Vascular Differentiation and Glucose Transporter Expression in Rat Gliomas: Effects of Steroids

Christopher Guerin, MD,*† Johannes E. A. Wolff, MD,‡¶ John Laterra, MD, PhD,†‡¶ Lester R. Drewes, PhD,** Henry Brem, MD,*† and Gary W. Goldstein, MD‡§¶

The GLUT1 isoform of the glucose transporter is normally expressed at high levels in differentiated brain vessels that also express a permeability barrier. In contrast, malignant brain neoplasms have relatively undifferentiated vessels that are highly permeable, proliferate to high vascular densities, and often lose GLUT1 expression. Using the rat intracerebral 9L glioma model, we investigated whether dexamethasone-induced changes in permeability are associated with the appearance of other differentiated vascular properties. The percentage of vessels expressing immunohistochemically detectable GLUT1 (74.2 ± 6.1%) and the tumor vessel density as assessed by laminin immunostaining (282 ± 37 vessels/mm^2) did not vary with control tumor size. Dexamethasone treatment resulted in an 83% reduction of vascular permeability to intravenous Evans blue, an increased percentage of vessels expressing GLUT1 (106.4 ± 10.5%), lower vascular density (102 ± 64 vessels/mm^2), and smaller tumor size (control cross-sectional area, 17.0 ± 3.4 mm^2; treated, 4.6 ± 1.0 mm^2). Essentially all vessels became GLUT1-positive after dexamethasone treatment. Increased GLUT1 expression by glioma vessels in association with the appearance of other signs of differentiation (low vascular density, slow tumor growth) suggests that immunostaining for GLUT1 may identify neoplasms that are biologically less aggressive.

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Microvessels of the normal brain are highly differentiated. Structural specializations such as tight junctions impose a permeability barrier that restricts the entry of polar substances into the brain [1]. These permeability restrictions necessitate the presence of biochemical specializations to maintain the delivery of compounds required for normal function [2]. These endothelial specializations comprise the structural and biochemical components of the normal blood-brain barrier.

In contrast, the microvessels of brain neoplasms are relatively undifferentiated. Abnormalities of tumor vasculature include vasoproliferation; structural alterations, resulting in abnormally high permeability; and the loss of expression of enzymes, transporters, and other biochemical characteristics seen in more differentiated vessels [3–7]. Most of these abnormalities increase in incidence or severity as the tumor grade increases [5–9]. Thus, the state of tumor vessel differentiation seems to bear a relationship to the malignancy of the tumor.

One characteristic of normal brain endothelium that is frequently lost in malignant brain neoplasms is glucose transporter expression. The GLUT1 isoform of the glucose transporter is normally expressed at high levels in brain vessels that also express a permeability barrier [10–12]. In contrast, vessels in brain areas lacking a permeability barrier (e.g., area postrema, median eminence) do not normally express GLUT1 [11, 12]. GLUT1 apparently serves to maintain brain glucose supplies in the face of restricted permeability, and is part of the biochemical component of the blood-brain barrier. Therefore, it has been hypothesized that GLUT1 can serve as a marker of barrier endothelium, since its expression appears to be linked to permeability restrictions [13, 14].

The increased permeability that is characteristic of undifferentiated brain tumor vessels should eliminate the need for high endothelial GLUT1 levels by allowing free diffusion of glucose into the parenchyma. We and others have shown that vessels in most human glioblastomas and metastases are GLUT1-negative [5, 15]. However, we have also shown that most human

From the Departments of *Neurosurgery, †Oncology, ‡Neurology, and §Pediatrics, The Johns Hopkins University School of Medicine, and ¶The Kennedy Research Institute, Baltimore, MD; and the **Department of Biochemistry, University of Minnesota School of Medicine, Duluth, MN.

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Address correspondence to Dr Guerin, Department of Neurosurgery, Johns Hopkins Hospital, Meyer 7-109, 600 North Wolfe Street, Baltimore, MD 21205.

anaplastic astrocytomas and experimental rat gliomas express GLUT1 in their vessels despite the presence of high vascular permeability [5, 16]. Thus, it appears that microvessels in gliomas can express GLUT1 independently of permeability restrictions.

