

**Featured Article****Hypoxia Is Important in the Biology and Aggression of Human Glial Brain Tumors**

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**ABSTRACT**

We investigated whether increasing levels of tissue hypoxia, measured by the binding of EF5 [2-(2-nitro-1-*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide] or by Eppendorf needle electrodes, were associated with tumor aggressiveness in patients with previously untreated glial brain tumors. We hypothesized that more extensive and severe hypoxia would be present in tumor cells from patients bearing more clinically aggressive tumors. Hypoxia was measured with the 2-nitroimidazole imaging agent EF5 in 18 patients with supratentorial glial neoplasms. In 12 patients, needle electrode measurements were made intraoperatively. Time to recurrence was used as an indicator of tumor aggression and was analyzed as a function of EF5 binding, electrode values and recursive partitioning analysis (RPA) classification. On the basis of EF5 binding, WHO grade 2 tumors were characterized by modest cellular hypoxia ( $pO_{2s} \approx 10\%$ ) and grade 3 tumors by modest-to-moderate hypoxia ( $pO_{2s} \approx 10\% - 2.5\%$ ). Severe hypoxia ( $\approx 0.1\%$  oxygen) was present in 5 of 12 grade 4 tumors. A correlation between more rapid tumor recurrence and hypoxia was demonstrated with EF5 binding, but this relationship was not predicted by Eppendorf measurements.

Received 6/2/04; revised 8/13/04; accepted 9/15/04.

**Grant support:** Primary support was from NIH RO1 CA 75285 and NIH R21 CA 93007 (S. Evans). Partial support for patient care was from the Public Health Services Research Grant M01-RR00040.

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**INTRODUCTION**

Primary central nervous system tumors are among the most lethal and treatment-resistant cancers (1, 2). The American Cancer Society projected that there would be 17,000 new cases of primary malignant brain cancer and 13,100 brain tumor-related deaths in 2002 (3). The average estimated 5-year survival rates for primary brain tumors is 28% overall, but the range is large and is dominated by patients with low-grade tumors. The 5-year survival rate exceeds 85% for low-grade tumors but is less than 5% for patients with high-grade neoplasms such as glioblastoma multiforme (GBM). Despite very active investigation of new therapeutic approaches, only marginal improvements in outcome beyond that documented from surgery and radiation therapy have been reported. One explanation for the lack of progress in improving outcome is that the root cause(s) of treatment resistance remain unknown. Malignant progression of brain tumors is known to be characterized by molecular changes (4) and cytokine release (5), both of which are known to be modulated by hypoxia (6–13). Although the presence of low tissue oxygenation, based on electrode values, has been reported in human brain tumors (14–17), its role in the biological and clinical behavior of these neoplasms has been questioned. One reason for this is that Eppendorf electrode studies have not been able to demonstrate a significant difference in the level of hypoxia between low- and high-grade tumors (14–17).

We have reported on the clinical use of the 2-nitroimidazole agent EF5 [2-(2-nitro-1-*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide] as an *in vivo* hypoxia detector (17–23). EF5 is administered intravenously 24 to 48 hours preceding tumor biopsy (21, 23). Tissues obtained at biopsy are either frozen for immunohistochemical analyses or dissociated for flow cytometric analysis of binding (21, 24, 25). The quantitative methodology involved in the analysis of EF5 binding in human tissue allows the translation of this binding into tissue oxygen levels, and oxygen maps have been generated (17, 18, 26, 27). In a recent publication examining human brain tumor specimens, we showed that the level of EF5 binding corresponds to a semiquantitative measure of the extent of necrosis, as reviewed by a pathologist. This article also reports that the majority of tumor cells, even in GBM tumors, were modestly, not severely, hypoxic (17).

The goal of the studies presented herein was to determine whether hypoxia, as measured by EF5 binding or Eppendorf needle electrodes, was associated with tumor aggressiveness in primary human glial brain tumors. If hypoxia is important in this disease, it should be present at increasing levels as the clinical aggression of this disease increases. We tested this hypothesis because it is important to understand the mechanisms by which hypoxia affects tumor biology/physiology as well as to determine whether hypoxia could be a target for treatment interven-

tion. The latter is relevant if we want to use the presence of hypoxia to prescribe individual therapies. We considered and tested two possibilities: first, that tumors with regions of severe hypoxia (<0.1% oxygen; >30% EF5 binding) are more clinically aggressive. This was based on our previously published idea that the biology and clinical behavior of tumors would be defined by the most hypoxic cells (21). We also considered whether higher average levels of hypoxia, as measured by EF5 binding, were associated with increased biological aggressiveness. Our data confirm that more hypoxia, as measured by higher EF5 values (average or maximum) are associated with more biologically aggressive glial tumors, reflected in decreased time to tumor recurrence.

