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Imaging and Analytical Methods as Applied to the Evaluation of Vasculature and Hypoxia in Human Brain Tumors

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Abstract

Tissue hypoxia results from the interaction of cellular respiration, vascular oxygen carrying capacity, and vessel distribution. We studied the relationship between tumor vasculature and regions of low pO_2 using quantitative analysis of binding of the 2-nitroimidazole EF5 given to patients intravenously (21 mg/kg) approximately 24 h preceding surgery. We describe new computer algorithms for determining EF5 binding as a function of radial distance from individual blood vessels and converting this value to tissue pO_2 . Tissues from six human brain tumors were assessed. In a hemangiopericytoma, a WHO Grade 2 and WHO Grade 3 glial brain tumor, all tissue pO_2 values calculated by EF5 binding were >20 mmHg (described as “physiologically oxygenated”). In these three tumors, EF5 binding gradients (measured as a function of distance from each observed vessel) were low, with small positive and negative values averaging close to zero. Much lower tissue oxygen levels were found, including near some vessels, in glioblastomas. Gradients of EF5 binding away from vessels were larger in glioblastomas than in the low-grade tumors, but positive and negative values again averaged to near zero. Based on these preliminary data, we hypothesize a new paradigm for tumor blood flow in human brain tumors whereby in-flowing and out-flowing blood patterns may have contrasting effects on average tissue EF5 (and by inference, oxygen) gradients. Our studies also imply that neither distance to the nearest blood vessel nor distance from each observed blood vessel provide reliable estimates of tissue pO_2 .

INTRODUCTION

There is increasing awareness that the tumor microenvironment plays a central role in anti-tumor therapy resistance. Hypoxia has been shown to cause radiation and chemotherapy resistance as well as tumor aggressiveness (metastasis and invasion) resulting from genetic instability and selection (1-15). Hypoxia occurs in tissues as a result of an interaction of three physiological factors: (1) cellular oxygen consumption, (2) vascular oxygen carrying capacity, and (3) distribution of blood vessels. These three factors are balanced in normal tissue to prevent hypoxia, but this is not the case in tumors. Several categories of tumor hypoxia have been described. Chronic or diffusion-limited hypoxia, first described in 1955 by Thomlinson and Gray (16) describes the relatively simple situation of a steady state of oxygen supply and demand, with high pO_2 at the vessels that decreases steadily with increasing distance away from vessels due to diffusion-limited oxygen consumption. Acute or perfusion-limited hypoxia

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was introduced by Brown in 1979 (17) as a way to explain both rapid reoxygenation after radiation (18) and direct observations of flow variation in window-chamber tumors (19). Acute cycling of blood flow between “on” and “off” states was shown by Chaplin *et al.* in 1989 using temporally separated injections of perfused-vessel dyes, demonstrating that they did not always mark the same vessels (20). Dewhirst and colleagues used a window-chamber model containing very small (several mg) epithelial tumors to describe some variations in the classical hypoxia categories described above (21). In these studies, longitudinal arteriole gradients resulting from the asymmetrical origin of vessels in the window-chamber model (all from the fascia side) as well as spatial and temporal variations in capillary hematocrit were identified (22). Using the same model, Jain and colleagues indirectly measured strongly negative oxygen gradients as a function of radial distance from such arterioles (23). Tumors are also known to contain arterio-venous shunts that can modify the local pO_2 in unpredictable ways (24). Changes in oxygen resulting from modifications in vessel diameter and blood flow can vary with both short (minutes) and long (hours) cycle periods (21). Despite the long history of these descriptive terms, e.g. chronic, acute hypoxia, etc., the relative importance of each specific cause of hypoxia remains controversial. Nevertheless, all mechanisms of hypoxia development discussed above would predict an inverse relationship between tissue oxygenation and distance to the nearest blood vessel (DTNBV), although changes in perfusion would prevent any absolute relationship. Thus DTNBV has often been used as an indirect surrogate for hypoxia (25-28).

A more direct measurement of tissue hypoxia can be made at high spatial resolution using exogenously administered drugs that bind specifically to hypoxic cells. This approach was initially suggested by Chapman (29) when he proposed that 2-nitroimidazole agents, initially tested as hypoxic cell sensitizers, could be used in much lower concentrations as hypoxic cell markers. This technique depended on the ability of cellular enzymes with nitroreductase properties to reduce such chemicals, thereby activating them (e.g. to the 4e- reduction product, hydroxylamine) to allow their covalent binding to intracellular proteins. Oxygen inhibits this process in a standard kinetic fashion by reversing the initial one-electron reduction product (30). The 2-nitroimidazole agent EF5 has been developed, validated and tested as a quantitative hypoxia marker in tumors, non-neoplastic pathological conditions and normal tissue (31-33). Using this detection system, hypoxia patterns can be described and the pO_2 in tissue regions can be estimated. This methodology also allows examination of the spatial relationship between blood vessels and hypoxia, proliferating cells, apoptotic cells and other imageable features (34-36).

Herein we describe new algorithms for and provide examples of the relationship between tumor tissue pO_2 and blood vessel distribution as applied to human brain tumors. To reduce some ambiguities resulting from the use of DTNBV (see the Discussion), we adopted a vessel-centric approach (distance from each observed vessel, DFEOV). We report that the number and area of blood vessels as well as the varying patterns of hypoxia relative to blood vessels can be described quantitatively. We find that EF5 binding gradients away from vessels have both positive and negative values that average to near zero over macroscopic tumor regions. While a positive EF5 binding gradient implies, as described above, a negative oxygen gradient (expected), the implication of a negative EF5 binding gradient away from a blood vessel is a positive oxygen gradient (unexpected; see the Discussion). The presence of both positive and negative EF5 gradients, in roughly equal proportions, suggests a dramatic heterogeneity of oxygen availability from blood vessels in large tumors.

We provide preliminary data that support a hypothesis regarding a new tumor blood-flow model whereby blood vessels can be characterized, relative to the tumor, as having either in-flowing/oxygenated or out-flowing/deoxygenated blood. The spatial separation of these flow patterns (expected to be serial, unlike their countercurrent-flow paired counterparts in normal

tissue) can explain several features of hypoxia patterns in large tumors. A further implication of our data is that neither DTNBV nor DFEOV are good surrogates for hypoxia in brain tumors, especially glioblastomas.

