American Journal of Pathology, Vol. 140, No. 2, February 1992
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Vascular Expression of Glucose Transporter in Experimental Brain Neoplasms

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Vascular abnormalities in brain neoplasms are important to tumor biology and therapy. Glucose transporter (GLUT1) expression is a differentiated property of normal cerebral microvessels typically associated with expression of the blood-brain barrier. We investigated the relationship of GLUT1 expression to other vascular characteristics in F98, 9L, and C6 gliomas and Walker 256 carcinomas implanted into adult rat brains. The percentages of microvessels with immunohistochemically detectable GLUT1 were 95.5 ± 3.9 in F98, 60.9 ± 3.9 in 9L, 45.4 ± 5.6 in C6, and 1.2 ± 0.3 in Walker 256 (mean ± SEM). The percentage of GLUT1-positive vessels in F98 was not statistically different from that in normal brain. GLUT1 expression was not dependent on restricted permeability as all tumors were highly permeable to Evans blue. GLUT1 expression was unrelated to vascular density, vascular morphology, and parenchymal GFAP expression. The expression of GLUT1, a marker of cerebral endothelial differentiation, is a newly described property of glial tumor vessels that may have diagnostic and prognostic significance. (Am J Pathol 1992, 140:417–425)

The vasculature of brain neoplasms is characterized by a number of abnormal properties which are important to tumor biology and therapy. Previous studies have demonstrated a relationship between blood-brain barrier dysfunction, assessed by contrast enhanced imaging, and tumor grade.[1,2] Higher grade tumors demonstrate greater degrees of vascular proliferation and have morphologically abnormal vessels, properties that are believed to account for their high permeability.[2–5] Despite this apparent relationship between the state of the vasculature and the behavior of a neoplasm, few studies have attempted to define more precisely potentially important biochemical changes which may be present in tumor microvessels.[6–8] If loss of barrier-associated endothelial properties like tight junctions and permeability restrictions are related to tumor behavior, then it may be valuable to assess more comprehensively other barrier-associated properties in tumor vessels.

Normal brain vessels, from which intrinsic brain neoplasms derive their vascular supply, express a complex array of endothelial specializations that comprise the blood-brain barrier (BBB). Structural specializations (e.g., tight junctions) impose a permeability barrier, which restricts the diffusion of polar compounds into brain parenchyma.[9] Biochemical specializations, such as transporters and enzymes, serve to regulate the flux of compounds across the endothelium.[10] Given the restricted permeability of brain vessels, the biochemical components of the BBB are necessary to maintain the delivery of compounds required for normal neurologic function.

One such biochemical specialization is the glucose transporter. A glucose transporter isoform (GLUT1) has been localized to the plasma membrane of endothelial cells in only those brain vessels having restricted permeability.[11–13] Endothelia of the circumventricular organs, whose vessels are permeable to molecules as large as proteins, do not express GLUT1.[11,12] It has been proposed that endothelial GLUT1 expression is linked to restricted permeability, to maintain adequate brain glucose supplies.[14,15] In the highly permeable vessels of brain neoplasms, the need for specific endothelial glucose transporters may be decreased or eliminated, as glucose

Supported by National Institutes of Health research grants CA-52857 (H. B.) and NS-27229 (L. R. D). C. Guerin is a National Research Service Awardee (CA-09574). J. Laterra is a Clinical Investigator Development Awardee (NS-01329).

Accepted for publication August 27, 1991.

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should freely diffuse into the parenchyma. However, we recently reported that human anaplastic astrocytomas express high vascular GLUT1 levels despite increased permeability demonstrated by contrast enhanced imaging studies.[16] Thus, independent mechanisms appear to regulate the expression of the glucose transporter and permeability restrictions in these microvessels.

To investigate further the biochemical state of brain tumor vessels and its relationship to other vascular properties, we assessed GLUT1 expression in several experimental glial tumors, a nonglial tumor, and normal brain. We then examined the relationship of GLUT1 expression to vascular permeability, vascular density, vascular morphology, and parenchymal differentiation.