## MATERIALS AND METHODS

### Human Subjects

Written informed consent, approved by the University of Pennsylvania institutional review board, Clinical Trials and Scientific Monitoring Committee of the University of Pennsylvania Cancer Center, Cancer Therapeutics Evaluation Program and the National Cancer Institute was obtained from all of the patients entered on this study. Eligible patients were those undergoing therapeutic craniotomy for supratentorial primary malignant disease based on imaging criteria. Patients of all ethnic and gender groups were included.

### Needle Electrode Studies

These studies were done as reported previously (17). Briefly, patients were prepared for surgery by performing a routine craniotomy. After the dura was opened, tumor oxygenation was measured with the pO<sub>2</sub> histograph needle electrode (Eppendorf-Netherler-Hinz, GmbH, Hamburg, Germany), under direct visualization, ultrasound guidance, or intraoperative frameless stereotaxy (SMN, Carl Zeiss, Thornwood, NY, or Stealth, Medtronic, Minneapolis, MN.). Inhaled oxygen concentration at the time of electrode insertion was  $\leq 40\%$ . Two to four needle tracks were sampled with a total track length of 1 to 4 cm. The Eppendorf values for each patient's tumor were tabulated as median pO<sub>2</sub> and percentage of values <2.5 mm Hg, <5 mm Hg, and <10 mm Hg.

### EF5 Studies

The National Cancer Institute, Division of Cancer Treatment, Bethesda, MD, supplied EF5 in 100-mL vials containing 3 mg/mL EF5 and 5% dextrose in water with 2.4% alcohol. The drug solution was administered intravenously via a peripheral catheter at a rate of  $\sim 350$  mL/h to a total dose of 21 mg/kg. In patients in whom magnetic resonance imaging scans suggested moderate or severe tumor-associated cerebral edema, 20 mg of furosemide was administered intravenously preceding the EF5 infusion.

**Tissue Acquisition.** Approximately 24 hours (range, 18–30 hours) after completion of drug administration, the tumor was resected. Sterile tumor tissue was placed in iced EXCELL 610 medium (JRH Biosciences, Lenexa, KS) with 15% fetal calf serum. These tissues were immediately processed to determine “*in situ* EF5 binding” and “cube reference binding” (17, 19, 21, 22).

**Assessment of EF5 Binding** EF5 pharmacokinetic analysis, tissue sectioning, and staining and fluorescence photography of tumor sections for analysis of *in situ* binding have been reported previously (17, 19, 21, 23). *In vitro* incubation of tumor tissue with EF3, an analog of EF5, under low oxygen conditions was used to determine the cube reference binding in tumor tissue from each patient, as described previously (17, 19, 21).

Quantitative fluorescence microscopy was carried out as described previously (19, 21). Briefly, at the beginning and conclusion of each camera session, an image of a hemocytometer-loaded calibration dye in PBS with 1% paraformaldehyde was taken (Cy3 dye;  $A_{549\text{ nm}}$ , 1.25). The ratio of the exposure time of the camera to the mean fluorescence intensity of this image was used to correct the fluorescence intensities of the experimental images (based on their exposure time) for day-to-day variations in the lamp intensity (22). In photographed images of the tissue sections obtained from the tissue cubes, the median EF3-dependent fluorescence intensity in areas of highest binding was identified, and this value was used to represent the maximum binding in that tissue (17, 19, 21). This value is referred to as cube reference binding.

### Image Analysis and Quantification of *In situ* Binding

Each tumor section was counterstained with Hoechst 33342 to stain nuclei, and bitmap (black/white) masks were created. Images were analyzed with 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. The final EF5 absolute intensity values were calculated after corrections for lamp intensity, tissue section thickness, camera exposure time (17, 19–21), and EF5 drug exposure (23). The final reported value, “percentage cube reference binding,” was calculated by dividing the corrected *in situ* EF5 binding intensity by the cube reference binding value, multiplied by 100.