METHODS

Patient Eligibility

Eligible patients had radiographic evidence of a supratentorial tumor and were scheduled for surgery to remove or debulk the mass. Exclusion criteria included age <18 years or a history of peripheral neuropathy. The exclusion for peripheral neuropathy was based on previous reports that drugs similar to EF5 can themselves cause peripheral neuropathies when given in multiple, high doses (37). Patients who weighed >230 pounds, had severe cerebral edema, and/or had a pre-existing condition that precluded delivery of approximately 300-600 ml of fluid over 1-2 h were also excluded. The weight exclusion was based on the relatively low solubility of EF5, since infusion rates and time for drug delivery were limiting. Diuretics were administered to patients preceding EF5 administration if their neurosurgeon felt that they had MRI evidence of pre-existing mild to moderate cerebral edema due to their brain tumor but were otherwise eligible. The Institutional Review Board of the University of Pennsylvania, the Clinical Trials and Scientific Monitoring Committee at the Abramson Cancer Center, and the Cancer Therapeutics Evaluation Program of the National Cancer Institute approved the entry of patients to this study.

Drug Administration and Tissue Collection

EF5 was supplied by the National Cancer Institute, Division of Cancer Treatment in 100-ml vials containing 3 mg/ml in water with 5% dextrose and 2.4% ethanol; it was administered through a peripheral intravenous catheter to a dose of 21 mg/kg. Blood samples for drug levels to determine AUC (area under the curve, drug concentration integrated over time) were obtained 1 h after completion of drug administration and again at the time of surgery. The rationale and methods for this analysis have been described previously (38).

Sixteen to 24 h after completion of drug administration, the tumor was resected and placed in sterile, ice-cold EXCELL 610 medium (JRH Biosciences, Lenexa, KS) with Hepes buffer. These tissues were immediately brought to the laboratory. A small portion of tumor tissue was reserved to determine cube reference binding (CRB), as described briefly below. The remainder was frozen to determine the *in situ* binding, i.e., the EF5 binding that occurs within the patient between the time of drug injection and tumor removal.

EF5 Binding Quantification in Biopsy Sample

The quantitative analysis of tissue sections stained for EF5 (or EF3; see section on Cube Reference Binding below) has been described previously (32-34). For the present study, it was necessary to dual stain each section for blood vessels using anti-CD31 and for hypoxia using anti-EF5 antibodies. In preliminary experiments, it was determined that the additional procedure required for the CD31 staining did not affect the absolute EF5 binding (data not shown).

Frozen sections (10 µm thickness) were first stained for CD31 (BD Pharmingen no. 555444) using a primary mouse anti-human antibody (5 µg/ml) followed by a secondary stain with Cy5-labeled rat anti-mouse (Jackson ImmunoResearch no. 415-175-166; 5 µg/ml). Each staining period was followed by three 30-min rinses in phosphate-buffered saline (PBS). A brief additional fixation step was then performed to prevent dissociation of CD31 antibodies followed by blocking and staining for EF5 (Cy3-labeled ELK3-51 antibodies; 75 µg/ml). Due to the minor overlap of excitation and emission wavelengths for Cy3 and Cy5, it was sometimes

necessary to apply minor optical corrections to the CD31 image (see the Appendix). Prior to imaging and photography, the slides were flooded with 20 μM Hoechst 33342 to identify the location of all nuclei. The Hoechst dye stains cellular DNA, allowing the creation of a binary image that separates tissue (white pixels) from non-tissue regions (black pixels) (Fig. 1). Images of the tissue sections were captured using a cooled CCD camera (Photometrics Quantix, Pleasanton, CA) and fluorescence microscope (Nikon LabPhot, Nikon Corp.). Each set of photographs included an image of a hemocytometer filled with a known concentration of Cy3 dye. The average intensity of this image allowed correction for day-to-day variations in lamp intensity, while the variation in intensity over the image frame was used for field flattening (32,39). The former accounts for a substantial variation (factor of up to 4 over the lifetime of a typical lamp) and the latter encompasses a range of intensities from about 70% to 130% of average (data not shown). Image sets also included off-tissue portions that were used to correct for background (optical leakage and camera noise). Nonspecific antibody staining was identified and corrected for using a technique referred to as “Competed Stain” (CS) whereby specific antibody staining was inhibited by the inclusion of authentic EF5 in the antibody mixture (33). This system generated images where fluorescence was assessed on an absolute scale by accounting for the microscope’s lamp intensity and camera shutter exposure time. We have shown previously that the fluorescence signal was a direct measure of absolute EF5 binding (30,40). EF5 binding is inversely proportional to oxygen concentration but is also directly proportional to AUC. Therefore, the fluorescence signal obtained from the tissue sections was also normalized for the patient’s drug AUC, which was determined from HPLC analysis of plasma EF5 concentration (38). After the imaging of the Hoechst 33342 and the other stains, the slides were dried and processed with hematoxylin and eosin (H&E) for standard pathological analysis to confirm that the section was comprised primarily of viable tumor tissue.

Cube Reference Binding

To directly compare *in situ* EF5 binding between tissues, we determined the inherent tissue-specific maximum capacity to bind EF5 in a process referred to as cube reference binding (CRB) (33). For CRB determination, 2-mm tumor tissue pieces were exposed *in vitro* to 200 μM EF3 (a sister drug to EF5) under hypoxic conditions (0.2% oxygen) for 3 h (AUC = 600 $\mu\text{M}^*\text{h}$).² EF3 is identical to EF5 except that the two fluorine atoms on the carbon adjacent to the terminal carbon are replaced by hydrogen. Anti-EF3 antibodies do not recognize EF5 very well, so the EF3 calibration measurements can be made with relative independence from pre-existing EF5 adducts resulting from the *in situ* patient exposure to EF5 (39). The cubes were frozen, sectioned on the cryostat, stained with the appropriate monoclonal antibody (ELK5-A8), and imaged as described above. The cube tissue section with the brightest binding was analyzed for fluorescence intensity. These data were assessed as a cumulative frequency plot, and the 50% value was used for the final calculations (33). The final value of EF5 binding value for each point in the *in situ* tissue images (% CRB) was its absolute binding level (after subtraction of CS) divided by the CRB, normalized for AUC and multiplied by 100.