Despite this abnormal regulation of GLUT1 expression, transporter levels in glioma vessels may change in association with changes in vascular permeability. Dexamethasone is routinely used to reduce the vascular permeability in brain tumors. This restoration of barrier function suggests that such vessels are relatively more differentiated. In this report, we investigated whether dexamethasone-induced reductions in permeability are associated with the appearance of other differentiated vascular properties. We treated intracerebral 9L rat gliomas, tumors that express GLUT1 in a portion of their vessels, with dexamethasone and assessed the changes in permeability, GLUT1 expression, vascular density, and tumor growth.

Materials and Methods

Tumor Implantation and Dexamethasone Treatment

9L glioma cells [17] were obtained from Marvin Barker at the University of California at San Francisco Brain Tumor Research Center and were maintained in tissue culture until the time of injection into rat brains. Cells were grown in Dulbecco's modified Eagles medium (Mediatech, Washington, DC) with 10% fetal bovine serum (Hyclone, Logen, UT), containing 2 mM L-glutamine (Mediatech), 200 units/ml of penicillin G (Mediatech), and 200 μg/ml of streptomycin sulfate (Mediatech); incubated at 37°C in 5% carbon dioxide–95% air; and passaged at confluence by trypsinization.

Immediately prior to injection, cells were trypsinized, counted with a Coulter counter (Coulter Electronics, Hialeah, FL), and diluted in media at the appropriate cell concentration. Adult male Fischer 344 rats (Harlan, Indianapolis, IN) were anesthetized by intraperitoneal injection of 3 ml/kg of a solution containing 25 mg/ml of ketamine (Parke-Davis, Morris Plains, NJ), 2.5 mg/ml of xylazine (Mobay, Shawnee, KS), and 14% ethanol in normal saline solution. After the head was shaved and the surgical site was prepared with 70% ethanol and povidone-iodine (Purdue Frederick, Norwalk, CT), a midline skin incision was made and with a burr, a 1.5-mm-diameter hole was drilled in the skull 3 mm lateral to the bregma. Animals were placed into a stereotactic frame and 10 μl of cell suspension containing 10^5 9L cells was injected into the left caudate-putamen. Wounds were closed with staples.

Treatment was begun on postimplant day 2, to allow the incision to seal and the tumor to establish itself prior to dexamethasone exposure. Until they were sacrificed, rats received daily intraperitoneal injections of either dexamethasone sodium phosphate (Elkins-Sinn, Cherry Hill, NJ) diluted in sterile normal saline solution, or an equivalent volume of sterile normal saline solution without the drug. The dexamethasone dose in all experiments was 3 mg/kg/day, which reduces vascular permeability in other models [18].

Quantitative Vascular Permeability Studies

A modification of the method of Farrel and colleagues [19] was used to assess vascular permeability. Animals received their final injection of dexamethasone or saline solution and 1 hour later were anesthetized as described above. Two milliliters per kilogram of 2% Evans blue (Fisher Scientific, Fair Lawn, NJ) in phosphate-buffered saline solution (PBS) (Sigma Chemical, St. Louis, MO) was injected intravenously and allowed to circulate for 1 hour. Animals were then perfused transcardially with 200 ml of PBS at 60 ml/min. Brains were removed, the brainstems discarded, and each hemisphere was separately quartered and extracted in formamide (Sigma) in the dark at room temperature for 4 days. The Evans blue content of the supernatant was determined by comparing the absorbance at 620 nm with a standard curve derived from solutions of Evans blue in formamide. The value in the contralateral hemispheres was subtracted from that of the tumor-affected hemispheres to correct for any retained intravascular Evans blue and nonspecific absorbance by extracted proteins. Results were normalized to tumor volume as determined below.

Assessment of Tumor Size

After the animals were anesthetized as described above, they were perfused transcardially with 200 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 maximum cross-sectional area of the tumors were embedded to paraffin and 5-micron-thick sections were cut and stained with hematoxylin-eosin.

