain physiologically.

MRH were also found in high-grade sarcoma and glioma tumors from human patients given EF5 [18,19]. In these cases, vessels were found within the hypoxic regions but an independent blood-flow measurement was not possible. EF5 binding can be assessed on a quantitative, analog scale, so we developed imaging algorithms to compute the gradients of EF5 binding away from individually identified vessels, grouped by their perivascular pO_2 . Unexpectedly, both positive and negative gradients were found, with averages over all observed vessels near zero, irrespective of the perivascular pO_2 . We hypothesized that such averaging might be possible if the overall tumor blood flow was organized to provide extended serial flow from well oxygenated inflow to poorly oxygenated outflow, and that these flow patterns were mixed spatially. This was termed the DIVOB model (differentiation of inflowing *versus* outflowing blood - [20]). Note the contrast between this postulated serial blood flow in tumors compared with the countercurrent parallel arterio-venous flow of vessels in normal tissue.

Our central hypothesis is that it is impossible to maintain the simple structure, illustrated in Figure 1a, for large tumors. Large tumors overtake and utilize pre-existing vessels. If the vessels are large (as with the epigastric model – Figure 1b), they can support large regions of tumor tissue. Thus, simple geometrical considerations require vascular flow along extended length vessels induced to grow within the tumor mass. In turn, gradients of oxygen and other nutrients will form along the path of flow (Figure 1c). Thus, both the vessels and the tissue regions fed can become successively more hypoxic. Herein, we present the first experimental data in support of the DIVOB model. We have investigated, in preliminary fashion, various invasive and non-invasive assays of blood flow to determine if they could predict MRH.

Experimental procedures

Tumor Initiation

All procedures were approved by the IACUC committee of the University of Pennsylvania. 9L gliosarcoma cells were originally obtained from Dr. Ken Wheeler. They have been routinely tested and found free of mycoplasma and rat pathogens. The 9L epigastric pedicle model was described in 1995 [14]. Briefly, two sutures are placed around the epigastric artery-vein pair approximately 10 mm peripheral to its junction with the femoral artery-vein. Both sutures were tightened to occlude blood flow. Tumor source material, in the form of

small tissue pieces (3–6 mg) were stored frozen in liquid nitrogen. A thawed tumor piece was tied to the more central suture, and the vessel-pair cut between the sutures. This process duplicated the original pedicle models of Gullino [21], but for a spontaneous tumor growing and overtaking an existing artery-vein pair it seemed that the tumor would cause neither complete occlusion of blood flow nor the severing of the vessels. Thus, the tumor-initiating process was modified by simply tying the tumor piece onto the epigastric vessel pair (traversing model). An encircling suture was double-knotted to ensure a stable location on the vessel, but not tied tightly enough to occlude blood flow. The tumor piece was then tied in place over the first knot. A drop of antibiotics was added, the fat pad replaced over the tumor and the access wound closed with surgical clips. Typically, the site of the original knot was identifiable by ultrasound even after substantial growth of the tumor to its experimental size (1–2 g), over a period of 2–3 weeks. The tumors will also grow at this site if the tumor piece is attached to the vessels using a surgical grade of superglue.

Immunohistochemistry

On the day prior to tumor collection, hair near the tumor was removed by Nair®. The next day the skin was marked to indicate the major axis of the tumor (along the direction of the epigastric vessels) and a second line at right angles marking the apparent maximum diameter of the tumor. Animals were anesthetized using isoflurane in medical air, at a concentration (typically 1.5–2.5%) that maintained consistent breathing rates of about 60/minute. Animals were placed supine on a warmed heating pad and temperature was monitored with a rectal thermometer. All drugs were administered by intravenous injection using an in-dwelling tail catheter. Animals received EF5 (30 mg/kg) at –180 minutes (all times with respect to tumor collection which was considered to be time ‘zero’), Hoechst 33342 (15 mg/kg) at –30 minutes and DiOC7 (0.15 mg/kg) at –5 minutes. Hoechst 33342 and EF5 were mixed at 200x and 100x, respectively, in 5% dextrose + 2.5% ethanol. Although DiOC7 has been administered to mice using a formulation of 100% dimethylsulfoxide [4], this was found to be toxic for rats (unpublished data). Thus, we formulated the DiOC7 (at 600x) in 50% glycerol with 10% ethanol. This appeared to have the least physiological impact (tail straightening, or breathing change on injection - for example compared with a PEG formulation). Some animals were also assessed by a macromolecular plasma marker; tetramethyl rhodamine labeled dextran 75K (Invitrogen).

