Hypoxia in a human intracerebral glioma model

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Object. The development of hypoxia in human gliomas is closely related to functional vasculature and the presence of hypoxia has important biological and therapeutic consequences. Assessment of hypoxia is necessary to understand its role in treatment response and to evaluate treatment strategies to improve tumor oxygenation. In this study, the authors report findings of their analysis of the degree of hypoxia in relation to other vascular parameters in a human intracerebral glioma xenograft.

Methods. In sections of tumor, hypoxic regions were identified immunohistochemically by using the hypoxic marker pimonidazole. The S-phase marker bromodeoxyuridine was used to detect cell proliferation, and the perfusion marker Hoechst 33342 was used to delineate perfused vessels. Vascular structures were stained with an endothelial marker.

Hypoxic tumor regions were clearly present in this human intracerebral glioma model. Hypoxic areas were usually found in nonperfused regions, whereas tumor cell proliferation was especially marked in perfused tumor areas. Furthermore, by using in situ hybridization the authors identified infiltrating tumor cells in the normal brain. This feature is often observed in gliomas in patients.

Conclusions. This model is a representative human glioma model that provides the researcher with the opportunity to examine the effects of treatments aimed at modifying the oxygenation status of a tumor.

KEY WORDS • hypoxia • glioma • vasculature • tumor model • nude mouse

MALIGNANT brain tumors, especially glioblastomas, are notorious for their resistance to therapy. This is emphasized by the fact that although new neurosurgical and radiotherapy techniques and chemotherapy agents have been developed in recent years, median survival rates for patients with these tumors have not improved. Apart from intrinsic properties of glioma cells involved in the resistance to radiotherapy and chemotherapy, the presence of hypoxia in these tumors may be an important factor in determining the resistance to radiation treatment. Hypoxic tumor regions in glioblastomas have been demonstrated in vivo and are probably the result of insufficient tumor perfusion due to significant differences in structure and function between normal and tumor vasculature. Currently, new treatment strategies such as carbogen inhalation combined with administration of nicotinamide are being developed to improve tumor oxygenation. To evaluate these new approaches in gliomas in humans, it is necessary to have models of this tumor type that contain hypoxic areas. To correlate the effects of therapy with the degree of hypoxia, a technique to detect and quantify hypoxic areas is also necessary. In this study, we present a human intracerebral glioma xenograft model that fulfills these requirements. In this model, hypoxic areas stained by the hypoxic marker pimonidazole were visualized immunohistochemically in relation to vasculature and tumor perfusion. An image analysis procedure was applied for quantitation of these parameters. This model demonstrates that hypoxia is present in human gliomas xenografted orthotopically. It provides the researcher with the opportunity to examine the effects of treatments aimed at quantitatively modifying the oxygenation status of a tumor in relation to other vascular parameters.

Materials and Methods

Animal Population

Nude mice were derived from the BonholdGard Denmark strain (BALB/c nu/nu mouse). Ten mice were used in the experiments. All animals were maintained in specific pathogen-free conditions in plastic cages equipped with air filters. They were kept in isolation rooms with controlled temperature, humidity, and lighting. The experimental procedures were approved by the local ethics committee for animal use.
Tumor Implantation

Tumors used in this study were derived from the E106 human glioma line (glioblastoma multiforme), which was passaged 20 to 21 times subcutaneously in nude mice. Genetic characterization of this xenograft showed that the genetic abnormalities found in gliomas are also present in this glioma xenograft.† For intracerebral implantation subcutaneous tumors were excised and minced mechanically to form a cell suspension. Six- to 8-week-old mice were anesthetized by injection of 50 to 60 mg/kg of pentobarbital. The skin on the top of the skull was incised and a hole in the skull was made in the left hemispheric region. Tumor cell suspensions of 0.01 ml containing 10⁶ cells were subsequently pressure injected using a needle with a 0.6-mm diameter. Injections were directed into the left hemisphere in the subcortical area above the lateral ventricle. The skin was closed with a clip; all surgical procedures were performed under sterile conditions. The mice were observed daily and tumors were harvested when the animals displayed neurological symptoms.

Tumor Markers

The presence of hypoxia in the tumors was determined by injecting the mouse intravenously via a lateral tail vein with 0.1 ml of saline solution containing 2 mg of the hypoxia marker, pimonidazole hydrochloride. This marker was administered 30 minutes before the animals were killed. The S-phase marker, BuDR, was administered intraperitoneally (2.5 mg in 0.5 ml of saline solution). This proliferation marker was given 15 minutes before the animals were killed.