At least four sections from each tumor, separated by at least 0.5 mm, were examined (28). The use of a calibrated fluorescence scale and a measure of the maximum possible binding of each tumor (cube reference binding) allowed a pixel-by-pixel analysis of the observed EF5 binding as a percentage of cube reference binding. Thus, the endpoints for EF5 binding presented herein reflect both the level and the area of binding (18, 26, 29). Data were summarized by providing a cumulative frequency (*CF*) analysis of all pixels. Selected points in the cumulative frequency curve were denoted by  $CF_{\#\#}$ . Thus,  $CF_{95} = 20$  would mean that 95% of the EF5 values in the image were at or below 20% of cube reference binding. Median EF5 binding would be denoted as  $CF_{50}$  (18).

To interpret the percentage cube reference binding as a hypoxia surrogate, we have previously performed *in vitro* studies to determine the relationship between EF5 binding and pO<sub>2</sub> (22, 25). We have converted EF5 binding to tissue pO<sub>2</sub> and defined a five-tier system to describe these levels: oxic, modestly hypoxic, moderately hypoxic, severely hypoxic, and anoxic (Table 1). Using these relationships, we have created two-dimensional tissue oxygen maps in a subset of patient tumors (17, 18). We have previously shown that the range of pO<sub>2</sub> values measured in brain tumors with this technique is similar to that of those measured by the Eppendorf electrode

Table 1 Conversion of EF5 binding to tissue pO<sub>2</sub>

Descriptor	Percentage of maximum EF5 binding	pO <sub>2</sub> (mm Hg)	pO <sub>2</sub> (%)	Color code (figures)
Physiologic conditions	1	75	10	Blue/green
Modest hypoxia	3	19	2.5	Red
Moderate hypoxia	10	4	0.5	Orange
Severe hypoxia	30	0.75	0.1	Yellow
Anoxia	100	0	0	White

technique, but that there is no correlation between these measurements in individual patients (17).

All of the tissue sections were reviewed by a neuropathologist (P. T. N.) at the Hospital of the University of Pennsylvania to assure that binding occurred in viable tumor tissue and to confirm histopathologic diagnosis and tumor staging.

### Recursive Partitioning Analysis

Recursive partitioning analysis (RPA) is a mathematical method of building a classification tree to separate groups of patients who had similar outcome. Curran *et al.* (30) analyzed three Radiation Therapy Oncology Group (RTOG) databases for primary brain tumors (study numbers 74-01, 79-18 and 83-02) and identified six subgroups of patients with statistically different outcomes based on clinical evaluation and tissue histology (30). Curran's original work has been validated by Scott *et al.* (31). For patients in RPA class 1, the median length of survival is 12.3-fold longer than for patients in RPA classification 6.

### Statistical Analysis

Patient characteristics for all 18 patients (all tumor grades) were summarized by using descriptive statistics. For continuous variables (such as age), mean, SD, and median were calculated. Patients were neither lost to follow-up nor experienced death without recurrence in this study. The relationship between time to recurrence and EF5 binding was analyzed by using the probability distribution for linear correlation coefficient.

## RESULTS

Eighteen patients with newly diagnosed supratentorial glial brain tumors were studied (Table 2). Each patient had a minimum of 6 months of follow-up unless they died before that time. Some hypoxia measurements on a subset of these patients have been previously reported (17). The mean and median follow-up was 403 and 350 days, respectively. The mean age ( $\pm$ SD) of the patients was 53.4 ( $\pm$ 16.5) years with 50% of the patients less than 50.5 years of age. The distribution for RPA analysis classification for the patients was 22% class 1, 6% class 2, 22% class 3, 28% class 4, and 22% class 5. Two tumors were histologically confirmed to be WHO grade 2 (astrocytoma with gemistocytic features and oligoastrocytoma); 3 were WHO grade 3 (anaplastic oligodendrogliomas or anaplastic mixed), 1 of the 3 was a malignant astrocytoma in a previously irradiated field; and 13 patients had WHO grade 4 tumors (GBMs). Adjuvant therapy was given to 14 of the 16 high-grade patients during and/or after radiation therapy. The remaining two patients with high-grade tumors did not receive adjuvant therapy