Methods

Cell Lines

The F98, C6, and 9L cell lines are all considered to be of glial origin, the former two being anaplastic gliomas and the latter a gliosarcoma.[17–19] C6 cells in culture express astroglial properties.[20] The Walker 256 is derived from a breast carcinoma, and allowed us to assess the effects of nonglial cells on brain-derived vessels.[21] Cells were maintained in tissue culture until brain injection. F98 and 9L cells were grown in Dulbecco's Modified Eagles Medium (DMEM; Mediatech, Washington, DC) with 10% fetal bovine serum (Hyclone, Logen, UT), C6 cells in DMEM with 10% bovine calf-serum (Hyclone), and Walker 256 cells in Medium 199 (Gibco, Grand Island, NY) with 5% horse serum (Gibco). All media contained 2mM L-glutamine (Mediatech), 200 U/ml penicillin G (Mediatech), and 200 μg/ml streptomycin sulfate (Mediatech). Cells were grown at 37°C in 5% CO_2/95% air and passaged at confluence by trypsinization. F98 cells were obtained from Joseph Goodman at the Ohio State University School of Medicine, 9L cells from Marvin Barker at the University of California at San Francisco Brain Tumor Research Center, and both C6 and Walker 256 cells were obtained from American Type Culture Collection (Rockville, MD).

Tumor Models

Cells were trypsinized, counted by Coulter Counter (Coulter Electronics, Hialeah, FL) and diluted in their respective media to achieve the appropriate cell concentration immediately prior to brain injection. Adult rats were anesthetized by intraperitoneal injection of 3 ml/kg of a solution of 25 mg/ml ketamine (Parke-Davis, Morris Plains, NJ), 2.5 mg/ml xylazine (Mobay, Shawnee, KS), and 14% ethanol in normal saline. Male Fisher 344 rats were used for F98 and 9L, male Wistar rats for C6, and female Sprague-Dawley rats for Walker 256 (Harlan, Indianapolis, IN). After shaving the head and preparing the surgical site with 70% ethanol and povidone iodine (Purdue Frederick, Norwalk, CT), a midline skin incision was made and a 1.5 mm diameter burr hole was drilled in the skull 3 mm lateral to bregma. Animals were placed into a stereotactic frame and ten microliters of cell suspension containing 100,000 F98 ($n = 9$), 9L ($n = 10$), or C6 ($n = 7$) cells or 350,000 Walker 256 ($n = 10$) cells were injected into the left caudate-putamen. Wounds were closed with staples.

Vascular Permeability Studies

Animals were studied when tumors were known to be large from prior studies: postoperative day (POD) 11 for F98, POD 9 for 9L, POD 12 for C6, and POD 13 for Walker 256. After anesthesia as described earlier, 2 ml/kg of 2% Evans blue (Fisher Scientific, Fair Lawn, NJ) in phosphate-buffered saline (PBS; Sigma Chemical, St. Louis, MO) was injected intravenously and allowed to circulate for 1 hour. Animals were then perfused transcardially with 150 ml of 10% neutral buffered formalin (Fisher) at 60 ml/min. Brains were removed and immersed in the same fixative overnight at 4°C. Coronal sections through the tumors were photographed.

Immunohistochemical Staining

Specimens were processed to paraffin blocks and serial 5-micron thick sections were stained with hematoxylin and eosin, or with rabbit antisera to glucose transporter (GLUT1), laminin (Gibco), or glial fibrillary acidic protein (GFAP; Accurate, Westbury, NY). Laminin antisera stains vascular basal lamina and is a reliable marker of vessels in brain neoplasms and normal brain.[22,23] Antiserum to GLUT1 was developed by one of us (L.R.D.) and identifies the carboxyl terminus of the "rat brain/human erythrocyte" glucose transporter. This antiserum has previously been shown to selectively stain blood-brain barrier vessels in normal brain, and to identify the appropriate molecular weight protein (M_r = 45–55,000 daltons) on Western blots.[11]

Sections were deparaffinized, rehydrated, and incubated for 15 minutes at room temperature with 1% hydrogen peroxide in methanol. Sections for laminin staining were digested for 15–20 minutes at 37°C with 0.2% pepsin (Calbiochem, LaJolla, CA) in 0.01N HCl just before peroxide incubation. Sections were incubated overnight at 4°C with a 1:1000 dilution of the relevant primary antisera in PBS containing 1% normal goat serum (Vector

Laboratories, Burlingame, CA). They were processed at room temperature by 30-minute incubation with 1:200 biotinylated goat anti-rabbit immunoglobulin (Vector) diluted in PBS containing 1.5% normal goat serum, 30-minute incubation with 1:50 ABC reagent (Vector) in PBS, and 15-minute incubation with 0.5 mg/ml 3,3'-diaminobenzidine (Sigma), and 0.01% hydrogen peroxide in 50 mM tris-buffered normal saline. Sections were counterstained with hematoxylin to optimize assessment of general tissue morphology, dehydrated, and mounted in Permount (Fisher). As a nuclear stain, hematoxylin does not interfere with the assessment of GLUT1 staining, since in brain microvessels GLUT1 is a plasma membrane protein.[11] Control slides had nonimmune rabbit serum (Vector) substituted for immune serum. Development time was the same for controls and experimental slides performed in parallel. All controls performed were negative.