Dynamic Contrast-Enhanced (DCE) MRI

DCE/MRI was performed on a 9.4T horizontal bore magnet interfaced to a Varian Direct drive console. Under isoflurane anesthesia, the animal was placed in a custom-designed trough and rectal temperature was used to monitor the core body temperature. Additionally, a respiratory pillow was attached under the abdomen to measure the respiratory rate of the animal. A vitamin E capsule was fixed on the skin (with superglue) in the proximity of the tumor for ease of identification of the tumor on MRI. A 24 gauge catheter was inserted into the tail vein and connected to plastic tubing for injection of the MRI contrast agent. The animal holder was then placed inside a 70 mm diameter transmit-receive volume coil such that the tumor was at the center of the coil. The coil was inserted in the magnet and the body temperature and respiration were monitored using a MRI compatible physiological monitoring device (SA Instruments, NY). The animal’s body temperature was controlled by

blowing warm air through the bore of the magnet. After initial localizer images, gradient echo images were acquired in the sagittal plane to localize a single 2mm thick section so as to include both the tumor as well as a major artery. The T1 of the tumor was measured using a TOMROP sequence as detailed elsewhere [22]. DCE-MRI was then performed at this slice section using a T1-weighted saturation recovery gradient echo imaging sequence (TR = 8 ms, TE = 2.75 ms) with a temporal resolution of about 4 s per image. After initial baseline images, a bolus of 300 microliters Gd-DTPA (0.2 mM) was injected while the images were being acquired for a total of 10 minutes. The signal intensity-time curves were converted into contrast agent concentration-time curves using the measured T1 relaxation values on a pixel-by-pixel basis. The arterial input function (AIF) was measured from the arteries proximal to the tumor. We used the shutter-speed model [23,24] often referred to as bolus enhanced relaxation overview (BOLERO) to estimate the forward transport constant K^{trans} , volume fraction of the extracellular space, v_e , and the mean lifetime of intracellular water protons, τ_{wi} .

Irradiation

Some animals were administered an X-ray dose of 17 Gy to half of the tumor, using a custom 'D'-shaped collimator attached to the small-animal-radiation-research-platform (SARRP) developed by Dr. Wong and colleagues at Johns Hopkins University [25], now marketed by Xtrahl. The tumor was collected 45 minutes following irradiation.

Tumor Collection and Immunohistochemistry

At the time of tumor collection, the isoflurane concentration was increased to a surgical plane, and the tumor was removed, while retaining the marked skin portion to preserve orientation. The tumor was cradled in a stainless-steel rectangular container (ID ~ 25mm × 25mm × 30 mm; using threads passing back and forth through tiny holes in the walls of the container) in the same orientation as was observed *in vivo*. The block was then filled with molten (~40°) 3% low melt agarose and the container held on ice until solidification of the agarose. After removal of the threads, the agarose block was removed, frozen on an aluminum block cooled by dry-ice, then stored at -75°. The surface of the frozen agarose block was also marked so that the overall orientation of the tumor could be maintained.

Tissue Sections and Staining

The location of the knot used to tie on the originating tumor piece was known fairly accurately for tumors assessed by ultrasound, but could only be guessed as being close to the maximum diameter for most tumors. Thus the middle third of the tumor was sectioned in its entirety, saving four each 10 micron sections every two mm. The saved sections preceding the knot, including the knot, and after the knot were then processed for IHC and imaging. In order to preserve the blood-flow dyes (Hoechst 33342, DiOC7 or Dextran), the sections had to be imaged before fixation or immunohistochemical (IHC) processing, since the initial dye localization is not stable in the presence of aqueous or other solvents. Thus slides were dried on a warm-plate (37°) in the dark immediately following sectioning. In our experience this precludes the use of paraformaldehyde fixation in order to maintain the ability to accurately quantify EF5 binding. Between the alternatives of graded ethanol (70%, 85%, 100%; 5

minutes each at 0°) *versus* acetone fixation (5 minutes at 0°), we found that either was acceptable for EF5 binding but assessment of CD31 required graded ethanol (data not shown). Thus after imaging the entire sections for Hoechst 33342 or DiOC7 (or dextran if included), the sections were fixed in graded ethanols, and dual stained for EF5 and CD31 (the latter if required to identify all vessels). None of the plasma markers were retained after fixation, so wavelengths were not limiting for anti-CD31 (primary; BD Pharmingen mouse-anti-rat # 550300 3 µg/ml; secondary Alexa 488 goat anti-mouse) or anti-EF5 (Cy5 direct-conjugated ELK3–51 primary). As previously introduced by our lab [13], we prevent interaction of the goat anti-mouse secondary for CD31 with anti-EF5 antibodies using a short paraformaldehyde fixation after CD31 secondary staining (4%, 20 minutes, 0° followed by 3 rinses with PBS containing 0.3% tween 20). Overall stability of the antibodies was additionally maintained post-staining by storing the stained slides in PBS with 1% paraformaldehyde. This allows imaging over a time that can be extended to several weeks [13]. Conversely, the section-containing slides could be stored at –80° in dessicant-containing storage tubes for several months before initiation of the staining and imaging process.

In our hands, staining for gH2AX requires an initial paraformaldehyde (pF) fixation, so it was necessary to use adjacent sections that were not pre-dried for this marker. Slides were fixed in 4% pF for 1 hr at 0°, and then additionally fixed in 70% ethanol overnight to gain access to DNA-associated antigens – this was in agreement with other DNA-associated events such as BrdUrd incorporation or TUNEL assay of apoptosis [26]. Staining for gH2AX was then accomplished using the Millipore 05–636 anti-gH2AX antibody (20 µg/ml), followed by staining for EF5 as per the above dual-stain procedure.