To interpret % CRB as a hypoxia surrogate, we have previously performed *in vitro* studies on the relationship between EF5 binding and $p\text{O}_2$ (30,39,40). We have studied numerous rodent and human cell lines and found consistency within and between species, irrespective of tumor type (39). Thus EF5 binding was related to $p\text{O}_2$ in human cells, as shown in Table 1. The binding values can be converted to two-dimensional tissue oxygen maps and/or used to assess other spatial parameters within the tissue sections (31,34,35,41).

² $\mu\text{M}^*\text{h}$ refers to the drug concentration integrated over time of exposure at 37°C. 600 $\mu\text{M}^*\text{h}$ was used because this approximated the *in situ* tumor drug exposure.

Combined Analysis of EF5 Binding and CD31

We have developed a series of algorithms to quantify the spatial relationship between blood vessels and hypoxia, as measured by CD31 and EF5, respectively (see Appendix). All programming for this procedure was done in Matlab 7.2. Batch processing was expedited by a powerful multi-processor computer, assembled by one of the authors (KWJ). There were six steps involved in the complete image analysis: image acquisition, Hoechst 33342 tissue mask creation, CD31 vessel identification and masking, generation of equally spaced, non-interfering contours around each vessel, EF5 quantification and data analysis (Fig. 1).

Data Analysis

The goals of the analyses were to (1) identify each vessel and determine its size, (2) segregate the blood vessels into various perivascular oxygen ranges (Table 1) based on the average EF5 binding in the band of tissue between the vessel's perimeter and the first contour, (3) calculate the gradient of EF5 binding averaged in the bands between successive contours around each vessel, and (4) estimate the variability of the observations per section and per patient described in (2) and (3) above. The technical details involved in the development and application of these algorithms are contained in the Appendix.

RESULTS

Twenty-three patients with brain tumors were entered on EF5 clinical trials at the Hospital of the University of Pennsylvania between 1/8/2000 and 3/27/2003. The six tumors used for the algorithm development and validation described herein were selected for study because they represented a range of brain tumor grades and histologies. Three to five tissue sections from each of the six patients' tumors [one hemangiopericytoma (HPCTA), one oligoastrocytoma (WHO Grade 2, OA), one anaplastic oligodendroglioma (WHO Grade 3, AO), two *de novo* glioblastomas (WHO Grade 4, glioblastoma), and one recurrent glioblastoma identified below as patients A-F, respectively] were stained for CD31 and EF5, then imaged and counterstained with H&E, as described above. Two slides were eliminated from further analysis because they contained >80% necrosis on H&E (one each from patient E and F). Additionally, two slides were eliminated because they contained very bright punctate artifacts in the face of weak CD31 staining. This artifact was confirmed to result from microprecipitation of the primary and/or secondary antibodies, since it could be removed by high-speed centrifugation of the antibodies (data not shown). Therefore, EF5 and CD31 were analyzed on 20 tissue sections from six patients; each tumor studied had at least three tissue sections evaluated (Table 2). Computer-generated tissue and blood vessel masks were compared visually to the original Hoechst 33342, H&E and CD31 images with the goal of confirming that the masks accurately represented vascular structures and viable tissue while excluding artifacts. After visual inspection, all Hoechst 33342 masks identifying viable tissue were considered acceptable for further analysis. Some of the CD31 masks required minor modifications. For example, on one slide, a region of necrosis and a very large vessel were over-masked; i.e., areas of tissue that should have been included in the analysis had been eliminated along with the necrosis. On another slide, there was a tissue region that was out of focus (the tissue was not well attached to the slide in this area) and the algorithm over-masked the adjacent tumor region. In both situations, manual changes were made to the mask. Once the CD31 masks were finalized, analysis of the density (vessels per mm² of tissue) and area of the vessels (μm²/vessel) on each slide was performed automatically (Table 2). The HPCTA tumor (patient A) was characterized by a high density of relatively small vessels. The WHO Grade 2 OA (patient B) and the WHO Grade 3 AO (patient C) contained a lower density of vessels that were larger than those of patient A. The slides evaluated suggested that the vessel density was higher in the lower-grade OA than the AO. There was substantial vascular heterogeneity among the three glioblastomas. One of the two *de novo* glioblastomas (patient D) was characterized by a high density of relatively small

vessels, whereas that from patient E had a lower density of much larger vessels. The recurrent glioblastoma (patient F) had an even lower density of similarly very large vessels (Table 2).

Analysis of the EF5 binding gradients away from individual vessels into the tumor tissue required the computer-generated construction of a series of equally spaced contours around each vessel. The EF5 binding values were then averaged for all pixels in each of the bands defined by the space between adjacent contours. To select the optimal parameters for this analysis, the spacing and number of contours around each vessel were varied and tested. Up to 20 contours with spacing of 5, 10 and 20 μm were considered. As the vessel density increased, the contours from a given vessel would intersect with those from nearby vessels and the number of intersections increased as the intercontour spacing increased. At contour intersection points, the intercontour bands were truncated so that pixels were assessed only once (see Appendix, Fig. 2 and Table 3). A maximum perivascular radius of 50 μm employing five equally spaced contours was selected since the use of five contours of 10 μm spacing compared to 10 contours of 5 μm spacing did not change the EF5 binding gradients but did significantly reduce the computation time (data not shown).

EF5 binding values (as a percentage of CRB) were averaged over the pixels comprising each intercontour band of each vessel. For example, all pixels between the 10- and 20- μm contours from the vessel periphery were used to define the average EF5 binding in the second band. Each vessel was thus characterized by average EF5 binding in the five bands with average distances of 5, 15, 25, 35 and 45 μm from the periphery of the vessel. When the average EF5 binding in each band was plotted as a function of these five distances, the resulting line could be characterized by a slope (or gradient) and an intercept.