Assessment of Staining

The total number of vessel profiles per high power field (0.0682 mm^2) was counted independently in adjacent serial sections stained for GLUT1 and laminin. Ten to thirty fields per specimen were counted, spanning the total area of solid tumor and excluding infiltrating border zones where tumor cells and normal brain intermingled. For each specimen, the percentage of GLUT1-positive vessels was calculated as the average number of GLUT1-positive profiles/field divided by the average number of laminin-positive profiles/field. Vascular density was determined for each specimen as the average number of laminin-positive profiles per field divided by the area of the field.

GFAP stained slides were assessed for the presence or absence of staining with particular attention to tumor cells and perivascular areas of tumor and normal brain.

Statistical Analysis

Single factor analyses of variance were performed to determine if differences existed among tumor types in the percentage of GLUT1-positive vessels or in vascular density. The Tukey test was used to determine which groups accounted for any observed differences. To assess the potential relationship between vascular density and the percentage of GLUT1-positive vessels within tumors, the correlation coefficient was calculated using data from all of the individual tumor specimens.[24]

Results

Expression of Glucose Transporter (GLUT1)

All tumors grew well in brain parenchyma, forming densely cellular tumors. They also simulated human neoplasms in that the gliomas invaded normal brain at their periphery, while the Walker 256 carcinoma was well demarcated as is characteristic of metastatic tumors. Assessment of GLUT1 expression was limited to areas of solid tumor, thus avoiding infiltrating border zones where vessels may be surrounded by a mixture of normal and neoplastic cells. Normal brain assessments were made in the cortical grey and caudate-putamen of contralateral hemispheres.

As previously reported,[11,12] essentially all impermeable normal brain vessels expressed GLUT1 (99.7 ± 1.5%, mean ± SEM; not significantly different from 100%) (Figures 1, 3). The percentage of GLUT1-positive vessels

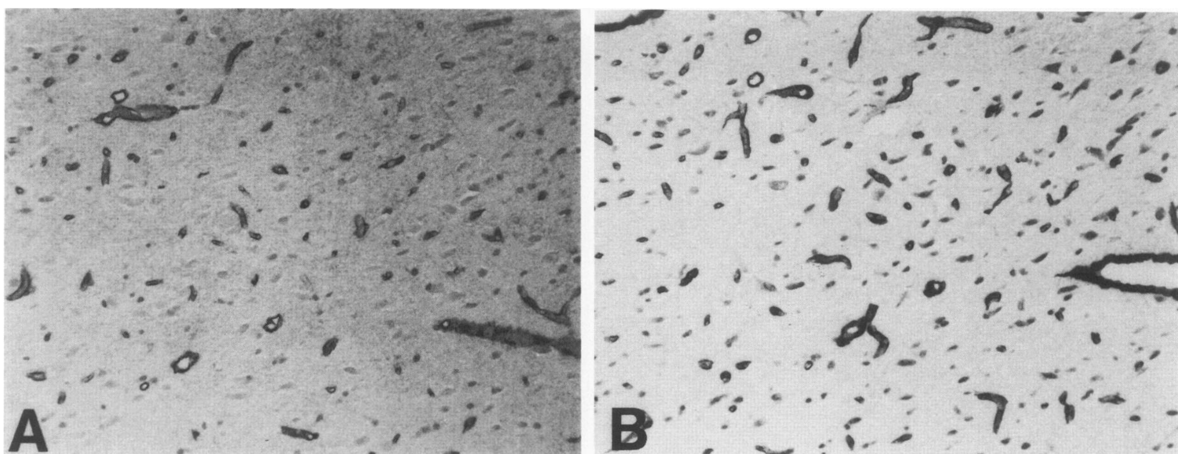
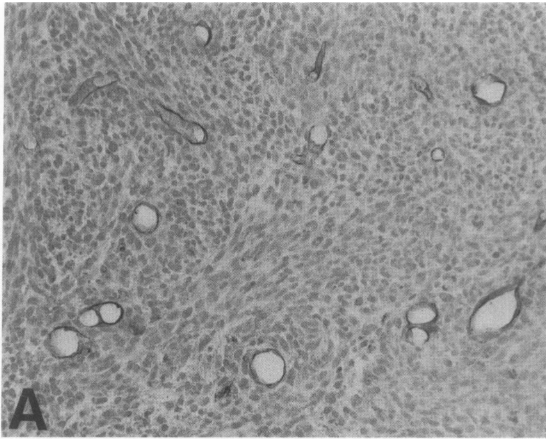