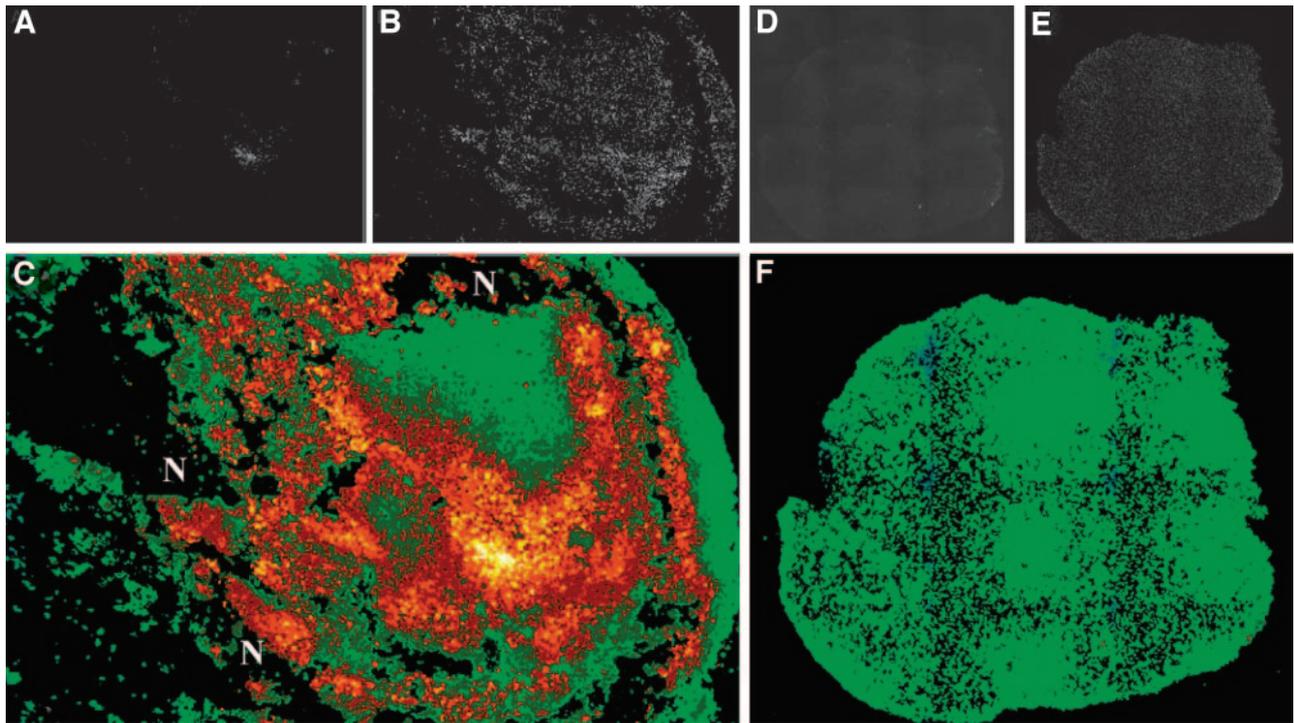
because their tumors recurred before radiation commenced or they died before radiation was completed. At the time of analysis, 12 of 18 of patients had experienced recurrence and 7 of the 12 had died because of their brain tumor.

We examined the spatial patterns of hypoxia based on an examination of immunohistochemical images of EF5 binding. In tumors with high EF5 binding, there was substantial heterogeneity characterized by large regions of oxia and smaller regions of hypoxia. EF5 staining was always found adjacent to necrotic areas, but regions of high EF5 binding, representing hypoxia, could be found in the absence of obvious necrosis. Figure 1A–C, is an example of a GBM with substantial hypoxia adjacent to regions of necrosis. However, the perinecrotic cells were not the most severely hypoxic cells in this tissue section. The most severely hypoxic region (*yellow* and *white* regions) was distant from necrosis, although three-dimensional studies were not done to determine whether necrosis was present in a closely adjacent tumor section. In low binding tumors, the binding patterns were dominated by homogeneous pO<sub>2</sub>s. Figure 1D–F shows the pO<sub>2</sub> levels in a grade 2 glial tumor. In contrast to the GBM, all cells are viable and at modest levels of hypoxia.

