

Comparative Measurements of Hypoxia in Human Brain Tumors Using Needle Electrodes and EF5 Binding

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ABSTRACT

Hypoxia is known to be an important prognostic marker in many human cancers. We report the use of two oxygen measurement techniques in human brain tumors and compare these data with semiquantitative histological end points. Oxygenation was measured using the Eppendorf needle electrode and/or EF5 binding in 28 brain tumors. These data were compared with necrosis, mitosis, and endothelial proliferation. In some tumors, absolute EF5 binding was converted to tissue pO₂ based on *in vitro* calibrations. Eppendorf electrode readings could not be used to identify WHO grade 1/2 versus WHO grade 3/4 tumors, they could not differentiate grade 3 versus grade 4 glial-derived neoplasms, nor did they correlate with necrosis or endothelial proliferation scores. EF5 binding increased as the tumor grade increased and was significantly associated with necrosis and endothelial proliferation. There was no statistically significant correlation between the two hypoxia detection techniques, although both methods indicated similar absolute ranges of tissue pO₂. There was substantial inter- and intratumoral heterogeneity of EF5 binding in WHO grade 4 glial neoplasms. The majority of cells in glial-derived tumor had levels of hypoxia that were mild to moderate (defined herein as 10% to 0.5% pO₂) rather than severe (defined as approximately 0.1% pO₂). Immunohistochemical detection of EF5 binding tracks histological parameters in adult brain tumors, with increased binding associated with increasing necrosis and endothelial proliferation. The proportion of moderately to severely hypoxic cells is relatively low, even in the high-grade tumors. Human brain tumors are dominated by oxic to moderately hypoxic cells.

INTRODUCTION

An estimated 13,100 deaths were attributed to primary malignant brain tumors in the United States in 2002 (1). Glial-derived neoplasms (gliomas) are the most aggressive brain tumor type and account for 44% of all primary brain tumors. The most aggressive glial neoplasm is the glioblastoma multiforme (GBM; WHO grade 4),⁵ and this tumor type accounts for more than half of the primary brain tumors seen in

the United States. Currently, the 2-year survival rate of GBM patients is <10%, irrespective of therapy (2). The cause of such treatment resistance is not well understood, but one known resistance factor, hypoxia, is present in human brain tumors (3–5). The histological features that define a GBM include the presence of pseudopalisading necrosis and proliferative blood vessels; the latter is referred to as endothelial proliferation by pathologists (6). The occurrence of hypoxia in such tumors would be consistent with these observations. The presence and level of hypoxia in primary brain tumors *without* necrosis (meningiomas and WHO grade 2 neoplasms such as astrocytomas and oligodendrogliomas) were unknown until polarographic needle electrodes were used to study a limited series of brain tumors (3–5). Despite the relatively small number of patients studied, substantial hypoxic heterogeneity between tumors of otherwise similar histology was shown. These data also demonstrated that the level of hypoxia in WHO grade 3 tumors overlapped those of WHO grade 4 tumors. This observation brought the prognostic and predictive value of hypoxia in brain tumors into question because WHO grade is a well-defined prognostic indicator in this disease. However, small studies on the use of hyperbaric oxygen (7, 8), RSR13 (9), and tirapazamine (10) have suggested that treatment for hypoxia can modify outcome of patients with malignant gliomas.

In other tumor sites, studies have demonstrated that hypoxia increases the likelihood of a poor prognosis. Local recurrence and metastasis are more common in patients with hypoxic cervical cancers after surgery alone or surgery plus radiation (11, 12). In patients with head and neck cancer, high levels of tumor hypoxia increased the likelihood of local recurrence (11, 13), and in soft tissue sarcomas, hypoxia was associated with metastasis of otherwise locally controlled tumors (14, 15). These studies emphasize the need to identify and quantify the presence of hypoxic cells in individual patients (16, 17). Therefore, we decided to study the presence and level of hypoxia in human glial tumors using another technique, 2-nitroimidazole binding (13, 18–20).

The reductive metabolism of these agents (*e.g.*, pimonidazole, EF5) leads to their activation and subsequent formation of intracellular covalent bonds with macromolecules (for review, see Ref. 21). This process is greatly inhibited as a function of increasing oxygen concentration. Intracellular adducts of EF5 can be detected using monoclonal antibodies and fluorescence immunohistochemical techniques. Extensive *in vitro* and preclinical studies have documented the quantitative nature of this detection technique and its ability to predict response to radiation therapy (21–27). We have previously described the clinical use of this agent in patients with squamous cell tumors and sarcomas (19, 20, 28, 29).

The purpose of the work reported herein is to assess the relationship between hypoxia, as measured by EF5 and the Eppendorf techniques, and known clinically relevant histological parameters in adult human brain tumors. The rationale for this work is that the histological parameters of mitosis, necrosis, and endothelial proliferation are generally accepted prognostic factors for adult brain tumor behavior. Also, these processes either depend on tissue oxygenation (prolifera-