Figure 1. *Normal brain stained with antisera to the glucose transporter (GLUT1)* **(A)** *or laminin* **(B)**. *For orientation, note the very similar vascular patterns in the upper left quadrants. (hematoxylin counterstain, magnification ×115).*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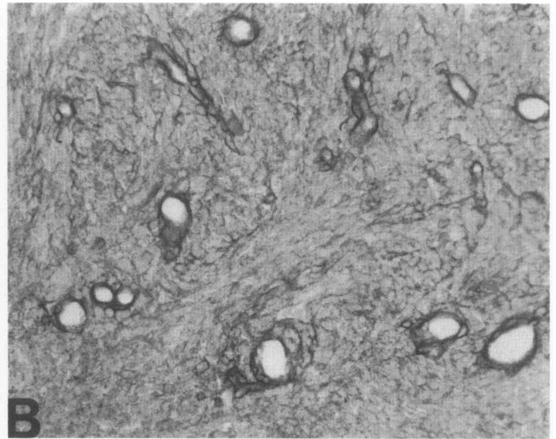
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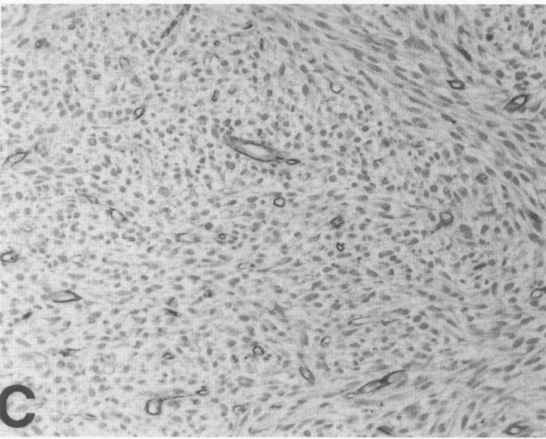
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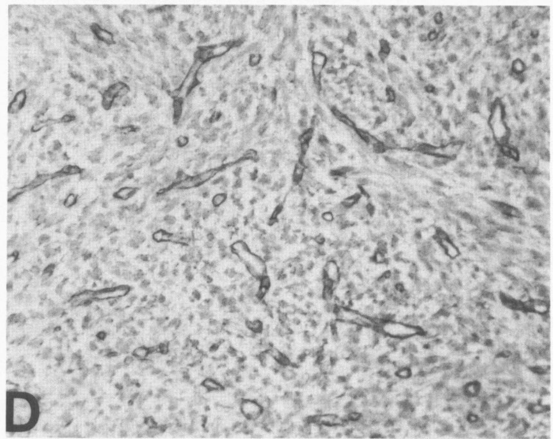
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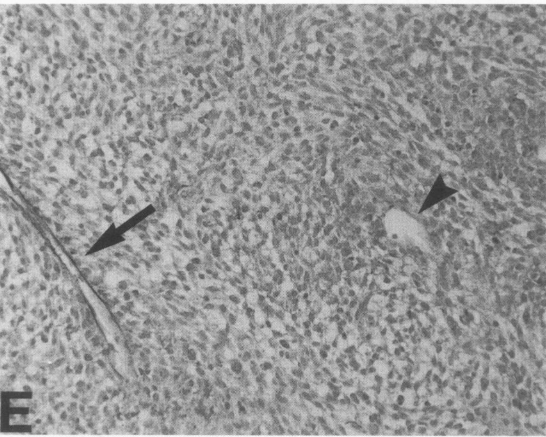
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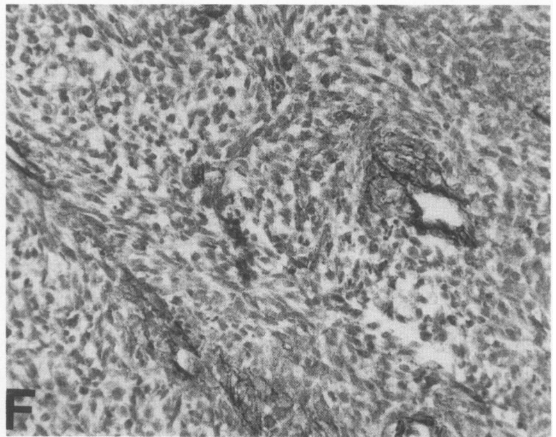
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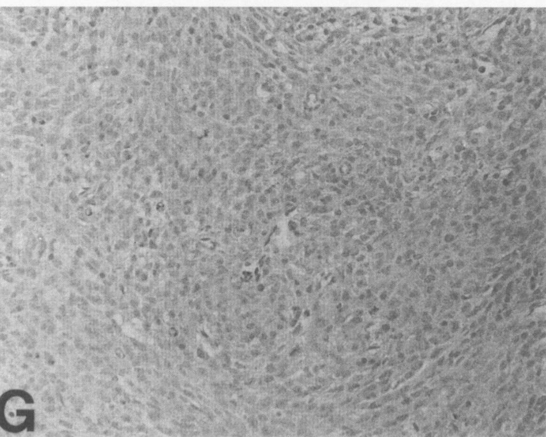
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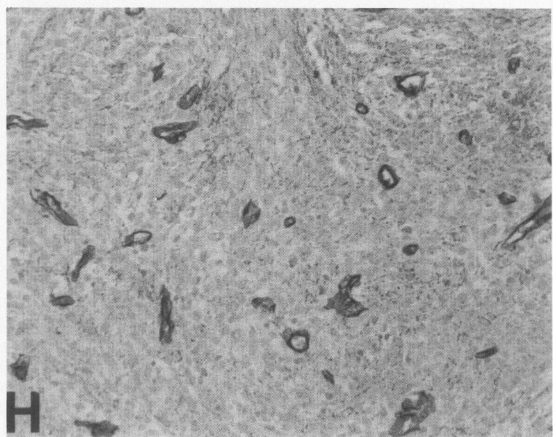
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for each tumor was significantly different from that of other tumors ($P < 0.001$; except 9L and C6: $P < 0.025$). Approximately half of the vessels in 9L and C6 gliomas were stained by GLUT1 antisera (60.9 ± 3.9% and 45.4 ± 5.6%, respectively) (Figures 2, 3). The nonglial Walker 256 carcinoma only expressed GLUT1 in a few vessels at the extreme periphery of solid tumor, accounting for about 1% of counted vessels (1.21 ± 0.31%, Figure 3). Vessels in central areas were consistently GLUT1-negative (Figure 2). Nearly all vessels of the F98 glioma expressed GLUT1 (95.5 ± 3.9%) (Figures 2, 3). In fact, the percentage of glucose transporter positive vessels in F98 tumors was not significantly different from that observed in normal brain ($P > 0.5$; pooled mean and 95% confidence interval 97.7 ± 4.6%). GLUT1 antisera did not stain tumor cells or brain parenchyma.