Images were made using a Nikon Eclipse 800 microscope, coupled to a 12-bit cooled CCD camera (Photometrics Quantix). The camera and automatic stage (Ludl) were controlled by IPLab algorithms developed by one of the authors (CK). Images were converted to 8 bit tif files without modification of the contrast and assembled and analyzed using MATLAB. All algorithms were developed by one of the co-authors (KWJ). Whole-tumor cross-sections were usually assessed using a 4x objective (3mm × 2mm field) whereas smaller regions were quantified using a 10× objective (1.2mm × 0.8mm field). As we have described previously, a separate collage of fields of each section was made, as required, for plasma marker(s), EF5, CD31 (or gH2AX), and then the section was flooded with Hoechst 33342 dye and re-imaged, which labels the nuclei of all intact cells (only a low level background fluorescence is seen in necrotic areas). This ‘Flooded Hoechst’ image (FH) was used as a tissue mask for any subsequent quantification. This should not be confused with the Hoechst used as a blood flow marker, administered just before tumor collection. Image analysis of EF5 binding and gH2AX intensity was done quantitatively using an analog scale, whereas the other markers were assessed in binary fashion using appropriately derived background and maximum intensity signals.

gH2AX Analysis and Quantification

Imaging at low resolution (10x objective) provides a measure of whole-nucleus Ab detection of gH2AX, not individual foci or their relative variation in size [27]. Using MATLAB

techniques similar to those quantifying Ki67 expression [28], the flooded Hoechst images were used to define the spatial coordinates of each nucleus. Note that for 10 micron thick sections, most nuclei will not be observed in their entirety. Thus, the observed 'nuclei' should really be regarded as nuclear fragments, and have a considerable range of sizes. Using the coordinates for each nuclear fragment, the intensity of EF5 binding and gH2AX intensity was recorded in a 5×5 pixel area (100 square microns). There were typically ~3000 partial nuclei per 10× field.

Results

We have assessed more than 25 epigastric 9L tumors. Of course, due to their size, it has not been possible to thoroughly image their characteristics throughout each tumor. However, focusing on the central portion, EF5 binding has varied from minimal in 4 tumors to the extremes shown in Figure 2. Of these 4 tumors, the upper pair indicates dramatic evidence of MRH, while the bottom pair contains high EF5-binding areas that are more consistent with diffusion-limited hypoxia. MRH occurs in about 25% of the tumors studied to date.

For the tumors with MRH, a fundamental feature has been, not dye mismatch, but rather the failure of either perfusion marking dye (DiOC7 or Hoechst 33342) to label vessels within the high EF5-binding areas. If one accepts the geometrical requirement of extended longitudinal blood-flow gradients in large tumors (1–2 gram; Figure 1c), a simple interpretation of this finding is that the perfusion-marking drugs are consumed, just like oxygen, along the blood flow gradients. Thus, vessels that are not marked by these dyes may indicate a lack of perfusion but also the absence of drug, due to consumption. Since the perfusion dyes are themselves known to be vasoactive [4,29], an additional concern was that there was essentially no blood flow in MRH due to infarcts occurring during the experiment. Thus we investigated various macromolecular dyes that could be injected just before tumor collection and therefore would demonstrate blood flow. Due to the relative infrequency of MRH and the high cost of the labeled markers, this turned out to be a complex task, especially since blood vessels tend to collapse during the process of tumor collection, making the amount of dye observable in the remaining blood negligible. Typically, agents such as Hoechst 33342 label several cell layers away from the vessel lumen, yet the vessels are observed as slits, with no lumen. Our general findings were that labeled rat antibodies and heterostarch both easily equilibrated with the tissue in regions of MRH (low level fluorescence throughout) thus making co-localization with vessels impossible to quantify. However, it was found that a charge-neutral dextran (75K) labeled with tetramethylrhodamine marked vessels that were not labeled by Hoechst 33342 (Figure 3).

MRH were first observed and the presence of ELG postulated based on detailed quantification of EF5 binding in small surgical biopsies of large human tumors [20]. A key observation in this report was that of both positive and negative gradients of EF5 binding away from individual vessels. This was thought to imply mixing of inflowing, oxygen rich, and outflowing, oxygen poor blood in nearby vessels. Due to the computational requirements of these measurements, and the availability of tissue from surgical biopsies, only tiny portions of human tumors had previously been assayed by these methods. Thus it was of interest to perform such studies on whole tumor cross-sections of the 9L epigastric

tumors studied herein. Mixed gradients were indeed found in tumors with MRH (data not shown), but more significantly, also in tumors that appeared to have diffusion-limited hypoxia – see Figure 4. In this cross-section more than 1000 vessels were observed (based on CD31 staining). About half of the vessels had positive EF5-binding gradients (as expected if the blood vessel were supplying oxygen) but, just as was found in the human studies [20], about half of the vessels had negative EF5 gradients. The most strongly negative and positive gradient vessels are indicated by the colors magenta and white, respectively, in an expanded portion of this section (Figure 4c). In retrospect, the geometrical necessity of long blood flow paths, and the mixing of inflowing and outflowing blood are simply a requirement for large tumors, and need not cooperate to produce MRH.