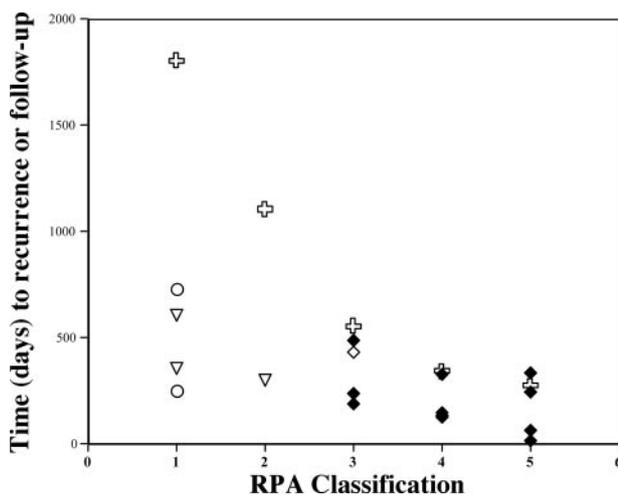
Table 2 Demographics

Category	
Age	
Mean $\pm$ SD	53.4 $\pm$ 16.5
Median	50.5
Range	22–77
Grade	
Grade 2	2
Grade 3	3
Grade 4	13
Histology	
Astrocytoma with gemistocytic features	1
Oligoastrocytoma	1
Anaplastic oligodendrogliomas	2
Anaplastic mixed	1
Glioblastoma multiforme	12
Malignant astrocytoma in previously irradiated site	1
RPA classification	
1	22%
2	6%
3	22%
4	28%
5	22%
6	0%
Follow-up time	
Mean $\pm$ SD	403 $\pm$ 256
Median	350
Range	57–762
Adjuvant therapy (chemotherapy) *	
Yes	14
No	2
Outcome at analysis	
Recurrent	12
Alive, recurrent	5
Dead due to recurrence	7
Nonrecurrent	6

\* High-grade tumors only.



**Fig. 1** EF5 binding in two tumors. *A–C*, EF5 binding in a WHO grade 4 glioma (GBM); *D–F*, EF5 binding in a patient with a WHO grade 2 glioma. *A, D*, EF5 binding; *B, E*, Hoescht 33342 binding (nuclei); *C, F*, the oxygen maps generated from the EF5 binding data. For each of these two patients, the presented images are from the tissue section with the highest EF5 binding. Regions that do not bind EF5 but contain nuclei on the Hoescht 33342 image represent viable, oxic tumor cells, whereas regions of necrosis are non-EF5 binding, but contain no, or very few nuclei (*A, C*). *C*, relatively high regional EF5 binding (*orange/yellow*) is adjacent to regions of necrosis (*N*; see Table 1 for color code for  $pO_2$ ). This image shows that substantial regions of severely hypoxic cells are distant from regions of necrosis. Microregional variations in the tissue  $pO_2$  can be discerned on these images. In contrast, the images (*D–F*) from the low-grade tumor show little EF5 binding (*D*) in regions with a viable cell population. *E*, these findings confirm that this tumor is dominantly oxic. The oxygen map (*F*) shows a homogeneous population of cells at tissue  $pO_2$ s in the range of  $\sim 10\%$  oxygen (*blue-green color*).



**Fig. 2** The time to recurrence or follow-up of patients with glial tumors versus their RPA classification. Circles, WHO grade 2 glioma tumors; triangles, WHO grade 3 glioma tumors; diamonds, WHO grade 4 glioma tumors. Solid symbols, patients who have experienced tumor recurrence; open symbols, patient without recurrence/progression at the time of analysis. Crosses, the expected outcomes based on published RPA data (30).

To confirm that the glial tumors in this group of patients had biological behavior that was similar to previously reported studies, we compared time of follow-up or recurrence with RPA classification (ref. 30; Fig. 2). Although all of the patients have not yet failed, the distribution of outcome of our patients was consistent with that reported by Curran *et al.* (30).

We assessed two separate end points for EF5 binding: (a) the  $CF_{95}$  averaged from at least four sections separated by at least 500  $\mu\text{m}$ , “average” (EF5\_Avg) and (b) the  $CF_{95}$  in the tissue section with the brightest binding, “maximum” (EF5\_Max). WHO grade 2 tumors were characterized by modest cellular hypoxia ( $pO_2$ s  $\approx 10\%$ ) and grade 3 tumors by modest to moderate hypoxia ( $pO_2$ s  $\approx 10\text{--}2.5\%$ ). Severe hypoxia ( $\approx 0.1\%$  oxygen) was present in 5 of 12 grade 4 tumors. There was substantial heterogeneity in both the EF5\_Max and the EF5\_Avg for high-grade tumors; several GBMs had intermediate levels of EF5\_Max binding, similar to that in some grade 3 tumors. As shown in Table 3, each successive increase in histologic grade (WHO grade 2  $\rightarrow$  grade 3  $\rightarrow$  grade 4) is associated with a decrease in average level of tissue  $pO_2$  (oxic  $\rightarrow$  modest hypoxia  $\rightarrow$  moderate hypoxia  $\rightarrow$  severe hypoxia).