Based on the EF5 binding average for the first band, each vessel was grouped into one of the five categories defined in Table 1. Since none of the vessels studied had $p\text{O}_2$ values corresponding to anoxia, only four categories were applicable: physiological oxygenation, mild hypoxia, moderate hypoxia and severe hypoxia. The HPCTA and the WHO Grade 2 and Grade 3 glial tumors contained only physiologically oxygenated vessels and tumor tissue (Table 2, Table 3, Fig. 3A-B). In contrast, various degrees of hypoxia were evident in the glioblastomas (Table 2, Table 3, Figs. 3C/D and E-G). In addition to physiologically oxygenated vessels and tissue, all three glioblastomas contained mild and moderate perivascular hypoxia. Severe perivascular hypoxia was found only in the recurrent glioblastoma (patient F, Table 2, Table 3, Fig. 3E-G). The significance of these differences in the glioblastomas cannot be determined in such a small cohort, but the heterogeneity warrants additional study.

Using the average EF5 binding value per intercontour band, the slopes (change of CRB per μm) and intercepts were calculated for each vessel (Table 3). These individual vessel values were then grouped and averaged for all vessels in each tissue section. Results for two of the glioblastomas are shown in Fig. 4. Although the average of the slopes was expected to be small for the physiologically oxygenated vessels, it was anticipated that the overall gradients would be much steeper for the more hypoxic vessels. Unexpectedly, the average slope remained low irrespective of the perivascular $p\text{O}_2$ (Table 3, Fig. 4). The basis for these low average slopes was not that the slope for individual vessels was uniformly low. Rather, the presence of both positive and negative slopes tended to average to near zero (Fig. 5, Table 4). Note that in Fig. 5, a logarithmic Y axis was required to encompass all values. Thus the slopes for the individual vessels were much larger than those for the averages, shown in Fig. 4.

DISCUSSION

In many medical fields including but not limited to tumor biology, there is considerable interest in the interaction between vasculature and tissue microenvironment. The importance of

analyzing the relationship between vessels and tissue oxygenation in tumors is driven by a number of factors: (1) the observation that hypoxia causes therapy resistance and is associated with tumor metastasis and invasion (1,8); (2) therapeutic drug delivery via the vasculature can be decreased by perfusion and diffusion limitations (42); and (3) the tumor vasculature itself has become a target of therapeutic manipulation. In normal tissues, the analysis of blood vessels is relatively straightforward because the vessels tend to be regular in shape, size and spatial distribution. In contrast, tumor vessel analyses are complicated by the heterogeneity of these factors.

In the absence of independently validated assays of tissue pO_2 , the parameter DTNBV has often been used as a surrogate for hypoxia, e.g., to test new endogenous or exogenous markers, assess cell division, apoptosis, etc. In analyses of two-dimensional tissue sections, the DTNBV metric is limited by the presence of unseen vessels outside the edges of the imaged area or in the third dimension. The original reference to diffusion-limited hypoxia based on tumor cords in lung cancer (16) suggested that the maximum oxygen diffusion distance was 100-150 μm . However, this value clearly depends on the vessel distribution (peripheral or central to the cord), tissue metabolic rate and perfusion limitations, as defined in the Introduction. Further complexities arise when a feature of interest (e.g. cell division, apoptosis) is influenced by factors in addition to oxygen. Indeed, the oxygen dependence of growth has not been defined for tumor cells *in vivo*. When cycling cells are measured by an exogenously administered agent (e.g. BrdUrd or IdUrd), drug availability can be compromised by diffusion and perfusion limitations and thus modify the evaluation of the end point (42). Begg *et al.* first described a number of parameters associated with vascular perfusion with respect to IdUrd labeling in human tumors (43). They suggested that blood vessels without surrounding IdUrd-labeled tumor cells could be an indication of acute hypoxia and reported a generally positive trend between fractional pimonidazole binding and DTNBV. Wijffels and colleagues also investigated tumor cell proliferation as a function of DTNBV in human head and neck tumors (27). They found a sharp decrease in labeling index with DTNBV, but this was highly variable in different tumor histologies. In a comparison of three glioma xenografts, Rijken *et al.* showed dramatic differences in distance from hypoxia to the nearest perfused vessels (up to 300 μm) (44). These studies support our contention that DTNBV does not provide a consistent measure of tissue hypoxia.

Many studies have investigated the relationship between vasculature (sometimes using an additional perfusion marker, e.g. intravenous Hoechst 33342 in animal models) and hypoxia using exogenous and/or endogenous markers (25-28,45). In these studies, hypoxia has been assumed to be a binary parameter (i.e. present or absent), typically identified by thresholding an immunohistochemically identifiable hypoxia marker (e.g. pimonidazole). This approach allows a great deal of flexibility in the staining methods and analysis but prevents the study of biological effects that vary with cellular pO_2 . A sophisticated analysis by Rijken *et al.* in glioma xenografts considered DTNBV with such a binary indication of hypoxia as monitored by either NITP or pimonidazole (44). The data were interpreted by the investigators to be consistent with diffusion-limited hypoxia with a small fraction of non-perfused vessels (i.e. no acute hypoxia). Roughly 5% of the total hypoxic area was seen within 50 μm of perfused vessels. Despite using a manually selected hypoxia threshold, this report is one of the very few to demonstrate gradients of hypoxia-marker binding as a function of distance from vasculature (although only for a single vessel and direction). In a related paper from the same laboratory, Ljungkvist *et al.* also interpreted their findings as representing only diffusion-limited hypoxia (46). In her study, dual hypoxia markers were injected at 150 (CCI-103F) and 30 (pimonidazole) min prior to tissue collection in SCCNij3 squamous cell xenografts. In control animals, the CCI-103F and pimonidazole markers delineated identical hypoxic areas. In contrast, therapeutic intervention after the CCI-103F injection dramatically modified the co-

localization of the two hypoxia markers. Thus, as with the Rijken study, the concept of acute cycling blood flow was not supported by these data.

A few studies have investigated co-localization of exogenous and endogenous hypoxia markers (e.g. pimonidazole and VEGF, HIF-1 α or CA9). As with the studies discussed above, binary assays were used to estimate hypoxia for each of the markers. Although there are too many papers to review thoroughly here, the relationship between hypoxia markers has generally shown good correlations in animal models (47,48) but poor or even negative correlations in clinical studies (49-54). Typically, the conclusion of such studies has been that the endogenous marker had some flaw with respect to the 2-nitroimidazole standard.