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⁵The WHO defines a grading system of central nervous system glial tumors that categorizes them from lowest (grade 1) to most malignant (grade 4) histopathological types (6). Grade 1 neoplasms are considered benign, *i.e.*, cured by total excision. Pilocytic astrocytomas and some gangliogliomas are examples of grade 1 tumors. Astrocytomas or oligodendrogliomas with low cell density and low mitoses are categorized as grade 2. Grade 3 glial neoplasms, anaplastic astrocytomas, or anaplastic oligodendrogliomas are characterized by endothelial proliferation and mitoses/high Ki-67 "proliferation index." Grade 4 glial neoplasms are designated as glioblastoma multiforme, with pathognomonic pseudopalisading necrosis and very high-grade histopathological features. Diffusely infiltrating glial neoplasms usually show differentiation along astrocytic or oligodendrocytic cell lines, but an admixture of morphological types among neoplastic cells is not uncommon. A prior history of exposure to high-dose ionizing radiation can be a confounding factor for histopathological changes (necrosis and cellular pleomorphism), and these may remain ungraded.

tion), may cause hypoxia (endothelial proliferation), or are associated with the presence of hypoxia (necrosis). Thus, we hypothesized that data obtained from an optimal oxygen measuring technique should parallel histological findings. We have specifically studied a heterogeneous group of patients and tumor types with different cell types and histological features to specifically explore the relationship between histology and oxygenation, irrespective of the histological cell type. As such, we determined (a) the correspondence between hypoxia as measured by EF5 or Eppendorf needle electrode and histological parameters (necrosis, mitosis, and endothelial proliferation) and (b) the relationship between hypoxia as measured by EF5 and that measured by the electrode technique.

MATERIALS AND METHODS

Human Subjects

Written informed consent, approved by the University of Pennsylvania Institutional Review Board, was obtained from all patients entered on this study. Eligible patients were those undergoing therapeutic craniotomy for tumor. Patients of all ethnic and gender groups were included.

Needle Electrode Studies

The agents used for general anesthesia for the craniotomy were determined at the discretion of the attending neuroanesthesiologist, for example, the inhalational agents nitrous oxide and isoflurane and the i.v. agents fentanyl and propofol. For this study, the intent was to keep the $\text{FiO}_2 \leq 40\%$ while maintaining the oxygen saturation at 100%.

Patients were prepared for tumor oxygenation measurements by performing a routine craniotomy for *de novo* tumors and reopening the previous craniotomy flap in recurrent tumors. After the dura was opened, tumor oxygenation was measured using the pO_2 Histogram needle electrode (Eppendorf-Netheler-Hinz, GmbH, Hamburg, Germany) within the tumor, under either direct visualization, ultrasound guidance, or using intraoperative frameless stereotaxy (SMN; Carl Zeiss, Thornwood, NY). Patients with deep-seated tumors were not studied with the electrode because direct observation of the needle entry point was not possible, ultrasound could not be used, and/or frameless stereotaxy was not helpful due to the movement of structures during dissection. Two to four Eppendorf tracks were sampled with a total track length of 1–2 cm. The electrode was programmed for a forward step of 0.7 mm followed by a 0.3-mm retraction at each data acquisition point along the track.

Analyses of Eppendorf values for each patient's tumor were performed for median pO_2 and percentage of values of <2.5 , <5 , and <10 mm Hg.

EF5 Studies

Drug Administration and Toxicity. The National Cancer Institute, Division of Cancer Treatment supplied EF5 in 100-ml vials containing 3 mg/ml EF5 and 5% dextrose in water plus 2.4% alcohol. The drug was administered via a peripheral i.v. catheter at a rate of approximately 350 ml/h (total dose, 21 mg/kg). In patients where magnetic resonance imaging scans suggested pre-existing moderate or severe cerebral tumor-associated edema, 20 mg of furosemide were administered i.v. preceding EF5 infusion.

EF5 Pharmacokinetic Analyses. The methods and analysis for EF5 pharmacokinetic analysis have been reported previously (28). In this study, the plasma concentration of EF5 was shown to follow a simple exponential decay after infusion, with a half-life in adults of 11.7 ± 2.6 h (average \pm SD). We account for individual variations in the drug half-life, which affects the drug area under the curve, by making a plasma EF5 measurement 1 h after drug infusion and at the end of surgery. Each measurement is made by high-performance liquid chromatography of the clear supernatant after dilution of the plasma 1 + 1 with 10% trichloroacetic acid and high-speed centrifugation. The area under the curve is calculated by assuming an exponential decay in drug concentration with time.

Tissue Acquisition. At 24–48 h (range, 18–55 h) after completion of drug administration, the tumor was either biopsied or resected. Sterile tumor tissue was obtained, placed in iced medium (ExCell 610 medium; JRH Biosciences)

with 15% FCS, and then immediately returned to the laboratory where it was processed to determine *in situ* EF5 binding and "Cube Reference" binding.