It is clearly very time-consuming to investigate, by immunohistochemistry, many large tumors especially with such a large number of potential stains and analyses. We thus began an investigation to determine whether it might be possible to predict MRH based on the non-invasive perfusion-imaging technique Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI – [22–24]). Since the parameter K^{trans} combines aspects of both blood flow and vascular permeability [22] the overall concern was that MRH would contain highly permeable vessels that would be interpreted similarly to regions with vessels having high blood flow. In the first two tumors studied with this method, this concern was confirmed (Figure 5). There was only a weak positive relationship between K^{trans} and blood flow (as monitored by DiOC7). Of course, we have already established in this model that we cannot count on DiOC7 (or Hoechst 33342) to monitor all blood flow (Figure 3). Conversely, there was a weak inverse relationship between K^{trans} and hypoxia. Unexpectedly, the intracellular water half-life, t_{wi} , a parameter that is established using the shutter-speed analysis of DCE-MRI, provides a stronger estimate of the aerobic regions of the tumor labelled by DiOC7 and v_e , the extracellular-extravascular (interstitial) volume fraction, is highest in the regions of high EF5 binding (Figure 5).

Our observations regarding MRH suggest an additional mechanism for the production of hypoxia. This appears best described as perfusion-related, but is quite different than cycling hypoxia. The mechanisms of hypoxia production are already complex, and the potential implications of ELG only increases this complexity. Therefore, we sought a novel experimental approach to ascertain the balance between cycling hypoxia and that arising from ELG. We reasoned that a determination of mismatches between EF5 binding, which averages hypoxia over several hrs, and a short term oxygen-dependent effect (DNA-damage from ionizing radiation) might allow the answer of this question. Furthermore, it is possible to assess the production of DNA-damage using an immunohistochemical approach via a quantitative assessment of gH2AX. Since hypoxic regions are known to be radioresistant, the expectation was that in an irradiated tumor, hypoxic regions should have relatively low gH2AX, while aerobic regions should have high gH2AX. Our first attempts to test this hypothesis quantitatively confirm this expectation (Figure 6). In this figure, a half-irradiated tumor was co-stained for both gH2AX and EF5-binding (panel a). In the whole tumor image, one can clearly see the vertical segregation of irradiated (left) and unirradiated (right) halves. On the unirradiated (right) side, there were a few scattered nuclei with high gH2AX and these were predominantly found in the regions with highest EF5 binding. This is

summarized by a scatterplot of all nuclei and nuclear fragments (panel b). On the irradiated side (left), the green color associated with high gH2AX did not co-localize with the red associated with high EF5 binding, since there is almost no 'yellow' which would reflect co-localization of these two processes. Interpretation of the scatterplots for two irradiated fields (aerobic area; panel c: and mixed hypoxic-aerobic area; panel d) is somewhat complex because in the aerobic (low EF5 binding) area there is a small but highly significant positive correlation between EF5 binding and gH2AX (see Table 1 for statistical summary). This finding is easily explained however, since as indicated above the variable having the greatest overall effect on gH2AX intensity is the amount of nuclear material sampled. For the mixed hypoxic-aerobic area (panel d) it is apparent that all the high EF5 binding nuclei correspond to low values of gH2AX. Despite this clear visual relationship, the same factors affecting the variables in panels b & c apply here, since some EF5 nuclei will be damaged even without radiation (see panel b), and the area analyzed consisted of mainly low EF5-binding cells. Thus, there remains a much lower, but positive correlation between EF5 binding and gH2AX overall. If the data pairs for each nuclear fragment are sorted for either EF5 binding or gH2AX intensity, and the top 300 values compared (about 10% of the total), the true relationship becomes clear (Table 1). That is, high EF5 binding is associated with low X-ray-induced DNA damage, and *vice versa*.

Discussion

In this report we have demonstrated the presence of MRH in 1–2g 9L gliosarcoma tumors grown with the epigastric artery-vein pair as their primary blood supply. The presence of MRH in this model recapitulates our previous observations in (much larger) human high-grade sarcomas and brain tumors (GBM) [18–20]. We suggest that the nature of blood flow in the 9L epigastric model lends itself to the production of very long paths of blood flow from the arterial inflow to venous outflow – indeed, this process appears geometrically inevitable. The ELG form simply because arterial inflow and venous outflow are not paired in tumors. What we did not completely appreciate in our first attempts to define such gradients [20] was their universal nature in all macroscopic tumors – however, it is only in tumors of greater than or equal to the size studied here that the impact of the gradients clearly distinguishes itself from that occurring in smaller tumors. Interestingly, in some of the first dye-mismatch experiments using murine tumors, Chaplin and colleagues noted that the intensity of the dyes varied dramatically from vessel-to-vessel and they suggested that this could be due not only to variations in vessel size, but also drug consumption along the length of the vessels. However, this required analog rather than binary image analysis techniques that were beyond the power of computers then available [30].