Our previous studies and the current analyses emphasize that the description of hypoxia as a binary property of tumors is much too simplistic (55). In rodent models, binding of EF5 as an analog variable has been validated against tumor cell killing by both radiation (56,57) and tirapazamine (58) in individual tumor-bearing animals. Similarly, EF5 binding values were found to vary inversely with the oxyhemoglobin saturation of nearby vessels (59,60). In these experiments, perfusion was monitored using DiOC7. Although EF5 binding has always been found to vary inversely with pO_2 , with a 50-200-fold dynamic range, it also varies with AUC (drug concentration integrated over time) and with the tissue nitroreductase level (38,39). In the studies described herein, these corrections (including optical aspects of the microscope light source) account for a total variation in fluorescence intensity of the anti-EF5 fluorescent antibody of several orders of magnitude (Table 5). It would be surprising if corrections of such a large magnitude could be ignored in the analysis of other exogenously administered hypoxia markers.

Using the quantitative methodology described herein, we have found that individual vessels in brain tumors can be enumerated and their characteristics described, specifically the approximate perivascular EF5 binding and the gradient of EF5 binding away from each vessel. In contrast to the more commonly described parameter of DTNBV, our algorithms consider binding between equally spaced contours radially enclosing each individual vessel as well as interference of contours from all neighboring vessels (i.e. distance *from* each observed vessel, not distance *to* the nearest vessel). This allows the characteristics of all (or any particular) vessels to be defined. Our expectation was that calibrated EF5 binding would generally increase (i.e. pO_2 decrease) with distance from each vessel, with the slopes increasing as the perivascular pO_2 decreased. The actual observed result was that there were similar numbers of vessels with positive and negative EF5 binding gradients, irrespective of the level of EF5 binding in the band of tissue between contours of 0 and 10 μ m.

Current blood flow models cannot readily explain negative EF5 binding gradients, since this implies a positive oxygen gradient (i.e., oxygen is flowing toward the vessel). For example, cycling blood flow should still produce positive EF5 binding gradients because during the time that blood flow was “on”, binding would be inhibited most near the blood vessel and drug would be delivered. During the “off” time, oxygen would be consumed much faster than EF5 (by approximately two orders of magnitude), and again, regions of tissue closest to the blood vessel would remain oxygenated for longer times compared to tissue regions at a distance from the vessel. Additionally, vessels with very low or zero flow or with low hematocrit would be expected to affect the local tissue pO_2 only minimally. This latter situation might apply to the subset of vessels specific to pseudopalisading regions of glioblastomas, since it has been proposed that these vessels are infarcted (61).

We hypothesize a novel yet very simple explanation for the presence of both positive and negative EF5 binding gradients. A detailed review of the literature reveals that tumor vessels are typically characterized in one of two ways: (1) as potential sources of nutrients, whether

by steady state or during the “on” stage of acute perfusion, or (2) non-perfused. This is embodied by the parameter DTNBV in that all (perfused) vessels are considered functionally the same. Such a characterization may be reasonable for very small tumors (only a few mm in diameter), especially when their vasculature arises from normal tissue at their periphery. However, in much larger tumors such as we are studying here, it is unlikely that vessels exist only as sources of oxygen and nutrients. A significant fraction of them must be considered to be “sinks”, having a similar function to veins in normal tissue. The close spatial pairing of the countercurrent arterial supply and venous drainage in normal tissue is lost in tumors, yet the overall function, nutrient supply and waste removal must be recapitulated. Thus the biological relevance of DTNBV is inherently hampered because it implies that all perfused vessels provide a similar function. We propose that the tumor vasculature may be better described by groups of vessels or vessel networks transporting blood into the tumor for nutrient supply (in-flowing) and out of the tumor for waste removal (out-flowing).

There are certainly some situations in which the differentiation of in-flowing and out-flowing blood is difficult. An important example is the window-chamber tumor model, where the feeding vessels and arterioles all arise from the tissue fascia side of the window (22). While many important observations regarding tumor blood flow have resulted from this unique model, it is important to realize that it is approximately two-dimensional. The total thickness of the chamber, including the normal tissue fascia (Z), is only 200 μm compared with a multi-millimeter extent in the plane of the chamber (X , Y). Additionally, the tumors are necessarily only a few mg in mass. Thus the important concept of longitudinal arteriole oxygen (nutrient) gradients developed in the window chamber model system (22) needs to be expanded conceptually for larger tumors, considering both in-flowing and out-flowing vessel functions.

A second example where our model may be difficult to apply is the tumor cord concept described by Thomlinson and Gray (16). This topology, vessels surrounding cords of tumor cells, simulates the consistent pattern typified by the Krogh cylinder model with peripheral rather than a central blood supply (62). However, each tumor cord is only a fraction of a millimeter in width, so it is not clear how this geometry can be multiplied in large tumors (grams in mice and rats to hundreds of grams in humans). The tumor equivalent for the geometry of the Krogh cylinder (a vessel down the center of the tumor cord) is even more problematic to scale, although it is known that tumor cells can migrate along and surround pre-existing vessels. Over 130 human tumors of varying histologies, stages and grades have been stained and analyzed for EF5 binding, but we have seen only a few instances of the cord structure postulated by Thomlinson and Gray (16). We recognize that most solid tumors, including those initially described by Thomlinson and Gray, are of epithelial origin, unlike the glial tumors described in the present study. Thus the relative importance of tumor histology in the significance of our blood flow model needs to be determined.

The $p\text{O}_2$ distributions seen in regions of the most severely hypoxic tumor, the recurrent glioblastoma (Fig. 3E-H) reveal a vessel pattern suggesting the type of cooperation between vessels predicted by our new model. On the lower left edge of the brightest region of EF5 binding, a group of four vessels (enclosed in box in Fig. 3E and expanded in Fig. 3G) appear to be contributing little or no oxygen, even though the two left-most vessels of the group are just contained within a region of lower binding (more oxygenated). The same can be said for the even larger vessels within the bright EF5 binding area (marked with * in Fig. 3E). In contrast, vessels at the lower left and upper right (marked with + in Fig. 3E) are clearly supplying oxygen since they inhibit EF5 binding over a substantial distance. A reasonable explanation for these observations is that the vessels adjacent to and within the bright EF5 binding region form part of the “return” blood flow and thus carry little oxygen. In the literature, the standard explanation for such a vessel/marker distribution would be that the vessels in hypoxia areas are not perfused. Although we have not measured perfusion directly in this study,

we suggest that an apparent lack of perfusion cannot easily be distinguished from a perfusion label's consumption along a long serial path of blood flow into and then out of the tumor. This possibility has broad implications for the interpretation of previously published preclinical and clinical studies on this topic. It is also very important for considerations of drug delivery.