Perfusion in the tumors was determined by injecting the mouse intravenously via a lateral tail vein with 0.1 ml of a saline solution containing 0.3 mg of the perfusion marker, Hoechst 33342. We had used this technique previously and found that it resulted in excellent demarcation of perfused tumor vessels.‡ To prevent diffusion of Hoechst 33342 into adjacent nonperfused vascular structures, each mouse was killed by cervical dislocation 1 minute after injection. The tumors were quickly removed, frozen, and stored in liquid nitrogen.

Immunohistochemical Procedures

For further analysis of hypoxia and vascular parameters, four to five frozen sections (each 5 μm thick) from central and peripheral tumor areas were prepared. To visualize the vasculature, sections were first incubated with the monoclonal rat anti–mouse antibody for mouse endothelium, ME 9F1,† after having undergone fixation for 10 minutes in acetone. The sections were incubated for 45 minutes at room temperature and then rinsed in PBS. They were subsequently incubated with a TRITC-labeled IgG goat anti–rat antibody for 30 minutes at room temperature, again rinsed in PBS, and incubated with a TRITC-labeled donkey anti–goat Ig antibody. This procedure resulted in an excellent fluorescent signal of the vascular pattern, which could be detected using a fluorescence microscope with 510- to 560-nm excitation and a 590-nm emission filter. There was minimal background staining, which did not interfere with the interpretation of the fluorescent signal. The nuclei of cells adjacent to the perfused vessels were intensely labeled by diffusion of the Hoechst 33342. Using the fluorescence microscope, Hoechst 33342 could be visualized under ultraviolet light, showing a blue fluorescence (excitation 365 nm, emission 420 nm) in the same sections as those stained with ME 9F1 antibodies.

To visualize the hypoxic marker, as a first step frozen sections were fixed in acetone for 10 minutes, washed in PBS (pH 7.4), and scanned for the Hoechst signal. Subsequently, these sections were incubated overnight at 40°C with rabbit antipimonidazole hydrochloride Ig (1:2000 in PBS-B, pH 7.4). The next day the sections were washed in PBS (pH 7.4) and incubated for 120 minutes at room temperature with a fluorescent antibody (fluorescein isothiocyanate–labeled donkey anti–rabbit Ig) and scanned for the hypoxia signal. Thereafter the same sections were stained for vasculature and again scanned.

Before the BuDR marker was visualized, frozen sections were scanned for the Hoechst signal and vasculature, as described earlier. Subsequently, DNA of the tissue sections was denatured by incubation with 0.2 N hydrochloric acid for 10 minutes. To neutralize the pH, sections were rinsed in 0.1 M borax for 10 minutes, followed by washing three times in PBS. Then the sections were incubated for 60 minutes at 37°C with Br-3, a mouse monoclonal antibody to BuDR (1:50 in PBS-B). After they were washed three times in PBS, the sections were incubated for 45 minutes at room temperature with a fluorescein isothiocyanate–labeled rabbit anti–mouse antibody (1:25 in PBS-B), again washed three times in PBS, stained for 15 minutes at room temperature with fast blue dye (1:1000 in PBS) to visualize all nuclei, and finally rinsed in PBS. This procedure was followed by scanning of proliferating nuclei (BuDR) and all nuclei.

Scanning Tumor Sections and Image Processing

The tumor sections were scanned with a computer-controlled motorized stepping stage on a fluorescence microscope with a high-resolution intensified solid-state camera for quantitative analysis. After scanning all fields (depending on tumor size), the area of interest was reconstructed from the separate processed images into one large composite image. This resulted in a set of three composite images for hypoxia in relation to the perfused vasculature and a set of four images for proliferation in relation to the perfused vasculature.

The tumor area was drawn interactively in a composite image.
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This area was used as a mask in further image analysis, excluding nontumor tissue and tumor necrosis from the analysis.

By combining the composite images, several parameters from each section could be calculated by the computer: the perfusion fraction (perfused vascular area/total vascular area), the relative vascular area (vascular area/total tumor area), the vessel density (number vessels/square millimeter), the hypoxic fraction (hypoxic area/total tumor area), and the labeling index. For each vessel a domain was determined and defined as the area in which a vascular structure was present, with boundaries equidistant to adjacent vascular structures. On the contours of these domains, the shortest distances between neighboring vascular structures were calculated and used as an estimation of the intervesselary distance in micrometers.

**Histological Investigation and In Situ Hybridization**

Sections adjacent to those sections used for qualitative analysis were stained with hematoxylin and eosin for histological examination.