For a major artery-vein pair such as the epigastric, one could argue that the same thing happens in normal tissue, since this vessel pair feeds various fat pads and other tissues over distances of several centimeters in the rat. However, in most normal tissues one finds perfectly organized, countercurrent pairs of ever smaller arteries and veins with the only unpaired flow occurring at the level of the capillary bed. If a similar organization of blood flow occurred in the epigastric tumors, one might expect a small number of larger vessels near the tumor center, their size decreasing and number increasing towards the tumor periphery. One might then expect the least degree and extent of hypoxia in the tumor center

under these circumstances. Our observations do not support any aspect of such a scenario. Tumor cross-sections proximal to, at, or distal to the original attachment site of the tumor-initiating piece on the epigastric vessels show a highly variable distribution of vessel sizes and densities throughout the tumor mass (data not shown). The locations and extent of tissue hypoxia suggest a random process that is, at present, unpredictable. Similarly, some tumors are characterized by MRH whereas others appear to be dominated by diffusion-limited hypoxia (hypoxia increasing with distance from vessels - see Figure 2, panels a & b *versus* c & d). We still do not understand the random nature of this process, which is under present investigation. There would be a clear advantage to be able to determine MRH using a non-invasive imaging modality, and therefore we have great interest in extending the present DCE-MRI experiments.

What appears to be incontrovertible is that extended longitudinal blood flow gradients must exist in these tumors due to their blood flow source (Figure 1c). This represents the logical extension of a phenomenon first described by Dewhirst in window chamber models (longitudinal arteriole gradients) [7]. Using this model, the Dewhirst team has uncovered many important properties of tumor vasculature, but the observations may be limited by the small thickness (0.1 mm) and mass (less than a few mg) of these tumors. Unlike tumor properties such as the oxygen-diffusion distance, the extent of vascular gradients would be expected to scale with tumor size - this aspect of tumor physiology has been largely ignored due to the predominant use of small, subcutaneous murine tumors and emphasis on the angiogenesis process that is critical for the initiation of microscopic tumors.

Additionally, studies of spontaneous human tumors have been severely limited by the availability of tissue - thus it has only been possible to investigate relatively small pieces of human sarcoma and GBM tumors using immunohistochemical analysis with EF5. Similarly, the largest human trial using pimonidazole was limited to small needle biopsies [31]. Thus, we would argue that detailed patterns of blood flow and hypoxia are virtually unknown in human tumors and remain limited in rat models or larger animal tumors.

There is certainly no consensus on the nature of tumor hypoxia in the literature. Indeed, spatial-resolution limitations of non-invasive techniques often force the emphasis of overall properties like 'tumor blood flow' as the critical parameter. However, diffusion-limited hypoxia does not require low blood flow, and in cases where both blood flow and hypoxia have been measured independently (e.g. both can be done using dynamic and static PET measurements with labeled EF5 -[32]) no relationship has been obvious. The issue of scale has generally been ignored. Thus, the cover of a recent issue of *The International Journal of Radiation Oncology, Biology, Physics* depicted a tumor as a large apple, with necrotic core and successive layers of better oxygenation towards the periphery [33]. This type of structure would be ideal from an imaging or therapeutic standpoint, but is not realistic physiologically [34].

The critical issue to be addressed is whether MRH and/or ELG represent new, clinically relevant phenomena, or whether they could be caused by a new manifestation of cycling hypoxia. Of fundamental importance is whether EF5 binding is accurately reporting stable pO_2 values or whether the results could be an artifact of a new type of cycling hypoxia that

includes coordinated changes in blood flow for hundreds of proximal vessels. This by itself would be an exciting new finding, but it raises a fundamental question: “Is tumor hypoxia like the ‘whack-a-mole’ game® where hypoxic regions pop in and out of existence on currently unknown spatial and temporal scales?” If so, the potential use of hypoxia imaging for diagnostic or prognostic purposes would be completely undermined, as would the potential use of image-guided radiotherapy (IGRT) to target the most hypoxic tumor regions with additional therapy. It is therefore clear that the relative presence and prevalence of cycling hypoxia *versus* ELG must be defined, on a time scale that extends from that for a single treatment (several minutes) to completion of therapy (several weeks). Our impressions for expectations and predictions of cycling hypoxia *versus* extended longitudinal gradients are summarized in Table 2.

The first three rows of the table (Ktrans DiOC7; Drug consumption; Macromolecular Loss) indicate areas where there is no available information regarding cycling hypoxia. The next two rows (MRH, mixed positive and negative EF5 gradients) indicate areas where the conventional interpretation of cycling hypoxia would not be expected to cause the observed effect. In contrast, all five effects have been found in the epigastric 9L tumors, and are a natural consequence of ELG. In terms of mechanism (row 6), the cause of cycling blood flow has not been determined. Dewhirst has suggested that temporal changes in hematocrit might prove an alternative explanation, since resumption of flow after stoppage is very problematic [6]. However, it is difficult to see how variable hematocrit would be compatible with the dye mismatch experiments and there has never been any indication that blood flow could stop and then resume in multiple coordinated vessels.

A key new finding in the present studies is the development of whole cell gH2AX as a short term indicator of hypoxia (effective only during the radiation exposure). The only data from the literature on this subject is from Dr. Peggy Olive’s group. It was the first to demonstrate that hypoxic areas (measured by coincident binding of pimonidazole) had increased damage in the absence of radiation (similar to earlier work looking at induction of p53 – [35]). However, their studies looked for a relationship between tumor cell cure and residual gH2AX foci 24 hr following radiation [36]. In contrast, our studies have emphasized initial damage production as modified by the well-known oxygen effect. Olive’s group has also published *in vitro* data that agree with our value for the oxygen enhancement ratio for gH2AX formation (~4 - data not shown).