In Fig. 3A and B, we demonstrate the overall relationship between time to progression/follow-up and EF5\_Max and EF5\_Avg, respectively. The  $r^2$  value and  $P$  value for EF5\_Max

Table 3 EF5 binding in glial brain tumors

WHO tumor grade	EF5_maximum (CF95)		EF5_average (CF95)		Eppendorf values (median)	
	Average $\pm$ SD	n	Average $\pm$ SD	n	Average $\pm$ SD	n
Grade 2	2.71 $\pm$ 1.75 (modest hypoxia)	2	2.2 $\pm$ 1.37 (modest hypoxia)	2		0
Grade 3	5.11 $\pm$ 1.97 (moderate hypoxia)	3	4.16 $\pm$ 0.97 (moderate hypoxia)	3	33.0 $\pm$ 31.9	2
Grade 4	40.22 $\pm$ 28.30 (severe hypoxia)	13	22.79 $\pm$ 21.05 (severe hypoxia)	13	20.0 $\pm$ 21.0	10

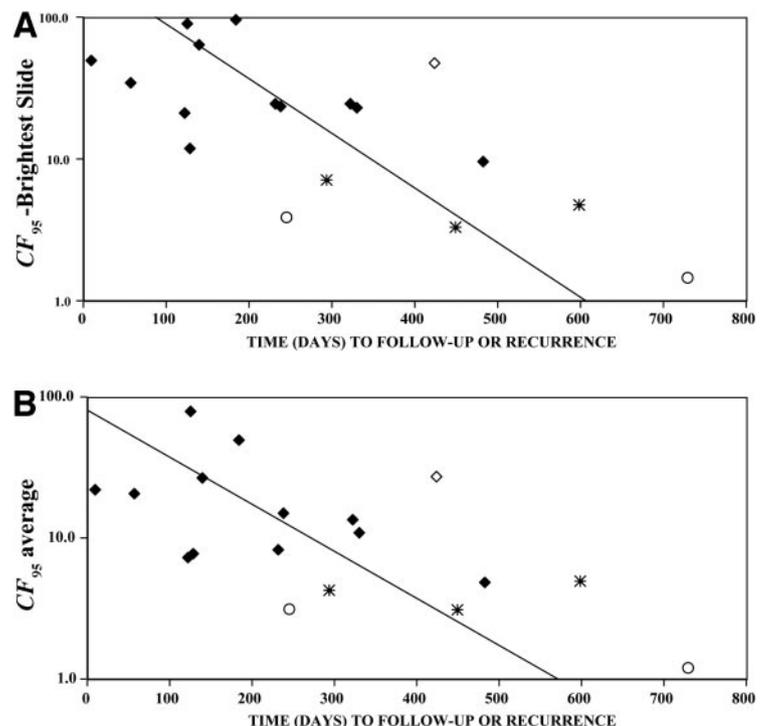
and EF5\_Avg, respectively, are 0.485 and  $P < 0.01$ , and 0.505 and  $P < 0.01$ . It is clear that tumors with a more clinically aggressive phenotype have higher EF5 binding, although for the grade 4 tumors, there is considerable intertumoral heterogeneity. Anecdotally, the patient whose GBM was characterized by oligodendroglial differentiation had the lowest binding of all of the GBM tumors, and this patient had the longest recurrence-free interval of the WHO grade 4 patients. GBM tumors with oligodendroglial differentiation are reported to be less biologically aggressive than the majority of GBM tumors (32). We have also studied two nonglial primary brain tumors, a hemangiopericytoma and a meningioma. Both of the tumors were completely oxyc (EF5 binding  $< 1\%$ ,  $pO_2 > 10\%$ ). These patients are alive and without recurrence 528 and 658 days post-operatively (these patients were not included in the present analysis).

In the 12 patients studied with the Eppendorf electrode, the mean  $\pm$  SD of the median, percentage values  $< 2.5$  mm Hg, 5 mm Hg, and 10 mm Hg were  $22.2 \pm 21.9$  mm Hg,  $5.5 \pm 9.4\%$ ,  $16.2 \pm 22.2\%$ , and  $36.7 \pm 30.7\%$ , respectively. A relationship between Eppendorf values and tumor aggression could not be demonstrated (data not shown).