Tumor vasculature is often described as disorganized and chaotic (63) as revealed by latex casts. These casts, primarily of murine tumor origin, generally demonstrate a highly irregular vascular mass comprising most of the tumor volume, with almost no space that could be occupied by cells (64,65). This relationship is not typical of the glial brain tumors described herein. We suggest that in large tumors, both in-flowing and out-flowing blood vessels serve the overall tumor mass. Because they are not paired as in normal tissue, it is not with equal efficiency; i.e., there could be some regions dominated by in-flowing blood and others dominated by out-flowing blood. Thus, in any given two-dimensional section, the distribution of vessels is likely to include a heterogeneous arrangement of in-flowing and out-flowing vessels or vessel networks arising from various regions within the tumor. This new paradigm requires considerable future testing and validation, but it does help to explain the large regions of moderate and severe hypoxia seen in the two glioblastomas depicted in Figs. 1 and 3. We have previously reported on several large high-grade extremity soft tissue sarcomas and found whole regions that appear to be at fairly consistent but intermediate oxygen levels, associated with multiple small vessels (66). Our blood flow model would explain these patterns if the vessels were sharing blood between more and less well-oxygenated regions of the tumor. Further understanding will require analyses to determine how spatially separate the various primary and secondary supply and return blood flows must be to create these patterns.

To summarize, our data do not support the assumption that pO_2 necessarily decreases as a function of distance from blood vessels. We propose that tumor vessels should not all be considered as functionally similar and hypothesize a new model of blood transport into and out of large tumors. Application of the computer algorithms presented in this report will allow further testing of this concept. Specifically, identification and analysis of individual vessels allows their differences as well as similarities to be studied. Neighborhood analysis and intersection of contours around vessels can be very useful in determining whether a vessel is isolated or can be considered as part of a group. Calculation of actual tissue pO_2 and gradients should assist in determining whether vessels are actually adding oxygen to the local environment. Although the tissue pO_2 will clearly be affected by vessels outside the plane and field of analysis, the oxygen levels and their gradients may allow the determination of tissue oxygen consumption even in the absence of 3D vessel information.

We plan to refine our analyses such that EF5 binding gradients can be evaluated in directed vectors away from each vessel, rather than averaging over all directions (as has been done in this report) because our images clearly show that EF5 binding distributions are not symmetrical around large vessels (Fig. 3). Finally, application of the methods described herein has led us to propose a new model of tumor blood flow, that may prove useful in improving our understanding of the microenvironment of human tumors.

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APPENDIX

Image Acquisition

For each slide containing a tissue section, a set of three co-registered images was used to perform the analysis: (1) EF5 binding *in situ* to stain for hypoxia, (2) Hoechst 33342 applied to the sections just before imaging to stain nuclei, and (3) CD31 to stain blood vessels (Fig. 1). Images and their corresponding calibration data were loaded in sequence from a directory hierarchy and an associated spreadsheet. Each image set was then processed in series. All EF5 images were calibrated to correct for camera variables, CRB and AUC (see the Methods). These calibration corrections were recorded on an associated spreadsheet. After this step, the resulting EF5 images contained biologically relevant and meaningful data points since the intensity values of the image could be converted to tissue pO_2 .

Creation of the Hoechst 33342 Tissue Mask and Background Subtraction

Tissue masking was essential to eliminate analysis of pixels from non-viable tissue regions. Use of the Hoechst 33342 dye to create a tissue mask has some key advantages and only a few limitations (described below). Hoechst 33342 brightly stains intact nuclei at wavelengths below that of the other dyes used, and thus it was not necessary to compensate for bleed-through caused by overlap in emission characteristics. It readily distinguishes between nuclei-containing viable cells from extracellular matrix and acellular regions. An important step was to “expand” the area of the nuclear stain to encompass the entire cell, i.e. the nucleus plus the cytoplasm. The challenge for the algorithm was to generate a tissue mask comprising the area around nuclei of viable cells in the face of widely varying cell densities, presence of partial nuclei and background staining due to apoptotic or necrosing cells (these tended to have relatively lower but more uniform intensities over large areas). Our procedure first reduced the background values using a top-hat filter. We used a structuring element that should be larger than any cell in the image to avoid eliminating useful data. After applying the top-hat filter to our test data set, the background values were found to be reduced and more homogeneous. This allowed a second, lower threshold value to be applied. The result from use of this threshold was not always sufficient to mask the image. Tissues where the cell nuclei were widely spaced generated very sparse maps or a mask containing many small “holes.” The algorithm examined these holes and re-thresholded against a still lower value. Using this technique, it was possible to determine which holes were likely to be on tissue and effectively rebuild the image. Occasionally, the top-hat filter reduced both the background off-tissue staining and the cytoplasmic ontissue staining. For these tissue sections, manual intervention to set the threshold was necessary.

Bleed-through of one image onto the other occurs because the emission ranges of the dyes and/or fluorescence cubes have some overlap. This problem would be insignificant if the EF5 signal (monoclonal antibody conjugated to Cy3) were always of similar intensity to the CD31 signal (secondary antibody conjugated to Cy5). However, the key difference between our methods and other approaches is that we quantify the intensity of the EF5 signal over a dynamic range of more than 100. Thus very bright EF5 binding may hide a weak CD31 signal and *vice versa*. In our analyses, we considered subtraction of a percentage of the CD31-stained image from the EF5 image and/or a percentage of the EF5 image from the CD31 image. To avoid interpreting a bright hypoxic region as a vessel, a subtraction of the EF5-Cy3 image from the CD31-Cy5 image was performed. The percentage of fluorescence subtracted was based on the relative intensity of the EF5 image with respect to the intensity of the CD31 image. Subsequently, corrections to the EF5 image were achieved by masking out the actual vessels, so that absolute EF5 signals were not changed.