Expression of the Permeability Barrier

To investigate its relationship to GLUT1 expression, vascular permeability was assessed by intravenous administration of Evans blue. All tumors, including F98 gliomas in which nearly all vessels were GLUT1-positive, were diffusely stained by Evans blue (Figure 4). This staining indicated that tumor vessels did not significantly restrict the diffusion of polar compounds into parenchyma, and thus these vessels were classified as highly permeable. In contrast, vessels of normal brain were impermeable since Evans blue did not diffuse into parenchyma. No areas of unstained tumor were observed and blue staining did not grossly extend beyond the tumor-brain interface.

Vascular Density and Morphology

Vascular density was determined for each tumor type using immunostaining for laminin, a reliable vascular marker in brain neoplasms and normal brain.[22,23] Tumor parenchyma did occasionally stain with antisera to laminin, but with a low intensity and pattern clearly different from that of the vascular basal lamina.

Vascular density varied significantly among the tumors ($P < 0.0005$; Figure 5), and all tumors were significantly different from each other except for F98 and C6. Vessels of F98 and C6 gliomas were also larger and more variable in size and shape compared with the others (Figure 2). However, all had significantly lower vascular density than normal brain ($P < 0.001$). There was no significant correlation between vascular density and the percentage of GLUT1-positive vessels among tumors ($r = -0.184$; $P > 0.5$).

Expression of Glial Fibrillary Acidic Protein (GFAP)

All tumors were stained with antisera to GFAP to assess whether they contained cells that expressed this marker of astrocytic differentiation. In addition, vessels were assessed for an association with perivascular GFAP-positive processes, which might arise from normal or neoplastic cells.

Despite the glial origin of C6, 9L, and F98 cells, only C6 tumors contained rare GFAP-positive cells. Several of these cells were undergoing mitosis, suggesting that they were tumor cells, though others may have been reactive astrocytes (Figure 6). Normal brain vessels were frequently seen to be ensheathed by GFAP-positive processes of astrocytes (Figure 6). However, no vessels within any of the tumors were associated with perivascular GFAP staining.

Discussion

We investigated glucose transporter expression by brain tumor vessels in a series of experimental neoplasms using immunohistochemical techniques. Expression of GLUT1 normally occurs in cerebral vessels which also express a permeability barrier.[11,12] Our study shows that brain tumor vessels also express GLUT1, that expression varies with tumor type, appears to be limited to tumors of glial origin, and occurs independently of restricted vascular permeability. GLUT1 expression was also unrelated to vascular density and vascular morphology, and was not associated with parenchymal GFAP expression. Thus, GLUT1 expression appears to be a newly described, differentiated property of certain glial tumor vessels that is seen despite the presence of other vascular characteristics associated with malignancy.