## DISCUSSION

We have investigated the presence and level of tissue hypoxia in human glial brain tumors by using two independent measurement methods. Our EF5 binding data show that the presence of more severe hypoxia in glial tumors correlates with a more aggressive clinical behavior, *e.g.*, these patients experience earlier recurrence. In this group of patients, Eppendorf electrode measurement values did not track tumor aggression. The small number of patient studied may account for our inability to identify a correlation. In the published literature, electrode values have been found to both predict (33–36) and not predict for outcome in other tumor sites (37–40). We have previously shown that Eppendorf electrode measurements do not correlate with the presence of necrosis in a group of brain tumors of various histologic types (17). The characteristics, advantages, and limitations of both the EF5 and the Eppendorf hypoxia measurement techniques are different, and, thus, differing conclusions resulting from these measurements on the same patients might be anticipated. The electrode takes a snapshot of the hypoxia at the moment of measurement. This value can be affected by anesthetic and physiologic transients accom-

Fig. 3 A, B, time to recurrence or follow-up is plotted against EF5 binding. A,  $CF_{95\_Max}$ , ( $r^2 = 0.485$  for all values;  $P = 0.01$ ). B,  $CF_{95\_Avg}$ , ( $r^2 = 0.505$  for all values;  $P = 0.01$ ). Circles, WHO grade 2 glial tumors; asterisks, WHO grade 3 glial tumors; diamonds, WHO grade 4 glial tumors. Solid diamonds, patients with tumors who have experienced recurrence; open diamonds, patient without recurrence/progression at the time of analysis.



panying the required surgical access to these tumors. In comparison, 2-nitroimidazole binding integrates the level of hypoxia that occurs between the time of drug injection and biopsy (*i.e.*, binding is irreversible and rate is directly proportional to the product of drug concentration and time, and inversely proportional to oxygen concentration). EF5 binding could theoretically underpredict hypoxia if there were any drug access limitations. However, validation studies in animal studies and the appearance of binding adjacent to necrosis and far from vessels suggests that, in brain tumors, this is not a problem (Fig. 1; refs. 19, 41). EF5 is quite lipophilic and has good access to normal and neoplastic brain (19, 23, 42). Issues regarding sampling limitations are common to both methods. However, these limitations are less operator dependent in the EF5 binding technique compared with the Eppendorf method.

Our data support the observation that hypoxia is associated with tumor aggression. But why should this be the case? Previous studies in women with cervical cancer, treated with surgery alone, have shown that if the tumors were preoperatively hypoxic, treatment failure was more likely (34). Similarly, the presence of hypoxia in locally cured high-grade sarcomas was associated with a greater likelihood of metastasis and death (35). The mechanisms involved may include hypoxic regulation of cytokine release (*e.g.*, VEGF; refs. 12, 43), regulation of tumor suppressors and oncogenes (*e.g.*, *p53*, *PTEN*; refs. 44–46), growth factor receptors (*e.g.*, EGFR; ref. 47) or induction of cell cycle perturbations and DNA overreplication (48). Although brain tumors do not metastasize, they are highly invasive locally. Similar principles for invasion and metastasis may apply because metastasis mediators are involved in both of these processes. For example, it is known that hypoxia modulates invasion-associated cytokines, such as matrix metalloproteins, and their inhibitors in tumor and normal cells (49–54). Other mechanisms under study include work from Cobbs *et al.* (55, 56) who have shown that a hypoxic glial tumor microenvironment can create posttranslational epigenetic modifications in tumor suppressors, such as *p53*. These changes can result in dysregulation of translational activities and downstream pathways in glial tumors (55, 56).

Our findings that hypoxia is important in tumor aggression invite a number of provocative questions. For example, in a subtotal resection, isn't any remaining tumor likely to be oxalic because it is at the normal tissue interface? Also, once any hypoxic tumor is removed, isn't its biological effect (such as cytokine release, and so forth) irrelevant. . . nothing can be done about it?