Maximal cross-sectional area was determined by computerized image analysis using the Microcomputer Imaging Device (MCID) software package of Imaging Research (Brock University, St. Catherines, Ontario, Canada), a Sierra Scientific (Sunnyvale, CA) high-resolution CCD camera, and a Compaq (Houston, TX) DeskPro 386/25 computer. Histological sections were digitized from the stained slides. Maximal cross-sectional area was measured by tracing the tumor border using the manual area format. Values proportional to tumor volume were obtained from the cube of the square root of the area [20].

Immunohistochemical Staining

Paraffin-embedded specimens were prepared as described above and serial 5-micron-thick sections were cut and stained with rabbit antisera to glucose transporter (GLUT1), laminin (Gibco, Grand Island, NY), or glial fibrillary acidic protein (GFAP) (Accurate, Westbury, NY). Laminin antiserum stains vascular basal lamina and is a reliable marker of vessels in brain neoplasms and normal brain [21]. Rabbit antiserum to GLUT1 was raised by one of us (L. R. D.) against a synthetic peptide identical to the carboxyl terminus of the "rat brain/human erythrocyte" glucose transporter, generally designated GLUT1 [11]. This antiserum was previously shown to specifically identify GLUT1 by several criteria: (1) It selectively stains microvessels of the blood-brain barrier in normal cerebrum, cerebellum, and peripheral nerve. (2) It does not stain permeable vessels of the adenohypophysis. (3) It identifies a protein of the appropriate molecular weight (relative molecular weight, 45,000–55,000) on Western blots of iso-

lated brain microvessels. (4) All staining is inhibitable by antisera adsorption with a GLUT1-specific carboxyl terminal peptide. (5) Immunoelectron microscopy specifically localizes staining to the luminal and abluminal endothelial cell plasma membranes, as predicted by physiological studies [11, 22].

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 to 20 minutes at 37°C with 0.2% pepsin (Calbiochem, LaJolla, CA) in 0.01 N hydrochloric acid just prior to peroxide incubation. Sections were incubated overnight at 4°C with a 1:1,000 dilution of the relevant primary antiserum 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 antirabbit immunoglobulin (Vector) diluted in PBS containing 1.5% normal goat serum, 30-minute incubation with 1:50 avidin-biotin complex (ABC) reagent (Vector) in PBS, and 15-minute incubation with 0.5 mg/ml of 3,3′-diaminobenzidine (Sigma) and 0.01% hydrogen peroxide in 50 mM Tris-buffered normal saline solution. Sections were counterstained with hematoxylin, dehydrated, and mounted in Permount (Fisher). For control specimens, immune serum was substituted with nonimmune rabbit serum (Vector) or immune serum that had been preadsorbed with 10 μM GLUT1-specific carboxyl terminal peptide. All control specimens 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. Five fields per specimen were counted independently by two observers. Counted fields spanned the total area of solid tumor and excluded infiltrating border zones where tumor cells and normal brain tissue intermingled. For each specimen, the percentage of GLUT1-positive vessels was calculated as the average number of GLUT1-positive profiles per field divided by the average number of laminin-positive profiles per 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 the tumor cells and the perivascular areas in the tumor and normal brain.

Statistical Analysis

For experiments with one experimental and one control group, two-tailed Student's *t* tests were used to determine whether differences existed between means. For experiments with multiple groups, single-factor analyses of variance were performed to determine whether differences in the percentage of GLUT1-positive vessels or in vascular density existed among groups. The Tukey test was used to determine which groups accounted for any observed differences. To assess the potential relationships between tumor size and the percentage of GLUT1-positive vessels or vascular density, correlation coefficients were calculated using data from all of the individual tumor specimens [23].

Results

GLUT1 Expression and Vascular Density in Relation to Vascular Permeability

To assess the relationships between GLUT1 expression, vascular density, and vascular permeability, all three variables were quantified in simultaneously implanted, intracerebral 9L gliomas. Fourteen rats were treated with daily intraperitoneal injections of dexamethasone (3 mg/kg/day) and 14 control rats received injections of normal saline solution. Permeability was quantified in 8 from each group and the other 6 were fixed for immunohistochemical staining and size determinations. Preliminary studies showed that dexamethasone treatment decreased the tumor size, and other tumor models are known to demonstrate lower permeability in smaller tumors [24, 25]. Therefore, treated tumors at 9 days after implantation were compared to smaller 6-day control tumors in order to eliminate possible size-related permeability reductions.