Assessment of EF5 Binding. The methods for EF5 pharmacokinetic analysis, tissue sectioning, staining, and fluorescence photography have been reported previously (18–20). Briefly, 10- μm tissue sections were briefly fixed in 4% paraformaldehyde and then blocked and stained with EF5 monoclonal antibody (ELK3-51) conjugated to Cy3 dye. Cube Reference binding was used to determine the maximum hypoxia marker binding in tumor from each patient (18). In this procedure, tissue cubes (mass of approximately 8 mg) were incubated *in vitro* in the related hypoxia marker EF3 under low (0.2%) oxygen concentration and frozen. Subsequently, sections were cut from these cubes and stained using an EF3-specific monoclonal antibody (ELK5-A8). A different marker and antibody are used so that *in situ* binding is not confused with reference binding (21). Fluorescence microscopy (Nikon LabPhot microscope with a 100-W high-pressure mercury arc lamp and Photometrics Quantix charge-coupled device digital camera) was carried out as described previously (18, 19) using a computer-controlled automatic stage (Ludl Electronic Products) and IP Lab Spectrum software (Scanalytics, Fairfax, VA). Immediately before photography, slides were treated with a solution of Hoechst 33342 (20 μM in PBS) to label tissue nuclei in the photographed images. Photographs were taken using filter cubes appropriate for Cy3 dye (EF5 or EF3) and Hoechst 33342 (nuclei). At the beginning and conclusion of each camera session, an image of hemocytometer-loaded calibration dye was taken for calibration of images (Cy3 dye in PBS with 1% paraformaldehyde; absorbance of 1.25 at 549 nm). Any variations in lamp intensity (a factor of up to 4 over the lifetime of the bulb) cause similar changes in the fluorescent light from the standard, allowing correction of all images to provide an absolute fluorescence scale (21). In photographed images of the cubes, the median EF3-dependent fluorescence intensity in areas of highest binding was used to represent the maximum binding for an *in vitro* drug exposure (area under the curve) of 600 $\mu\text{M} \times \text{h}$, as discussed above. With this information, and knowing the patient's drug area under the curve, *in situ* binding can be converted to an absolute percentage of Cube Reference binding.

Image Analysis and Quantification of *in Situ* Binding

Our prior studies have reported a relatively simple measure of EF5 binding, namely, the binding level of the most hypoxic region(s) of the brightest of a minimum of 4 slides/patient. Our present image analysis allows a more thorough documentation, which is a pixel-by-pixel analysis of the percentage of Cube Reference binding. This can be displayed as two-dimensional maps of EF5 binding or cumulative frequency analysis of total pixels. Using extensive data from *in vitro* studies (21), it is possible in principle to convert absolute EF5 binding into tissue pO_2 . We have denoted a 4-range map based on the EF5 binding values (Table 1).

For the purpose of this analysis, binary bitmap masks of tumor tissue were created after staining cell nuclei with Hoechst 33342 and imaging the sections. The resulting images were converted to a mask by a series of functions and filters in Adobe Photoshop. The paintbrush tool was used to remove artifacts such as folding and regions of necrosis (identified by the neuropathologist based on corresponding H&E-stained images). For each patient, analyses were performed on the slide with the highest EF5 fluorescence as stained with ELK3-51, based on our hypothesis that the most hypoxic cells in the tumor would define the biology.

Images were analyzed using routines written in MatLab (The MathWorks, Inc., Natick, MA), whereby EF5-dependent fluorescence intensity values were sampled within an image based on tissue identified by the Hoechst 33342 mask (29). As indicated above, the final EF5 intensity values were corrected for lamp intensity, Cube Reference binding, and patient EF5 drug exposure. The latter value was calculated from the area under the EF5 blood concentration curve for individual patients (28). Fluorescence due to nonspecific staining, as

Table 1 Approximate conversion of EF5 binding to tissue pO_2

Descriptor	pO_2 (%)	% Cube Reference binding	pO_2 (mm Hg)
Physiological conditions	10%	1%	75
Modest hypoxia	2.5%	3%	19
Moderate hypoxia	0.5%	10%	4
Severe hypoxia	0.1%	30%	0.75

Table 2 Parameters for semiquantitative pathological analysis

Histologic end point	Semiquantitative path score			
	0	1	2	3
Mitosis	0/HPF ^a	<1/HPF	<2/HPF	>2/HPF
Endothelial proliferation	0/HPF	<1/HPF	<5/HPF	>5/HPF
Necrosis	0/HPF	<1/HPF	<10/HPF	>10/HPF

^a HPF, high-power field (×400).

measured on a tissue section stained with Competed EF5 (antibody mixed with 0.5 mM authentic EF5), was subtracted from that due to specific binding (18, 19, 29). The result of these corrections provides an EF5 binding value for each pixel, expressed as a percentage of Cube Reference binding. To further characterize the image, cumulative frequency histograms were then calculated (29). The short form used to denote a specific value of interest is the percentage of maximum EF5 binding for a given cumulative frequency (CF): $CF_{(%) } \leq EF5_{(\% max)}$. Thus, $CF_{95} = EF5_{20}$ would mean that 95% of the EF5 values in the image were at or below 20% of maximum binding. The median EF5 binding would be represented by CF_{50} using this notation.

Histopathological Evaluation

A board-certified neuropathologist (A. R. J. or P. T. N.) reviewed the histopathology of all tumor specimens. The histopathological diagnosis was confirmed, and semiquantitative analyses (a 0–3 scale where 0 = none and 3 = extensive) of necrosis, endothelial proliferation, and mitotic index were recorded, per high-power field (×400). The approximate values used for these ordinal scales are shown in Table 2.

Statistics

Comparison of values between patient groups was assessed by *t* tests, and *P* ≤ 0.05 was considered significant.

RESULTS

Between March 1998 and December 2002, 31 patients signed University of Pennsylvania Institutional Review Board-approved informed consent allowing Eppendorf electrode measurements of their brain tumors with or without EF5 administration. All patients had radiographic evidence of a brain mass that was assessed to most likely represent a primary neoplasm.