High-grade gliomas are well known to be highly invasive and not amenable to a true "complete resection"; optimal resections are described as removing 95 to 98% of the tumor tissue (57). Assuming that the initial tumor was 2 cm in diameter, a 95% resection would leave almost 250 mg of tumor tissue behind. Could some of these cells be hypoxic and could they influence treatment response? We have found severe hypoxia in human tumor foci as small as 2 to 3 mm in diameter, that is, only a few milligrams (19, 58). Thus, it is easy to imagine that small regions of hypoxic tissue could be left behind. However, this would depend on the heterogeneity and scale of hypoxia throughout the total tumor volume. Additionally, during the 6-week interval between surgery and radiation, much smaller

cell collections could proliferate into hypoxic masses. Because the radiation dose at such peripheral sites is likely to be the lowest in the radiation field, and hypoxic cells are known to require up to three times the dose to kill aerobic cells, hypoxia as the major cause of local failure remains a distinct possibility. What about reoxygenation? Many investigators have shown that a subset of tumor treated with radiation do not reoxygenate/reperfuse, and some tumors may actually "re-hypoxiate" (59–65). Importantly, those tumors that do not reoxygenate are more likely to fail (60, 65), supporting the possibility that even large radiation doses would be insufficient to kill resistant cells. These observations are consistent with the report that 78% and 56% of unifocal tumors recurred within 2.0 and 1.0 cm, respectively, of the presurgical, initial tumor margin, defined as the enhancing edge of the tumor on computed tomography scan (66). Now that hypoxic regions can be prospectively identified by using non-invasive imaging methods (67–69), treatment methods such as intensity-modulated radiation therapy or proton beams, hypoxia modifying agents such as RSR13 (70), hyperbaric oxygen (71), dodecafluoropentane [DDFP (72)], and tirapazamine (73) become therapeutically relevant.

Because of the invasive nature of gliomas, there are a substantial number of patients with tumors that cannot be completely resected; small contrast-enhancing tumor deposits at the edge of the main tumor mass are commonly left behind because they are in eloquent or hard-to-reach areas. These areas might be assumed to be oxalic, based on the misconception that the distribution of hypoxia in human tumors is like that in multicellular tumor spheroids but on a much larger scale, *e.g.*, peripheral oxia with concentrically decreasing shells of hypoxia and central necrosis. This picture is erroneous because fluctuations in oxygen and other nutrients occur on the actual scale of spheroids, *e.g.*, submillimeter. This scale of metabolic fluctuation is not influenced by the presence of macroscopic regions of necrosis, which may or may not be located centrally. Because *in vivo* tumors have a distribution of blood vessels throughout, small regions of diffusion- and perfusion-limited hypoxia can be found in all regions of the tumor, even near the tumor-normal tissue interface. The considerations (discussed above) regarding the number of cells, and their likelihood of having a microenvironment that is unfavorable for therapy, applies to an even greater degree in these larger tumor deposits. This concept, although clearly observable in rodent tumors (74) is very difficult to document in human tumors. This is especially true in brain tumors, which are usually not removed in a mass, and the normal tissue-tumor interface is extremely difficult to identify intraoperatively.

Finally, there is the perception that hypoxia can cause tumor aggression (such as local invasion), but once this happens, nothing can be done. Indeed at the present time, there are no efficacious therapies for cells that have escaped brain tumors and are resistant to radiation, or are outside the radiation field. However, our data support a new and exciting possibility for the diagnosis and early intervention of brain tumor progression. As shown in Figure 3, moderately or severely hypoxic cells do not occur in either grade 2 or grade 3 glial tumors nor in benign nonglial tumors (data not shown). Although these observations are based on a very small patient group, the data are consistent with an accumulation of hypoxia leading to the development

of a more aggressive phenotype. Monitoring the development of hypoxia with noninvasive methods, *e.g.*,  $^{18}\text{F}$ -labeled EF5, Cu-60 diacetyl-bis(N (4) methylthiosemicarbazone), or [ $^{18}\text{F}$ ]fluoromisonidazole, may have an important role in determining the timing and type of therapy (67–69).

To conclude, the Eppendorf electrode method may not be a suitable measuring technique of hypoxia in brain tumors, but immunohistochemical analyses based on EF5 binding predicts tumor biology and perhaps clinical outcome. A larger number of patients will need to be studied to determine whether hypoxia is a prognostic factor for grade 4 glial tumors. In the future, such studies will include the noninvasive measurement of hypoxia by using [ $^{18}\text{F}$ ]-labeled EF5 (75–77).

## ACKNOWLEDGMENTS

Special thanks to Dr. Eli Glatstein for his comments and suggestions.

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*Clin Cancer Res* 2004;10:8177-8184.

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