We have looked in preliminary fashion at the relationship between gH2AX and EF5 staining in two other tumor types –9L subcutaneous (shoulder) and R3230 mammary carcinoma, the latter grown orthotopically (mammary fat pad). In all cases investigated to date, an inverse relationship has been found. Thus, although much further work is required, this new assay does not support cycling hypoxia as a major factor in rat tumors of a size of 0.5–2 g.

We suggest several important implications of ELG and MRH:

- a. Metastasis; One of the least understood consequences of tumor hypoxia is enhanced metastasis [37]. On the one hand, if hypoxic cells were themselves metastasizing, they would have to invade through aerobic tissue (thus becoming ‘reoxygenated’) and then further invade through an aerobic vessel. On the other

hand, if hypoxic tissue coexists with perfused, hypoxic vessels, as we have found in these tumors, then the hypoxic tumor cells would have intimate access to vessels permeabilized by hypoxia-induced VEGF, produced locally. We have observed this type of local permeability enhancement in that most labelled macromolecular markers (albumin, antibodies, heterostarch) are not localized to vessels in regions of MRH. Indeed, this finding was initially interpreted to mean that MRH tissue was not perfused, but raised the problem of how EF5 could gain access for the observed labeling. Our finding that vessels within MRH are labeled by TRITC-labeled dextran (overall neutral charge) but not other high molecular mass markers can reasonably be interpreted to mean that many vessels within MRH are both perfused and highly permeable.

- b.** The presence of MRH and the attendant vascular permeability that occurs randomly in hypoxic tumors provides a possible explanation for the co-operative therapeutic importance of both hypoxia and interstitial tumor pressure, described for cervix cancer by Fyles, Milosavic and colleagues [38,39]. It may be possible to exploit this permeability to administer macromolecular therapeutics, because they would have selective access to MRH.
- c.** Drug consumption along blood flow. Initially, the failure of either Hoechst 33342 or DiOC7 to label vessels in MRH was interpreted (as indicated above) to mean that the tissue was not perfused. However, since MRH was only found in a fraction of the total number of tumors studied, it could also have been possible that we were observing cycling blood flow on a massive scale. Alternatively, blood flow could have been present shortly after induction of anesthesia, when the EF5 was injected, and then ceasing shortly thereafter. Such local infarcts are often observed in human GBM tumors [40]. Labeling of such vessels by TRITC-dextran suggests a different mechanism, but all of the perfusion markers can only image perfusion at a single time. In the interpretation offered by ELG, reactive drugs are simply consumed along the path of blood flow. If this interpretation were correct, it would mean that other rapidly metabolized drugs might also be consumed along the path of blood flow. Thus, both diffusion and perfusion problems could limit the effectiveness of therapeutic drugs. Although we have only looked at this in preliminary fashion, it has been found that adriamycin (which can also be detected by fluorescence microscopy) has very limited access to the tissue of these tumors (data not shown).
- d.** Our results have important implications for the use of non-invasive imaging to further characterize hypoxia prior to therapy. First, the presence of MRH would allow much stronger signals for the relatively low spatial resolution of PET, and could allow IGRT to target sub-tumoral regions. We observed this in our initial characterization of hypoxia in human glioblastomas using PET, with ^{18}F -labelled EF5, and IHC with unlabelled drug [41]. Although we did not have a short-term assay for hypoxia (i.e. X-ray induced gH2AX formation) the uptake of ^{18}F -EF5 over a relatively short period (3 hr) agreed with the overall EF5 binding detected by immunohistochemistry, at surgery, 24 hrs following unlabelled EF5 injection. Secondly, our data for DCE-MRI are consistent with the conflicting aspects of flow and permeability inherent with this technique. Our new findings about the possible

use of t_{aui} and v_e will require much further study. It may even be possible to combine the characteristics of all three parameters to obtain a more complete understanding of hypoxia and blood flow in large tumors. Interestingly, in head and neck cancer in humans a significant relationship has been found between patient outcome (local treatment response as well as survival) and K^{trans} [42]. More recent analysis that has re-evaluated these patients has suggested an even better relationship between t_{aui} and outcome, consistent with the findings of the current study (Quon and Poptani, unpublished data).

Conclusions

1. MRH is found in 9L epigastric tumors and is associated with an apparent perfusion deficit that involves extended longitudinal blood flow gradients on an enormous scale.
2. In view of the singular source of blood flow (epigastric artery-vein pair) such extended blood flow gradients are predicted by simple geometrical considerations (see Figure 1). However, there is no need for a singular blood supply for such gradients to exist. As such the 9L epigastric model may be regarded as a basic unit that is repeated in larger tumors, with multiple blood sources
3. Such blood flow gradients could cause the consumption of rapidly reacting substances (such as the DNA-binding dyes used as vascular perfusion markers, chemotherapy agents, etc.) along the lengths of the gradients. This is a macroscopic version of the longitudinal arteriole gradients first demonstrated by Dewhirst and colleagues in much smaller tumors grown in window chambers [7].
4. Large tumors may have fundamentally different properties than small tumors. Nevertheless, much smaller tumors may still harbor a vascular architecture similar to what has been described herein.
5. To date, all observations of MRH have been in human sarcomas and glioblastomas, and the rat gliosarcoma described in this report. Thus, it remains unclear how our observations and hypotheses will apply to epithelial tumors and non-orthotopic (subcutaneous) tumors. Much further study is required to elucidate this important issue.