Three patients (not receiving EF5) were unable to have electrode measurements because a FiO_2 of ≤40% could not be maintained safely. The needle electrode was found to be damaged during the postmeasurement calibrations in a fourth patient, and intraoperative bleeding prevented Eppendorf measurements in a fifth patient. Two of these patients had also received EF5, so only the values from this assay were available. 2-Nitroimidazole binding studies with EF5 were successfully performed in all 16 of the patients who consented to this study.

Six patients had recurrent tumors; three of these were anaplastic astrocytomas (AAs; WHO grade 3), and three were GBM (WHO grade 4). Of the 22 patients with newly diagnosed tumors, 1 had a meningioma, 1 had a hemangiopericytoma, 1 had an astrocytoma (WHO grade 2), 4 had AA (WHO grade 3), 1 had an anaplastic oligodendroglioma (WHO grade 3), 1 had a high-grade astrocytic tumor in a previously irradiated site, and 13 had GBM (WHO grade 4; Table 3). The oxygen levels using either Eppendorf or EF5 binding in the recurrent patients were not distinguishable from that of the *de novo* patients, so these groups were combined for further analyses (data not shown).

Eppendorf studies were performed in 26 patients [1 meningioma, 1 hemangiopericytoma, 1 malignant glioma, 8 WHO grade 3 (AA or anaplastic oligodendroglioma), and 15 WHO grade 4 (GBM) neoplasms]. Neither the percentage of values < 2.5, 5, or 10 mm Hg nor

median Eppendorf readings were useful in differentiating low-grade (WHO grade 1 and 2) from high-grade tumors (WHO grade 3 and 4; Fig. 1) or in distinguishing WHO grade 3 from WHO grade 4 tumors.

Necrosis and endothelial cell proliferation are negative prognostic factors for glial-derived neoplasms (6) and have been subjectively associated with the presence of hypoxia. In principle, these are independent parameters, but most tumors studied herein had similar scores for both indicators. However, there was some crossover between WHO grade 3 and 4 tumors with respect to these combined parameters. Thus, we evaluated the relationship between these assays, as determined by the pathologist, and Eppendorf readings. The pathological scales were grouped into two categories (0 and 1) versus (2 and 3) to provide roughly even numbers of patients/group.

In three of the GBM tumors, the Eppendorf values did not report the presence of hypoxia despite the presence of substantial necrosis (+3

Table 3 Patient and tumor characteristics

Variable	N (%)
Age (yrs)	N = 28
Mean ± SD	47.3 ± 16.9
Median	44
Tumor histology	N = 28
Hemangiopericytoma	1 (3.6%)
Meningioma	1 (3.6%)
Astrocytoma (WHO grade 2)	1 (3.6%)
Anaplastic astrocytoma (WHO grade 3)	7 (25%)
Anaplastic oligodendroglioma (WHO grade 3)	1 (3.6%)
Glioblastoma multiforme (grade 4)	16 (57.1%)
Malignant astrocytoma	1 (3.6%)
Tumor type	N = 28
Recurrent	6 (21.4%)
<i>De novo</i>	21 (75%)
New tumor in previously irradiated site	1 (3.6%)
EF5 data	N = 16
CF_{50} ; average ± SD	4.67 ± 5.28
CF_{95} ; average ± SD	40.16 ± 39.26
Eppendorf measurement	N = 26
Median; average ± SD	22.24 ± 22.03
% <2.5 mm Hg; average ± SD	15.76 ± 23.8
% <5; mm Hg; average ± SD	24.92 ± 27.17
% <10; mm Hg; average ± SD	39.88 ± 31.0

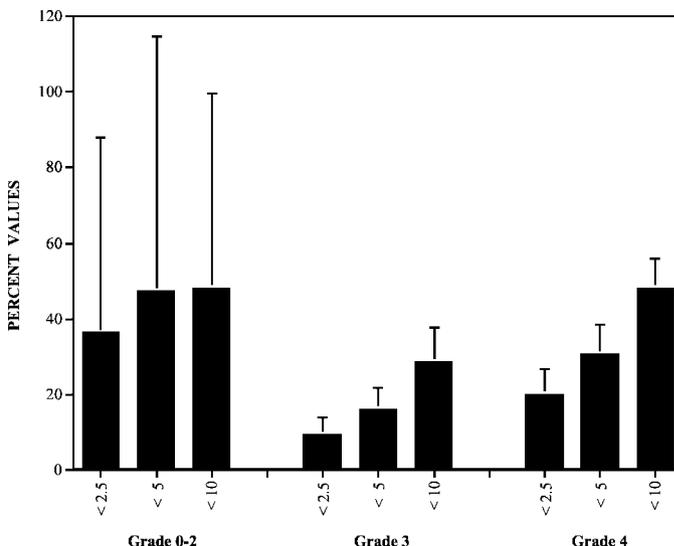


Fig. 1. Eppendorf electrode readings in human brain tumors. Three end points (percentage of values < 2.5, 5, and 10 mm Hg) are shown. No statistically significant difference between the level of hypoxia for benign (called grade 0 for consistency; *n* = 2) or WHO grade 1–2 (*n* = 1), WHO grade 3 (anaplastic astrocytoma/anaplastic oligodendroglioma; *n* = 8), and WHO grade 4 (glioblastoma multiforme; *n* = 11) tumors was seen. SE limits are illustrated for each category. The large SE for the lower-grade tumors and benign tumors was dominated by an apparently severely hypoxic hemangiopericytoma (see text for discussion).