In addition to the brain, impermeable vessels in the normal eye, peripheral nerve, and testis have all been previously shown to express high levels of GLUT1, unlike more permeable vessels elsewhere in the body.[14,25]

Figure 2. *Representative sections of neoplasms stained with antisera to GLUT1* **(A,C,E,G)**, *or the vascular marker laminin* **(B,D,F,H)**. **A,B**: *F98 glioma;* **C,D**: *9L gliosarcoma;* **E,F**: *C6 glioma;* **G,H**: *Walker 256 carcinoma. Essentially all F98 glioma vessels are GLUT1-positive. A positive (arrow) and a negative (arrowhead) vessel are clearly seen in the C6 glioma. Vessels in the nonglial Walker 256 are GLUT1-negative. F98 and C6 glioma vessels are less dense and more variable in size and shape than vessels in other tumors (hematoxylin counterstain, magnification ×110).*

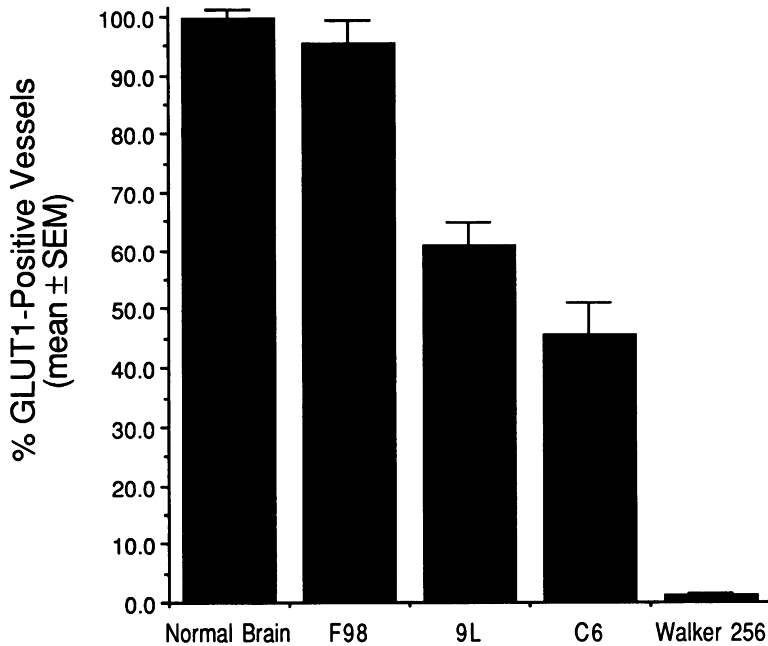

Figure 3. *Percentage of GLUT1-positive vessels in normal brain and brain neoplasms. Only glial tumors express GLUT1 in a significant proportion of vessels. The percentage of GLUT1-positive vessels in F98 gliomas was not significantly different from that observed in normal brain.*

Therefore, GLUT1 has been proposed as a possible histologic marker of barrier vessels, with strict linkage of GLUT1 expression to restricted permeability.[14,15] In brain tumors, high vascular permeability might thus lead to decreased transporter expression. Recently, Harik and Roessmann[26] found essentially no human brain tumors that expressed vascular GLUT1. However, only a small number of specimens from each tumor type were studied and vascular permeability was not specifically assessed. In the present study, vessels within all of these tumors were highly permeable, as demonstrated by the free diffusion of Evans blue into parenchyma. In C6 and 9L tumors, unstained vessels may have accounted for elevated permeability, whereas vessels expressing GLUT1 were impermeable. However, in F98 tumors high vascular permeability occurred despite essentially all vessels expressing GLUT1. In fact, the percentage of vessels expressing GLUT1 in permeable F98 tumors and impermeable normal brain was not statistically different. This finding is in accordance with our previous report that essentially all vessels in 80% of human anaplastic astrocytomas express high GLUT1 levels despite elevated permeability demonstrated by contrast enhanced imaging.[16] Therefore, we conclude that in tumor vessels