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a.

b.

c.

Figure 1.

a. Depiction of static, diffusion limited hypoxia. Tumor cords form between vessels (smallest white circles) surrounded by successive annuli of tissue with decreasing oxygenation (darker rings). Cells become anoxic and/or necrotic when the distance between vessels is larger than twice the oxygen diffusion distance (double arrow – 250 – 400 microns) (adapted from [43]). This picture cannot be accurate for large tumors because the source of oxygenated blood must come from outside the tumor, or from preexisting vessels taken over by the tumor. Thus, oxygen gradients must form along the path of blood flow. In

the lower right, cycling hypoxia is depicted. The white line indicates a stoppage of flow, whereby the entire tissue cord would become hypoxic (black 'X'). In rodent tumors, blood vessels typically have a much closer spacing, suggesting that hypoxic regions should never be larger than about 300 μm in diameter.

b. Photo of epigastric tumor, showing the femoral and epigastric vessel junction, just left of tumor. Almost all vessels arise from the original epigastric because the inguinal region is protected by fat pads towards the periphery, and the abdominal wall centrally.

c. Highly schematic diagram depicting a central disk from an ovoid tumor, supplied by a major artery-vein pair near its center (A-V). Illustrated at 12:30 o'clock is a tumor microvolume MV1 with distance 'd1' from the artery-vein. Although there are an infinite number of possible (branched) paths from the artery to MV1, none can be shorter than 'd1'. A multiply-branched circumferential blood flow path is depicted, travelling clockwise as indicated by the curved lines. At each branch, only one path is considered. Of course, such a path would not have to be so consistent, nor would it need to be confined to the plane of the paper. Note that blood flowing out of MV1 might then be the inflowing blood for MV2, and so on for MV3, etc. before returning to the venous outflow. Thus, there could also be much shorter paths supplying MV3 allowing the proximity of inflowing and outflowing blood. A large human tumor might grow to incorporate many major vessel pairs. To simplify this possible complexity, we suggest use of the epigastric tumor implant in rats, thus limiting the boundary conditions of the blood supply to one major vessel-pair.

Figure 2.

Examples of whole-tumor cross sections from the middle segments of pedicle (panel a, upper-left) and traversing-vessel (panels b, c, d) epigastric tumors showing EF5 binding. As seen from the scale bar (1 mm) these tumors are about 4 to 8 mm in diameter. The pedicle tumor's maximum EF5 binding (brightness adjusted to allow similar visibility to the other panels) is at a level of only 10% maximum (moderate hypoxia), and is spread throughout the tumor whereas that of the traversing vessel tumors (b,c, & d) is close to 75% maximum (severe hypoxia) and is seen predominantly in the left half of the tumor (panel b) or with shapes more characteristic of diffusion-limited hypoxia (panels c & d). Binding from endogenously administered Hoechst (not shown) was apparent only in the regions of low EF5 binding for all tumors investigated to date even though there are hundreds of vessel in the regions of macroscopic hypoxia (not shown for image clarity).

Figure 3.

Image of border between macroscopic area of EF5 binding (left side) and low binding (right) from the tumor depicted in Figure 1b. In the region of low EF5 binding (more aerobic) both Hoechst and TRITC-dextran are observed (pseudocolored red and green, respectively) whereas in the macroscopic region of high EF5 binding, only the TRITC-dextran (green) is observed. Since Hoechst penetrates several cell layers away from the vessels, some small spots of Hoechst only are also seen. This is a common observation when comparing CD31 vs. Hoechst labeling and indicates an out of plane vessel.

Figure 4.

Panel a: 4×6 10× field of 5mm × 8mm epigastric tumor cross-section. Vessels are identified (light green) and contours formed around each vessel (5 contours, each separated from the next by 20μ) – the paths between the contours are pseudocolored white through increasingly dark grey. At the observed resolution of this image, only the innermost (white) path is clearly distinguished. Black pixels contain no nuclear stain, and blue areas are more than 100 μ from the nearest vessel. Average EF5 binding is calculated for the path between each pair of contours, and gradient of EF5 binding computed for each vessel (see reference 20). Panel b: Same region of tumor. EF5 binding has been converted to color-coded levels of oxygenation (green, 75–24 mm of Hg, physiological oxia; dark green, 24–7.5 mm of Hg, mild hypoxia; red, 7.5–2.4 mm of Hg, moderate hypoxia; orange, 2.4–0.75 mm of Hg, severe hypoxia). Vessels with the largest positive EF5 gradients are colored white, while those with largest negative EF5 gradients are colored magenta. Note that this tumor appears to have ‘normal’ diffusion-limited hypoxia. Thus, mixed positive and negative EF5 gradients are not directly linked with MRH but rather appear to be caused by the geometry of blood flow in large tumors.

Figure 5.