semiquantitation of necrosis). In addition, there were two to four tumors (depending on the Eppendorf end point considered) in which significant degrees of hypoxia were observed, but no histological necrosis was reported (0 semiquantitation of necrosis; AA tumors). The Eppendorf readings in the hemangiopericytoma (0 necrosis) showed a very low median pO_2 and a high proportion of values < 2.5 mm Hg. These readings may have been caused by bleeding during the surgery; the tumor had invaded the cranium, and in the process of removing the bone, substantial disruption of the vasculature occurred. Because of these anomalies, there was no relationship between any of the Eppendorf measures and necrosis score ($P > 0.25$). The data for percentage of values < 2.5 mm Hg was most discriminatory and is presented in Fig. 2 (*left pair*). If the apparently anomalous values for the hemangiopericytoma were eliminated, there was a borderline significance between these specific Eppendorf readings and necrosis score (Fig. 2, *middle pair*; $P = \sim 0.07$). However, this did not improve the relationships between necrosis score and any of the other Eppendorf measures (median pO_2 , percentage of values < 5 or 10 mm Hg).

As indicated above, the tumors with high scores for endothelial proliferation generally also had high scores for necrosis, and *vice versa*. However, in three GBM tumors, the score for endothelial proliferation was much lower than that for necrosis. Nevertheless, there was no significant correlation of endothelial proliferation with any Eppendorf measurement.

Using EF5 binding as the analysis method, benign (meningioma and hemangiopericytoma) and GBM tumors could be easily segregated. However, there were not enough patients with WHO grade 3 tumors (AA or anaplastic oligodendroglioma) to investigate whether EF5 binding could distinguish this group of patients from that with WHO grade 4 tumors (GBM). EF5 binding was extremely low in the meningioma, hemangiopericytoma, and the WHO grade 2 astrocytoma, as compared with the GBMs and AAs. (Fig. 2; *right pair*; $P \ll 0.01$). The hemangiopericytoma, which appeared to be severely hypoxic by Eppendorf measure, showed only background levels of EF5 binding, suggesting that the tumor was completely oxia. This was supported by the extensive, evenly distributed pattern of blood vessels, which is a characteristic of this tumor type (Fig. 3). Conversely, for the three GBM tumors, where high pO_2 values were recorded by the Eppendorf (all of which had +3 necrosis), EF5 binding supported

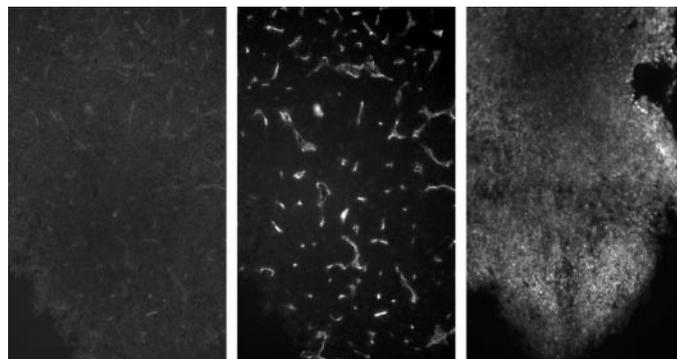


Fig. 3. The hemangiopericytoma, whose Eppendorf values were consistent with severe hypoxia, was characterized by very low EF5 binding, consistent with physiological oxia. The *middle panel* illustrates the prominent, evenly spaced blood vessels (stained by anti-CD31 antibodies). The *left panel* shows EF5 binding, barely distinguishable from the background (*bottom left corner*). The *right panel* illustrates Cube Reference binding for this tissue, documenting its ability to bind 2-nitroimidazoles under hypoxic conditions. The obvious difference in brightness between the *left* and *right panels* is actually much greater than it appears because the camera shutter's exposure time for the *right panel* (cube) was 30-fold shorter than that for the *in situ* binding (*left panel*). All images represent a tissue section size of 0.7×1.05 mm.

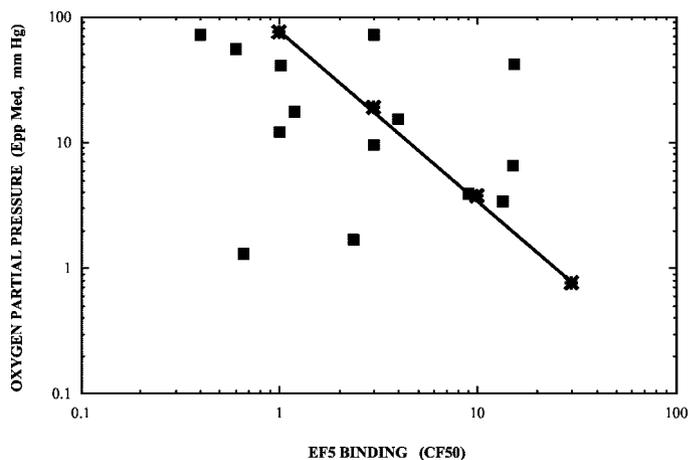


Fig. 4. Comparison between EF5 binding values (CF_{50}) and Eppendorf values (median) in 14 tumors where both values were obtained (■). Although there was a trend for the two values to agree, the correlation coefficients are poor ($r = 0.26$; $P > 0.1$). Shown on the same figure (*asterisks*) is the relationship between CF_{50} values and tissue pO_2 , as explained in Table 1. Experimental points (■) above this line indicate tumors where the Eppendorf readings are higher than predicted by average EF5 binding, and *vice versa*.