To generate a definitive vessel mask, it was essential to account for the variability in the CD31 stain and to eliminate small artifacts. We began with a similar approach as outlined above for the Hoechst 33342 masking. A top-hat filter was applied to reduce the background and ensure a higher contrast between vessel and non-vessel pixels. While this method was effective for ignoring background variations that might confuse standard thresholding approaches, it did not always provide a clear mask of the vascular structures, because it often failed to capture the contiguous pixels of each vessel. The problem of selecting a threshold value was approached by applying a built-in Matlab function called “graythresh” that uses Otsu’s method (67). The threshold value was selected by minimizing intraclass variance of the vessel and non-vessel regions. Matlab also provided an effectiveness metric (EM) as output of this function. The EM approximated how successful an application of the threshold value was in producing a correct result, and we used this information to adjust the suggested threshold. The threshold yielded by Otsu was often overly discriminatory for CD31 images. A multiplication of the suggested threshold value by the EM was useful to identify the tissue vasculature more accurately. The resulting “basic” CD31 image typically identified all the vessels but sometimes failed to generate contiguous entities. To complete the mask, we applied a procedure that examined each individual vessel object to determine whether it should be attached to nearby vessel objects. Temporary lines 25 pixels in length were drawn between discontinuous CD31 positive points, beginning on the exteriors of objects (vessels). If the average intensity value of the original image along these lines was greater than half the original threshold, the line was added to the mask. Adding lines had the effect of connecting vessel objects that, based on the H&E image, should have remained contiguous. In instances where a vessel was ring shaped, the program would often connect the ring. For “C” shaped vessels, lines were added that “connected” the ends to also form rings. The program then “filled in” the rings to avoid analyzing points in the interior space of vessels for EF5 binding. Further changes, identified by visual inspection, could be added by hand, but this was seldom necessary.

After the lines were drawn and interior spaces filled in, we applied a clean-up step that erased any lines that were one pixel wide. Further, very small (<15 pixel area) objects (likely to be artifacts because they were smaller than most nuclei) were removed from the mask. At this point, the masking procedure typically had identified the great majority of vessels, although some vessel pixels and bleed-through could still be present around the edges of identified vessels.

Regions for EF5 Analysis

Once individual vessels were identified on the CD31 image, each vessel of each image was assigned an identifier for the purposes of subsequent analysis. The generation of the EF5 values surrounding each vessel proceeded by identifying the first vessel to be analyzed (the primary vessel) and all neighboring (secondary) vessels. A predetermined number of contours were drawn around the primary vessel; the number of contours and their spacing could be changed as program parameters. The intercontour bands comprised the tissue areas that were then analyzed for EF5 binding. However, portions of these bands may not have been valid for data collection because they were off tissue or there was competitive influence from one or more secondary vessels. We used the Hoechst 33342 mask to eliminate off-tissue regions. To avoid the effects of competitive influence, we sequentially “grew” the secondary vessel contours at an equal rate to the primary vessel. In areas of overlap, the bands associated from each vessel were truncated so that they were not assessed multiple times (Fig. 2).

EF5 binding values (as corrected percentage of CRB) were averaged over the pixels comprising each intercontour band of each vessel. So, for example, all pixels between the 10- and 20- μ m contours from the vessel periphery were used to define the average EF5 binding in the second band. Each vessel was thus characterized by average EF5 binding in the five bands with average

distances of 5, 15, 25, 35 and 45 μm from the periphery of the vessel. If average EF5 binding in each band was plotted as a function of these five distances, the resulting line could be characterized by a slope (or gradient) and an intercept. Based on the EF5 binding average for the first band, each vessel was grouped into one of the five categories as defined in Table 1. Since none of the vessels had $p\text{O}_2$ values corresponding to anoxia, only four categories were applicable: oxygenated, mild hypoxia, moderate hypoxia and severe hypoxia. These data and associated images were saved to disk for analysis.

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FIG. 1.

Steps in image analysis: Top row: left, uncalibrated EF5 image; right, calibrated EF5 image. Middle row: left, non-optimized CD31 image (vessels); right, CD31 mask. Bottom row: left, Hoechst 33342 image (nuclei); right, tissue mask. Data are based on a tissue section from recurrent glioblastoma, patient F. See the Methods for details of calibration and masking.

FIG. 2.

Influence of neighboring vessels. For each individual blood vessel, EF5 binding information (percentage cube reference binding, CRB) was collected as a series of 20 10-μm-wide bands between adjacent contours. The algorithm builds contours for an individual vessel along with its neighboring vessels. Contours and bands are truncated when they intersect those from neighboring vessels, and the process is repeated for each identified vessel. The percentage of vessels with at least some portion of each band unencumbered by neighboring vessels is analyzed and plotted on the Y axis as a function of distance from the blood vessel (X axis). For the HPCTA (patient A, triangles), less than 10% of vessels can be analyzed beyond 50 μm total perivascular distance. This plot is almost a vertical line because of the large number of vessels in the tissue section that are in proximity to each other. Therefore, data are not shown for the remainder of the bands. In contrast, 90% of vessels have a complete contour analysis out to 50 μm for the glioblastoma (patient D, circles) while all vessels can be analyzed for the recurrent glioblastoma at the same distance (patient F, squares).

FIG. 3.

Tissue oxygen maps and vessel contours: Examples of tissue oxygen maps (panels A, C, E; left side) and vessel contour depiction (panels B, D, F, H; right side) for three tumors. Panels A, B: HPCTA, patient A; panels C, D: glioblastoma, patient D; panels E-H: recurrent glioblastoma, patient F. In the left panels, vessels are blue and EF5 binding is color coded as physiological oxygenation (light green), mild hypoxia (dark green), moderate hypoxia (red), severe hypoxia (yellow) and anoxia (white). In the right panels, vessels are blue, the tissue mask is green, and the vessel contours, starting at the vessel perimeter, are white with shades of gray for the intervening tissue bands at increasing distance (see expansion of inset from panel F in panel H). On the lower left edge of the brightest region of EF5 binding, a group of

four vessels (encircled in box in panel E, expanded in G) appear to be contributing no oxygen to the tissue, although the two left-most vessels of this group are contained in a region of lower binding (more oxygenated). The same can be said for the even larger vessels within the bright EF5 binding area (panel E, marked with *). In contrast, vessels at the lower left and upper right (panel E, labeled with +) are clearly supplying oxygen since they inhibit EF5 binding over a substantial distance (~200 μm). A possible explanation for this observation is that the vessels adjacent to and within the bright EF5 binding region form part of the out-flowing blood flow and thus carry little oxygen. Scale bar = 250 μm .