This multi-panel figure illustrates our work towards characterizing hypoxia and blood flow in a 9L tumor ($13 \times 17 \times 15$ mm³) grown on the epigastric artery/vein. The upper panel shows an immunohistochemical (IHC) section, cut from the same region of tumor that is also seen on the MRI images (lower panels). The IHC section is 10 microns thick whereas the MRI voxel depth is 2 mm. In the upper panel EF5 binding, as a surrogate for tissue hypoxia, is shown in red, all vessels as identified by anti-CD31 antibody in green and labeled vessels, marked by an intravenous injection of DiOC7 before the tumor was removed, in blue. The

region of very high density vessels at the lower edge of the section is skin. An image of all intact nuclei (measured by flooding the section with Hoechst 33342) showing a substantial region of partial necrosis in the central lower half of the tumor, indicated by the white polygon; N). The MRI panels (left) show an axial section (contrast enhanced T1 weighted image; P) of the animal (tail to the left and tumor in lower right). DCE MRI was performed using the Shutter Speed Model and the three parametric maps (right) represent K^{trans} , τ_{ui} and v_e . τ_{ui} provides the best estimate of DiOC7 labeling, while v_e (fractional volume of the extravascular, extracellular space) provides a reasonable association with EF5 binding.

Figure 6.

Partial tumor cross-section (5×7 10x fields) of an epigastric tumor that had been irradiated on its left side 45 minutes prior to tissue collection. The section was jointly stained for EF5 binding (red) and γ H2AX (green). These are the unmodified images coming directly from the camera, and the variable light intensity of the microscope, decreasing from left to right of each 10x field, is obvious. γ H2AX was generally low on the right half of the tumor and high on the left half of the tumor – centerpoint indicated by white bars. However, γ H2AX intensity was diminished in regions of high EF5 binding, otherwise the observed color would be yellow (red + green). To quantify this relationship, individual fields were assessed after correcting for the variable excitation light and background. A single 10× field (1.2mm × 0.8mm) from a mixed hypoxic unirradiated region (panel b) an aerobic irradiated region (panel c) and a mixed hypoxic irradiated region (panel d) were further quantified to collect EF5 and γ H2AX intensities from each (partial) nucleus, as defined by flooded Hoechst stain (approximately 3000 partial nuclei per field). For unirradiated tissue, the overall γ H2AX was low, but was positively associated with the most hypoxia, as previously reported [36]. The overall positive relationship seen in the irradiated aerobic field (panel c) was due to the variability in the amount of nuclear and cytoplasmic tissue identified per Hoechst positive partial nucleus. This relationship was greatly diminished in the irradiated field containing

high EF5 binding (panel d) and one can observe that all of the high EF5 binding events were associated with low γ H2AX. These relationships were further quantified after sorting the data pairs according to either EF5 or γ H2AX (see Table 1).

Table 1

Data pair analysis (EF5 versus γ H2AX) from the half-irradiated tumor depicted in Figure 6. Three of the 25 fields were analyzed detailing the signals from EF5 and γ H2AX for every partial nucleus detected. Note that when selecting for the top γ H2AX, the most aerobic levels of EF5 binding (low) are found, whereas when selecting for the top EF5 binding, the lowest levels of γ H2AX (radioresistant) are observed. Thus, cells with high EF5 binding, (as averaged over the three hr drug exposure period) are the same as the most radioresistant cells, selected over a period of less than 10 minutes, and vice versa

Selected Field		EF5 ($\pm$ SD)	γ H2AX($\pm$ SD)
Control; Mixed Hypoxia	Total (n = 3281)	0.089 $\pm$ 0.107	0.057 $\pm$ 0.033
	Top 300 EF5	0.376 $\pm$ 0.140	0.085 $\pm$ 0.029
	Top 300 γ H2AX	0.106 $\pm$ 0.118	0.055 $\pm$ 0.025
17 Gy; no Hypoxia	Total (n = 3554)	0.087 $\pm$ 0.032	0.210 $\pm$ 0.127
	Top 300 EF5	0.156 $\pm$ 0.025	0.399 $\pm$ 0.152
	Top 300 γ H2AX	0.135 $\pm$ 0.032	0.478 $\pm$ 0.095
17 Gy; Mixed Hypoxia	Total (n = 2936)	0.105 $\pm$ 0.088	0.153 $\pm$ 0.102
	Top 300 EF5	0.320 $\pm$ 0.119	0.132 $\pm$ 0.103
	Top 300 γ H2AX	0.112 $\pm$ 0.055	0.359 $\pm$ 0.091

Table 2

Predictions of cycling hypoxia versus extended longitudinal gradients. These have been ordered from 'unknown' to 'not likely', and end (based on the γ H2AX data), to known. Thus, cells with high EF5 binding, (as averaged over the three hr drug exposure period) are the same as the most radioresistant cells, selected over a period of less than 10 minutes, and vice versa

Properties	Cycling Hypoxia	Ext. Long. Gradients
Ktrans _ Blood Flow	Unknown	Yes
Drug Consumption	Unknown	Found
Macromolecular Loss	Unknown	Yes
Macro-Regional Hypoxia	Not Likely	Yes
+ve & -ve EF5 Gradients	Not Likely	Yes
Mechanism	Unknown	Geometrical Necessity
Dye Mismatch	Isolated Vessels	Isolated Vessels & Regions
EF5 Bind ~ 1/gH2AX	No - Expect Mismatch	Yes - found