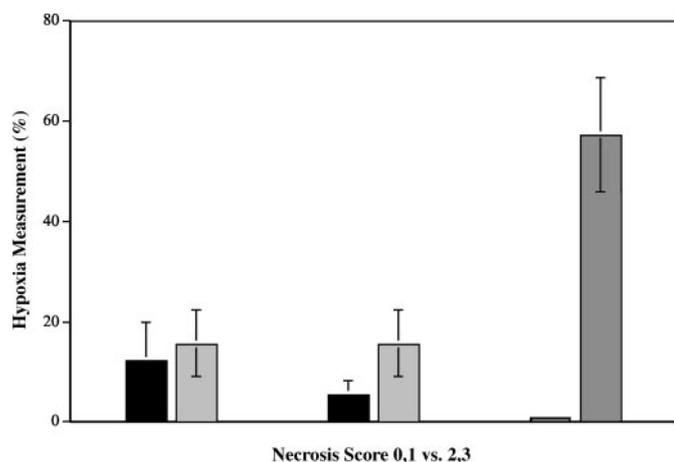


Fig. 2. Comparison between the presence of necrosis and two hypoxia detection methods, Eppendorf needle electrode measurements and EF5 binding. For each pair of values, the *black bar* represents necrosis scores of 0 and 1, whereas the *gray bars* represent necrosis scores of 2 and 3. The *left pair* (percentage of Eppendorf values ≤ 2.5 mm Hg) compares all tumors (not significantly different, $P > 0.2$), whereas the *middle pair* (percentage of Eppendorf values ≤ 2.5 mm Hg) compares the same group of tumors, with the exception that the hemangiopericytoma was eliminated. The *right pair* illustrates EF5 binding (CF_{95} values) for the two necrosis categories ($P \ll 0.01$).

substantial hypoxia, with CF_{95} values of 18%, 28%, and 106%. Although the last of these values (106%) seems anomalous, there are several corrections to the raw fluorescence value (described in "Materials and Methods"), each with associated errors.

The relationship between hypoxia and mitosis was random using either hypoxia-measuring system (data not shown).

To assess whether the EF5 and Eppendorf readings were giving similar pO_2 readings in a given tumor, we compared their median values in the 14 tumors having both assays [median Eppendorf values (in mm Hg) against median EF5 binding (CF_{50}); Fig. 4, *squares*]. Because EF5 binding is inversely dependent on oxygen, and Eppendorf readings are directly dependent on oxygen, these are the only measures that can be directly compared. For individual patients, median Eppendorf values and CF_{50} are not significantly correlated with each other ($r = 0.26$; $P > 0.2$). To apply an oxygen scale to the CF_{50} measurements, the values from Table 1 are also plotted on the same figure (*asterisks*). Interestingly, this analysis shows that the two measures (Eppendorf mean and oxygen tension corresponding to the CF_{50}) comprise a similar biological range in terms of absolute tissue