FIG. 4.

Average gradient of EF5 binding away from vessels for one tissue section each in a *de novo* glioblastoma, patient D (upper panel), and recurrent glioblastoma, patient F (lower panel). These analyses correspond to the tumor images for each patient in Fig. 3. Each line represents a different category of perivascular pO_2 (physiologically oxygenated, squares; mild hypoxia, diamonds; moderate hypoxia, circles; severe hypoxia, triangles). The recurrent glioblastoma (patient F, lower panel) has a relatively small number of vessels ($n = 69$, also see Fig. 5, Table 4) with no oxygenated vessels and only two severely hypoxic vessels (both shown individually). Glioblastoma patient D (upper panel) had no severely hypoxic vessels (Table 2). The slope is calculated per pixel, with each pixel = 1.75 μm.

FIG. 5.

Graphic illustration of vessel gradients assessed on three slides from the recurrent glioblastoma from patient F. On each slide, oxygenated vessels are squares, mildly hypoxic vessels are diamonds, moderately hypoxic vessels are circles, and severely hypoxic vessels are triangles. Sixty-nine vessels were examined (also see Tables 2, 4). Note that the *Y* axis of this figure is on a log scale.

TABLE 1Approximate Conversion of EF5 Binding to Tissue pO_2

Descriptor	Percentage CRB ^a	pO_2 (mmHg)	Percentage oxygen ^b
Physiological oxygenation	1-3	100-20	12.5-2.5
Mild hypoxia	3-10	20-4	2.5-0.5
Moderate hypoxia	10-30	4-0.75	0.5-0.1
Severe hypoxia	30-100	0.75-0	0.1-0
Anoxia	100	0	0.0

^a Cube reference binding (see Methods).^b Percentage oxygen refers to percentage of one atmosphere of dry gas at 37°C since the gases used for *in vitro* experiments were dry (i.e., humidity correction adds to the total pressure but not to the oxygen partial pressure).

TABLE 2

Summary of Tumor Grade and Vessel Findings

Patient ID: Histology	Tumor grade	N	Vessels/mm ²	Vessel area (μm ²) ± SD	Number of vessels analyzed		
					Oxygenated	Mild hypoxia	Severe hypoxia
A: HPCTA	NA	4	244	416 ± 878	2405	0	0
B: OA	2	3	70	800 ± 1358	493	0	0
C: AO	3	4	16	610 ± 1094	177	0	0
D: GB	4	3	127	548 ± 1029	313	360	104
E: GB	4	3	24	3330 ± 6395	272	34	2
F: recurrent GB	4	3	11	3385 ± 5929	9	47	11

Notes. N = number of slides studied. The image size per slide was typically 2 × 2 fields (10× objective) of 600 × 400 pixels (2.9 mm², each pixel was 3.1 μm²). NA, not applicable. AO, anaplastic oligodendroglioma. OA, oligoastrocytoma. HPCTA, hemangiopericytoma. GB, glioblastoma.

TABLE 3
Summary of Parameters for the Lines Describing Average EF5 Binding per Band, Averaged for all Vessels with Similar Perivascular EF5 Binding

Patient ID/Histology	Band no.	Oxygenated		Mild hypoxia		Moderate hypoxia		Severe hypoxia	
		Intercept	Slope	Intercept	Slope	Intercept	Slope	Intercept	Slope
A/HPCTA	1-5	0.3	-0.9	-	-	-	-	-	-
	2-5	0.3	-0.3	-	-	-	-	-	-
B/OA	1-5	1.2	-0.7	-	-	-	-	-	-
	2-5	1.2	-0.3	-	-	-	-	-	-
C/AO	1-5	1.0	-5.8	-	-	-	-	-	-
	2-5	1.0	-16.8	-	-	-	-	-	-
D/GB	1-5	1.7	-2.4	5.9	-5.3	13.2	-43.6	-	-
	2-5	1.6	-1.0	5.8	-3.4	13.5	-51.0	-	-
E/GB	1-5	1.2	3.9	5.0	-52.1	18.0	-153.4	-	-
	2-5	1.2	9.9	5.1	-7.2	14.1	-54.2	-	-
F/r-GB	1-5	2.4	5.9	5.5	16.5	13.9	71.4	33.6	9.9
	2-5	2.3	10.3	5.4	18.8	14.3	60.9	33.1	11.3

Notes. Analyses presented using bands 1 to 5 compared to 2 to 5. Sections without vessels in the pO_2 category of interest are indicated by —. Intercepts and slopes listed as % CRB and slopes as % CRB per μm multiplied by 1000 (to make numbers more readable). AO, anaplastic oligodendroglioma. OA, oligoastrocytoma. HPCTA, hemangiopericytoma. GB, glioblastoma, r-GB, recurrent glioblastoma.

TABLE 4

Quantitative Data Supporting Fig. 5

Specific oxygen level	Tissue section left		Tissue section middle		Tissue section right	
	No. of vessels with + slope	No. of vessels with - slope	No. of vessels with + slope	No. of vessels with - slope	No. of vessels with + slope	No. of vessels with - slope
Oxygenated	0	0	0	0	5	4
Mild hypoxia	12	12	4	6	10	3
Moderate hypoxia	5	1	2	0	1	2
Severe hypoxia	1	0	0	0	0	1
Total vessels examined	18	13	6	6	16	10

TABLE 5

Range of Correction Factors Required to Calibrate EF5 Binding to Percentage Cube Reference Binding (CRB) for the Six Patients of the Present Study

No. of patients = 6	Optics	Competed stain background and mean signal	AUC	CRB
Correction factor range	2.8	Combined ~1000	1.9	7.2

Notes. The competed stain (CS) protocol is critical for determining whether low levels of apparent EF5 binding in well-oxygenated tissue represent actual signal or nonspecific binding and/or tissue autofluorescence. The optics correction refers to day-to-day variations in the lamp output, while the variations in signal correspond to measured differences in fluorescence, corrected for camera exposure time. The most consistent corrected parameter is drug AUC, but even this entails a nearly twofold variability. Part of this arises from actual interpatient variations in drug half-life, and part arises from variations in the time between drug administration and tissue biopsy.