In situ hybridization was performed using a human chromosome 1 centromere probe to determine the human origin of the cells. In situ hybridization was performed in the manner described by Poddeighe et al., with minor modifications. Briefly, 4-mm frozen sections were mounted on a slide, air dried, and fixed in 1% formaldehyde/70% ethanol for 20 minutes at −20°C. Protein digestion was accomplished using pepsin hydrochloride. After dehydrating the slides, they were postfixed in 1% formaldehyde/PBS (5 minutes at room temperature). The centromere probe for chromosome 1 (pUC1.77) was labeled by nick translation with biotin and dissolved in hybridization solution (final concentration 1 ng/µl probe in 60% formamide and 10% detrann sulfate in 2× SSC, pH 7). The probe was applied to the slides under a coverslip and denatured simultaneously with the targeted DNA (3 minutes at 70°C). After an overnight hybridization at 37°C, posthybridization washes were performed at 42°C in 60% formamide/2× SSC, pH 7, three times for 5 minutes each and in 2× SSC alone three times at 5 minutes each. The biotinylated probe was detected using a mouse antibiotin, biotin-labeled horse anti–mouse IgG and a final incubation with biotinylated rabbit anti–mouse antibody. Peroxidase was visualized using 3,3-diaminobenzidine tetrahydrochloride, and the signal was amplified with copper sulfate. The slides were counterstained with hematoxylin and mounted in Permount.

**Sources of Supplies and Equipment**

The BrdU, Hoechst 33342, and fast blue dye were obtained from Sigma Chemical Co. (St. Louis, MO). The TRITC-labeled goat anti–rabbit and donkey anti–mouse IgG antibodies were acquired from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The Br-3 mouse monoclonal antibody to BrdU was purchased from Caltag Laboratories (San Francisco, CA) and the fluorescein isothiocyanate–labeled rabbit anti–mouse antibody from Dako A/S (Glostrup, Denmark).

Scanning of tumor sections and image processing were accomplished using a fluorescence microscope acquired from Carl Zeiss BV (Weesp, The Netherlands) and an MXRi solid-state camera from Adimec BV (Eindhoven, The Netherlands).

**Results**

**Tumor Growth and Histological Characteristics**

Six weeks after tumor implantation, the mice began to demonstrate neurological symptoms and weight loss and were killed. Examination of their brains revealed circumscribed tumors in one or both hemispheres, usually adjacent to the lateral ventricle. Midline shift was sometimes present with signs of hydrocephalus. In hematoxylin and eosin–stained sections, the highly cellular tumors demonstrated a compact, solid growth pattern in one or both lateral ventricles and, focally, in the leptomeninges, there was dispersed perivascular or more diffuse infiltration into the surrounding brain tissue. The markedly polymorphic nuclei in the tumor cells were surrounded by a rim of eosinophilic cytoplasm. There were no signs of cellular processes or a fine fibrillar eosinophilic background between the tumor cells. Signs of mitosis, including atypical forms, were frequent. Areas of necrosis and florid microvascular proliferation were absent. Dispersed individual tumor cells contained pyknosis and shrinkage of their nuclei with hyperesinophilic changes in the cytoplasm, which were consistent with hypoxic damage. In the hematoxylin and eosin–stained sections it was difficult to differentiate between infiltrating tumor cells and preexistent neuronal and glial cells, whereas in the sections on which in situ hybridization for the chromosome 1 centromere had been performed, such infiltrating tumor cells were clearly demarcated by one or two spots in their nucleus (Fig. 1).

**Tumor Vasculature and Perfusion**

In Table 1 an example of vascular parameters in a whole tumor section, which were analyzed using the image analysis system, is presented. Figure 2 shows the vascular architecture of normal and brain tumor vessels in combination with tumor perfusion and hypoxia. Usually, we found tumor vessels to be irregularly shaped and larger in size with increased lumina, compared with normal vessels. This resulted in larger vascular areas. Normal brain vessels were homogeneously distributed, whereas brain tumor vessels were heterogeneously spread over the tumor area. Intervascular distances between tumor vessels were frequently found to be considerably larger than intervesselary distances in normal brain tissue (Table 1). As a consequence, vascular density was lower in brain tumor areas in comparison with normal brain. Perfused tumor vessels were delineated by the marker Hoechst 33342. This marker was almost absent in normal brain vessels due to an intact blood–brain barrier in the normal brain. Some tumor...
vessels were not stained by Hoechst 33342, indicating that not all tumor vessels were perfused during administration of this marker (perfusion factor 0.92).

**Hypoxic Areas**

In the xenografted human gliomas, hypoxic areas could be visualized by using the hypoxia marker pimonidazole, as shown in Fig. 2. In the section investigated, the hypoxic area covered 11% of the total tumor area. The median distance of hypoxic tumor cells from perfused vessels, as measured by the image analysis system, was 143.6 μm. Usually, an unstained area separated the hypoxic zone from the area delineated by the perfusion marker, Hoechst 33342. Histologically, the hypoxic areas did not contain necrotic material, but appeared to be composed of vital cells.