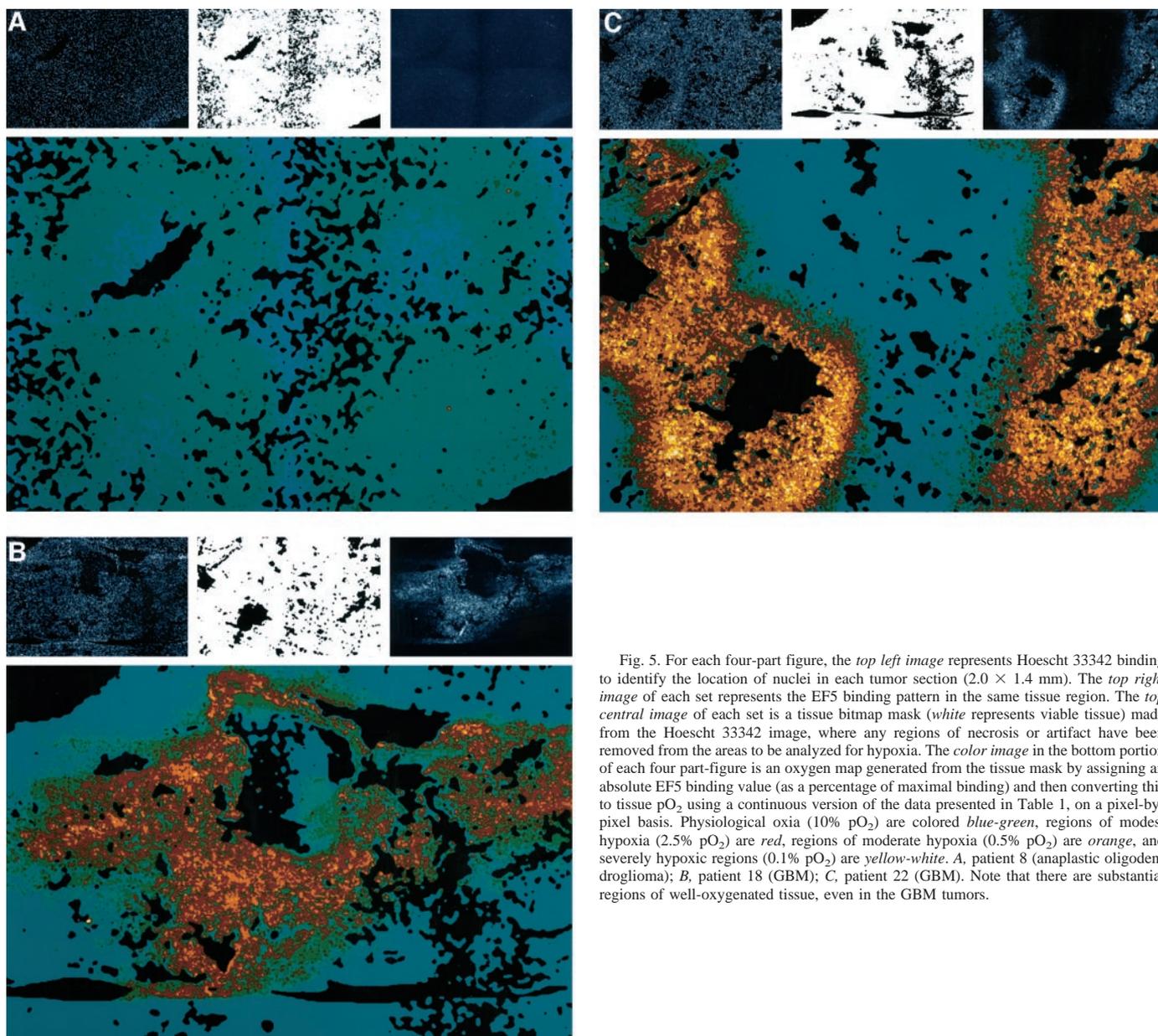


Fig. 5. For each four-part figure, the *top left image* represents Hoescht 33342 binding to identify the location of nuclei in each tumor section (2.0×1.4 mm). The *top right image* of each set represents the EF5 binding pattern in the same tissue region. The *top central image* of each set is a tissue bitmap mask (*white* represents viable tissue) made from the Hoescht 33342 image, where any regions of necrosis or artifact have been removed from the areas to be analyzed for hypoxia. The *color image* in the bottom portion of each four part-figure is an oxygen map generated from the tissue mask by assigning an absolute EF5 binding value (as a percentage of maximal binding) and then converting this to tissue pO_2 using a continuous version of the data presented in Table 1, on a pixel-by-pixel basis. Physiological oxia (10% pO_2) are colored *blue-green*, regions of modest hypoxia (2.5% pO_2) are *red*, regions of moderate hypoxia (0.5% pO_2) are *orange*, and severely hypoxic regions (0.1% pO_2) are *yellow-white*. A, patient 8 (anaplastic oligodendroglioma); B, patient 18 (GBM); C, patient 22 (GBM). Note that there are substantial regions of well-oxygenated tissue, even in the GBM tumors.

pO_2 . Although there is not a third “gold standard” to compare with these estimates, this similar range does provide a measure of validation for the conversions of EF5 to tissue pO_2 and further suggests that the Eppendorf values are not biased in this group of tumors (for example, by necrosis; Ref. 30).

We also compared the two more “severe” assays of hypoxia ($CF_{0.5}$ versus percentage of Eppendorf values < 2.5 mm Hg). Again, there was no correlation between these measures, and there was no improvement by using the other Eppendorf end points (percentage values ≤ 5 or 10 mm Hg). We are continuing to investigate these relationships and will also be testing each of the hypoxia measures as predictors of patient outcome.

Detailed examination of tissue sections from these tumors demonstrated that in tumors where there was no necrosis, EF5 binding outside of the physiological to modest hypoxia range was not seen (Table 1; Fig. 5A). Conversely, strong EF5 binding was always present in tumors that contained necrotic regions. This binding was often in the areas of the pseudopalisading cells (Table 1; Fig. 5, B and C). However, the absolute pO_2 levels in these regions varied between

patients. In Fig. 5B, modest to moderate hypoxia is seen adjacent to regions of necrosis, whereas in Fig. 5C, moderate to severe hypoxia is shown. Both of these patients had histological diagnoses of GBM.

DISCUSSION

Primary central nervous system tumors are among the most lethal and difficult to treat cancers. Depending on factors such as patient age, Karnofsky performance status, tumor histology, extent of surgical resection, and radiation therapy dose, median survival ranges from 12 to 72 months for patients with WHO grade 3 tumors versus 6–18 months for patients with WHO grade 4 tumors (31). Because of these short overall survival times, any measure to differentiate patients who will respond relatively well could be important. There is evidence that hypoxia is present in brain tumors, and, based on studies in other tumor types, it is reasonable that it plays a role in the heterogeneity of treatment response observed in these patients. However, in agreement with previous studies, our data show that there is substantial overlap of the electrode measurements in glial tumors of varying

grade (Refs. 3–5; Fig. 1). The reasons for this lack of specificity may include sampling error, technical problems with the electrode (*e.g.*, the blood-brain barrier could cause problems with the function of the reference electrode), modification of blood flow during surgical access, or the possibility that the Eppendorf might overpredict the presence of hypoxia by interpreting necrosis as hypoxia (30). Using necrosis alone as an indicator of the presence of hypoxia in the data set presented, at least one-fifth of the present tumors were “miscategorized” by the Eppendorf electrode. Two of these tumors were described by the Eppendorf as having hypoxic regions, despite having no necrosis on pathological examination. In the case of the hemangiopericytoma, this was almost certainly related to interruption of local blood flow during surgical access because the tumor had negligible EF5 binding and was replete with blood vessels (Fig. 3).