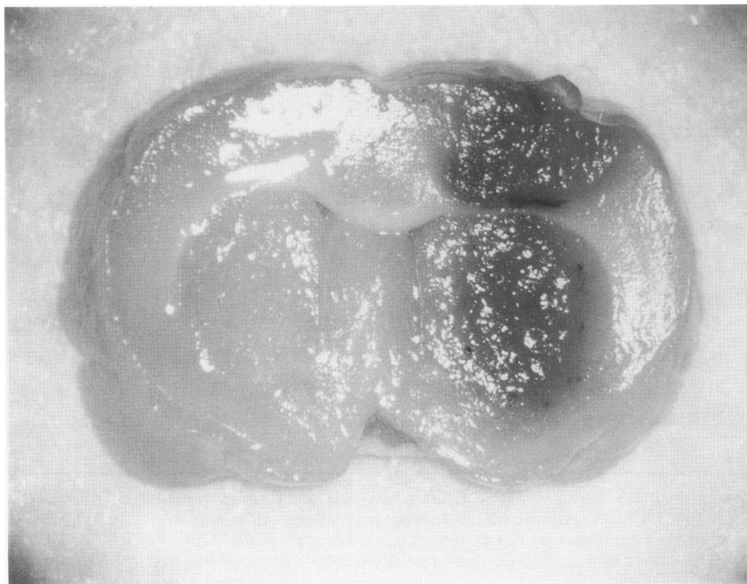

Figure 4. *Coronal brain section containing an F98 glioma after intravenous injection of Evans blue. The tumor is stained diffusely blue, indicating absence of the permeability barrier. (magnification ×50).*

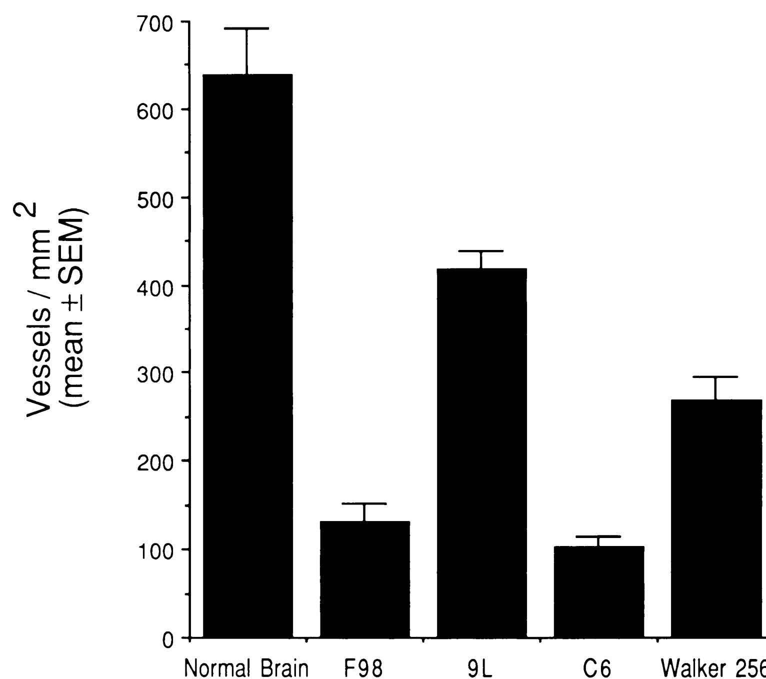

Figure 5. *Vascular density of normal brain and brain neoplasms. Vascular density varied among tumor types, but was always less than that of normal brain.*

GLUT1 expression is not strictly linked to restricted vascular permeability, but is regulated by independent mechanisms.

The staining techniques used in this study were chosen to optimize normal brain microvessel staining, thus defining a threshold level of detection. Since this technique does not quantify GLUT1 protein levels, it is possible that positively stained tumor vessels contain less GLUT1 protein than do normal brain vessels. In addition, differences in the detection threshold in other studies (e.g., due to different antibody titer or source) may account for apparently conflicting findings. Ideally, glucose transporter levels could be quantitated by cytochalasin B binding or immunoblotting methods.[12,13] These techniques require the isolation of tumor microvessels uncontaminated by normal brain vessels, which is technically difficult in the case of small experimental rat gliomas.

Although the cellular and molecular mechanisms mediating endothelial GLUT1 expression remain unknown, its expression was essentially limited to tumors of glial origin in our studies of both rat and human neoplasms.[16] A significant proportion of vessels in F98, 9L, and C6 gliomas expressed transporter. In Walker 256 carcinomas, staining was only observed in a few vessels near the tumor-brain interface. Likewise, we previously showed that human astroglial tumors express vascular