**Tumor Proliferation**

Cell proliferation was visualized using the S-phase marker, BUdR. The labeling index was 9% in the section investigated (Table 1). Labeled cells were present not only in the tumor but also in a widespread area surrounding the tumor border in normal brain tissue. The human nature of these cells was verified using in situ hybridization. This indicates that the glioma cells had migrated over considerable distances into normal brain tissue. The images we obtained showed most BUdR-labeled cells near blood vessels and, very infrequently, in hypoxic or necrotic areas of tumor (Fig. 3).

**Discussion**

Immunohistochemical examination performed using the hypoxia marker, pimonidazole, as demonstrated in this study, offers us the opportunity to study the distribution of hypoxia in relation to vascular architecture and proliferation. Moreover, the human intracerebral glioma xenograft model selected for this study allows us to study an orthotopically xenografted tumor, approaching as closely as possible the original microenvironment of the tumor.

The hypoxia marker used in this study, pimonidazole, is a nitroimidazole derivative that is bound to hypoxic cells by the process of nitroreduction. Nitroreductive activity has been demonstrated in glioma cells and it has also been shown that nitroimidazole compounds “recognize” hypoxic areas with oxygen tensions of 5 to 10 mm Hg in glioma tumor models. Using radiolabeled nitroimida- zole derivatives, hypoxic areas in patients with glioma as well as in glioma models can be detected. Our results indicate that, similar to gliomas growing in patients, hypoxic cell regions are also present in the intracerebral tumor model. These observations are in agreement with the observations of Parliament and colleagues, who implanted hypoxic cells of the M006 human glioma cell line subcutaneously and intracerebrally in nude rats and labeled them in situ with the hypoxia marker [3H]misonidazole. In their study, the labeled cells were found adjacent to necrotic areas. In our tumor model, hypoxic areas were present at regular distances within the perfused tumor areas, emphasizing the close relationship between tumor perfusion and the development of hypoxia, which has been stressed by many investigators. The median distance of hypoxic areas to perfused vessels was 143.6 μm. This value is in agreement with data from the literature indicating that hypoxic areas begin to appear at distances beyond 100 μm from a blood vessel as a result of the oxygen consumption rate and limitations in oxygen diffusion.

The relevance of hypoxia for tumor growth and therapy response has recently been reviewed by Gulledge and Dewhirst. They emphasized that hypoxic areas have been
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shown to be more resistant to radiotherapy and chemotherapy than other areas. Moreover, hypoxia induces vascular endothelial growth factor production in tumor cells, thus stimulating angiogenesis and promoting tumor growth. Hypoxia increases mutagenicity in tumors, which also provides the tumor with a growth advantage and a mechanism by which to develop therapy resistance. The inadequate delivery of oxygen to certain tumor areas is largely a consequence of reduced perfusion and altered geometric dimensions of tumor vasculature in those areas. Differences in vascular architecture between brain tumors and normal brain have been reported by Plate and Mennel, and by Wesseling, et al. These differences include endothelial cell proliferation, larger vessel sizes, and altered vessel shape and distribution. In our glioma model, we also observed large irregularly shaped tumor vessels, in contrast to the smaller and more regularly shaped normal brain vessels. The tumor vessels were also more heterogeneously distributed and intervascular distances between tumor vessels were larger than those observed between normal brain vessels, sometimes beyond the oxygen diffusion limit, thus contributing to the development of hypoxic tumor areas. Infrequently, hypoxia was present in the direct neighborhood of Hoechst-stained vessel structures. This may be due to intermittent perfusion, resulting in acutely hypoxic areas, a mechanism demonstrated by Trotter and associates.

In this study the labeling pattern produced by BUdR was clearly related to the blood vessels, with more labeled cells observed to be adjacent to perfused and nonperfused vessel structures. Furthermore, the labeling index was in most cases higher around perfused vessels than around nonperfused vessels, emphasizing the close relationship between tumor perfusion and cell proliferation. Our observation concerning the relationship between the labeling index of tumor cells and their position relative to perfused vascular structures also supports the reports of Hirst and Denekamp, Kligerman, et al., Tannock, and Tannock and Steel, who demonstrated that the rate of cell proliferation decreased rapidly with distance from the vessel, in parallel with the decrease in oxygen content. Tannock demonstrated that dividing cells traveled from well-nourished regions around capillaries to poorly nourished regions, and, finally, moved into necrotic zones.

In the present tumor model, in situ hybridization demonstrated that human glioma cells were able to infiltrate the normal brain parenchyma over varying distances in a manner resembling the growth of gliomas in patients. This finding contrasts with the circumscribed growth pattern usually found in intracerebrally xenografted tumor lines. The infiltrative growth pattern of human gliomas has been usually found in intracerebrally xenografted tumor lines. In vitro analysis of extracelullar matrix components in the normal brain by invading glioma cells. Int J Cancer 75:864–872, 1998


References


Conclusions

In this study a human intracerebral glioma model is pre-


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