Conversely, the Eppendorf electrode described five GBM tumors as oxic (median $pO_2 > 15$ mm Hg), despite the presence of substantial necrosis on histopathology. In each of these tumors, the Eppendorf study was performed using imaging guidance to ensure that the tissue sampled was tumor not normal brain. In this situation, it is possible that tumor heterogeneity prevented a representative characterization by the Eppendorf electrode. Alternately, necrosis could be a poor indicator of hypoxia. However, all GBM tumors had high EF5 binding adjacent to necrotic regions. Other explanations include the possibility that surgical intervention (removing the cranium and incising the dura), preoperative use of diuretic agents, or anesthetic agent and/or inhaled gases with greater than aerobic oxygen content prevented the determination of a pO_2 profile that properly represented the preoperative tumor state. Similar considerations apply to the lack of association between the parameters of Eppendorf oxygen measurement and endothelial proliferation.

Unlike the Eppendorf measurements, which necessarily reflect the tumor microenvironment at the time of surgery (and sometimes modified by the surgical procedures), EF5 binding represents the cumulative presence and levels of hypoxia over the time period between drug injection and tumor removal, usually 24 h. Binding at a given oxygen concentration is dependent on drug concentration. Because the half-life of EF5 in humans is 11.7 h (28), the EF5 binding rate is maximal shortly after drug injection and decreases continuously thereafter. Thus, any physiological changes to the tumor during surgery would minimally affect the binding patterns or levels. In the present study, the highest EF5 binding was consistently seen in GBM tumors, which are defined by necrosis and high endothelial cell proliferation.

We have converted absolute EF5 binding levels to absolute tissue pO_2 using calculations and data arising from experiments *in vitro*. Although this process is difficult to validate in a completely independent fashion, several aspects of the present studies suggest that this process may be feasible. For example, there was no indication of binding above background in the heavily vascularized hemangiopericytoma, even though the tissue was capable of high binding under hypoxic conditions (Cube Reference binding; Fig. 3). Similarly, we have seen only background levels of EF5 binding in normal tissues that are removed along with the surgical specimens. This has proven to be the case in all tumor types studied to date (data not shown). Conversely, the viable tumor regions adjacent to necrosis in GBM tumors were characterized by the highest EF5 binding levels. Furthermore, we have previously shown (32–34), using similar methods, that in normal tissue studies with induced interruptions of blood flow, cellular apoptosis has only been found in regions corresponding to maximal EF5 binding. If this conversion of EF5 binding to tissue pO_2 is correct, some interesting implications arise. Oxic regions are found in all tumors, and the maximum level of hypoxia varied between tumors. The most important observation, however, is that the vast majority of brain tumor cells exist at oxygen levels that range from

oxic to modestly hypoxic. This result is unexpected because most previous studies have emphasized severe hypoxia as the cause of treatment resistance. Recently, some authors have pointed out the importance of moderate hypoxia (22, 35), especially combined with other causes of tumor treatment resistance such as nonprotein thiols (36). We have previously described a cervical tumor in which only moderate levels of hypoxia were present adjacent to regions of necrosis/keratinization (18). Herein, we further demonstrate that, in the small number of patients studied, the highest EF5 binding was always present adjacent to regions of necrosis. However, the level of binding in these regions varies (*e.g.*, the degree of hypoxia adjacent to the necrosis).

Necrosis, endothelial proliferation, and high EF5 binding as a surrogate of hypoxia all tended to be conspicuous in biologically aggressive brain tumors. A causal relationship is possible because in brain tumor cells, hypoxia-responsive genes involved in angiogenesis are transcriptionally activated (37). Pietsch *et al.* (38) suggest that the frequent finding of vascular endothelial growth factor protein in GBMs may be one of the causes of neovascularization. We and others have shown that vascular endothelial growth factor is up-regulated in hypoxic tumors, including brain tumors (39, 40). Another potential causal association between hypoxia and endothelial proliferation is the observation that GLUT3 mRNA is up-regulated in proliferative tumor vessels (41). Studies in various model systems, including tumors (42), confirm the importance of hypoxia in modulating GLUT transporters, including GLUT1 and GLUT3.

We wanted to determine whether either hypoxia measurement technique tended to have specific biases in “reading” tissue oxygenation. The difficulty is that for brain tumors, there is no gold standard for hypoxia measurement against which to compare. When we compared the Eppendorf and EF5 techniques we found that although both methods provided similar average tissue pO_2 values, the correlation between values in individual tumors was poor. This observation is under current investigation. There are now quite a few studies that have used 2-nitroimidazoles to assess hypoxia in human tumors, several of which have been compared with alternative oxygen measurement techniques (18–20, 30, 43–45). However, this is the first report of 2-nitroimidazole binding in human brain tumors, and, as a link to previous studies, the corresponding Eppendorf electrode values are presented. Our data support previous observations that the electrode readings are not useful in separating WHO grade 3 from WHO grade 4 glial-derived neoplasms. We will be following all patients in whom oxygen measurements have been made to determine possible relationships with patient outcome.

To our knowledge, the present study is the first attempt to use 2-nitroimidazole binding assays to generate tissue “oxygen maps.” An unexpected outcome of this analysis is our finding that brain tumors are more commonly characterized by regions of moderate rather than severe hypoxia. This observation has substantial ramifications for tumor biology and treatment.

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