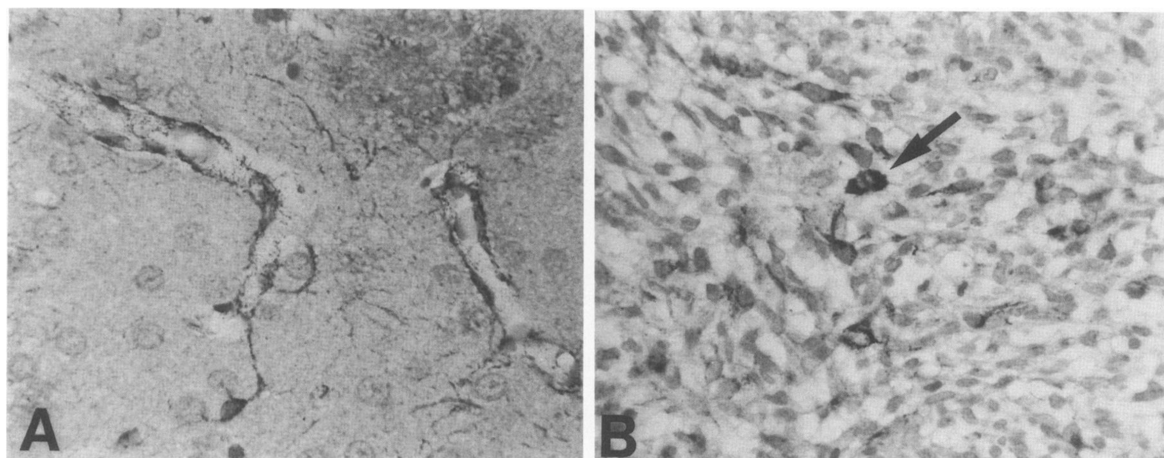

Figure 6. *Normal brain* (A) *and C6 glioma* (B) *stained with GFAP antiserum. Normal brain vessels are ensheathed by GFAP-positive astrocytic processes. C6 glioma parenchyma contains rare GFAP-positive cells, one of which is undergoing mitosis (arrow). (hematoxylin counterstain, magnification ×270).*

GLUT1 whereas metastases do not.[16] Recent studies have shown that parenchymal transplants of normal brain can induce endothelial expression of barrier properties *in vivo.*[27,28] *In vitro,* isolated astrocytes can induce biochemical and structural barrier properties in endothelial cells.[29–31] Since GFAP is a standard marker of astrocytic differentiation, we investigated whether tumors with transporter-positive vessels also expressed GFAP. Although normal brain vessels were frequently ensheathed by GFAP-positive processes, this was not observed within tumors. Only C6 tumor parenchyma contained GFAP-positive cells, but they were rare and not associated with vessels. If astroglial-derived cells induce vascular glucose transporter, then that induction must persist even with loss of GFAP expression. It is unlikely that brain vessels growing into tumors simply retain GLUT1 expression since essentially all vessels in the nonbrain derived Walker 256 were transporter-negative. It is also unlikely that there is a progressive loss of GLUT1, which is more rapid in some tumors than in others, since we have shown that the percentage of GLUT1-positive vessels in 9L gliomas remains constant as tumors grow.[32] Thus, an ongoing inductive influence by glial tumor cells on microvessels appears to be required for GLUT1 expression.

Vascular density has been correlated with the malignancy of neoplasms[33,34] and the proliferation of morphologically abnormal vessels is characteristic of gliomas.[3–5] The tumors studied here varied greatly in vascular density, but there was no correlation between vessel density and the percentage of vessels expressing GLUT1. F98 and C6 gliomas had statistically identical vascular densities, yet the vessels of the latter expressed GLUT1 half as often as the former. However, since our study does not assess the actual amount of GLUT1 protein expressed by positive cells, we cannot rule out more subtle quantitative relationships between GLUT1 expression and vascular density. Vascular morphology was also most abnormal in F98 gliomas, with wide variation in the size and shape of vascular profiles. These characteristics of F98 vasculature have been noted in previous quantitative studies.[35] Since all of these vessels were shown to express GLUT1, such expression is not limited to tumor vessels having a normal appearance. A similar finding was noted in human malignant gliomas where foci of endothelial proliferation were often seen to express GLUT1.[16]

Our findings have implications for the biology of both malignant gliomas and the blood-brain barrier. The expression of glucose transporter by tumor vessels independently of permeability restrictions suggests that endothelial specializations comprising the blood-brain barrier are induced and maintained by a complex set of signals, rather than a single inductive event. We show that despite the loss of permeability restrictions, glucose transporter expression can be maintained in malignant gliomas. Immunostaining for the glucose transporter may help in distinguishing glial from nonglial tumors, since its expression has been limited to tumors of glial origin.[16] Of more importance, the fact that a tumor parenchyma retains the ability to induce endothelial glucose transporters may indicate that it is more differentiated than a tumor of similar appearance with negative vessels. Therefore, glucose transporter expression may provide additional diagnostic and prognostic information for the classification of brain neoplasms.

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