Dexamethasone therapy reduced vascular permeability 82.7% as evidenced by the lower content of Evans blue in treated tumors after intravenous injection of the dye (Table). The maximal cross-sectional area of control tumors was smaller than that of treated tumors (mean ± standard error of mean [SEM], 1.67 ± 0.49 mm^2 versus 4.67 ± 1.07 mm^2). Although permeability was significantly reduced, it was not normalized, and treated tumors were still visibly blue compared to normal brain tissue.

As predicted by prior studies, GLUT1 antiserum stained microvessels in normal brain tissue and stained the impermeable choroid plexus epithelium, but not the permeable plexus endothelium [11, 12]. Tumor cells and normal brain parenchyma were unstained. Microvessels in all tumors were stained by GLUT1 antiserum. In control tumors, about 80% of the vessels were GLUT1-positive. However, dexamethasone-treated tu-

Effects of Dexamethasone Treatment on 9L Gliomas[a]

	Permeability (ng of Evans blue/mm^3)	%GLUT1-Positive Vessels	Vascular Density (vessels/mm^2)
Dexamethasone	123 ± 13[b]	98.3 ± 2.7[b]	179 ± 15[b]
Control	713 ± 138	80.0 ± 1.1	476 ± 53

[a]Values are expressed as mean ± standard error of mean. For permeability values, n = 8; for other values, n = 6.
[b]$p < 0.001$ compared to control values.

mors expressed GLUT1 in essentially all observed vessels (see Table). The percentage of GLUT1-positive vessels in treated tumors was significantly higher than that observed in control tumors ($p < 0.001$). All positive staining in the tumor and normal areas was completely inhibited by the adsorption of antiserum with 10 μM GLUT1-specific carboxyl terminal peptide.

Vascular density was determined by immunostaining for laminin, a basement membrane glycoprotein that reliably identifies brain tumor vessels [21]. Laminin antiserum did occasionally stain tumor parenchyma, but with a low intensity and diffuse pattern clearly different from that of microvessels. In dexamethasone-treated tumors, vascular density was significantly lower than in control tumors ($p < 0.001$; see Table).

GLUT1 Expression and Vascular Density in Relation to Tumor Growth

We investigated whether the observed differences in GLUT1 expression and vascular density after dexamethasone treatment were due to differences in tumor size. Control tumors were studied at several time points (postimplant days 6, 8, and 9; 6 rats each day), and compared to simultaneously implanted tumors treated with 3 mg/kg/day of dexamethasone (postimplant day 9; n = 6). Time points were chosen based on the 10-day median length of survival of untreated tumor-bearing rats in our laboratory. Control tumors grew well during this period (Fig 1). The maximum cross-sectional area of the treated tumors at 9 days after implantation (mean ± SEM, 4.6 ± 1.0 mm^2) was smaller than that of control tumors at 9 days (17.0 ± 3.4 mm^2). Treated tumors at 9 days were equivalent in size to 6-day control tumors (3.8 ± 0.9 mm^2). Thus, in this experiment, dexamethasone-treated tumors could be compared to control tumors either of the same size or on the same postimplant day.

Vessels in all tumors demonstrated positive staining with GLUT1 antiserum (Fig 2). There was no significant difference in the percentage of GLUT1-positive vessels among control tumors studied at 6, 8, or 9 days after implantation, with a mean of 74.2 ± 6.1% (pooled mean ± 95% confidence interval) (Figs 2, 3). There was also no correlation between individual tumor size and the percentage of GLUT1-positive vessels in control tumors ($r = 0.118$, $p > 0.5$). However, dexamethasone-treated tumors again expressed GLUT1 in essentially all observed vessels (106.4 ± 10.5%) (see Figs 2, 3). This staining was completely inhibited by the adsorption of antiserum with the carboxyl terminal peptide of GLUT1. The percentage of GLUT1-positive vessels in treated tumors was significantly higher than that observed in all control tumors, regardless of the size or postimplant day ($p < 0.001$ versus day 8 and 9 control tumors, $p < 0.025$ versus day 6).

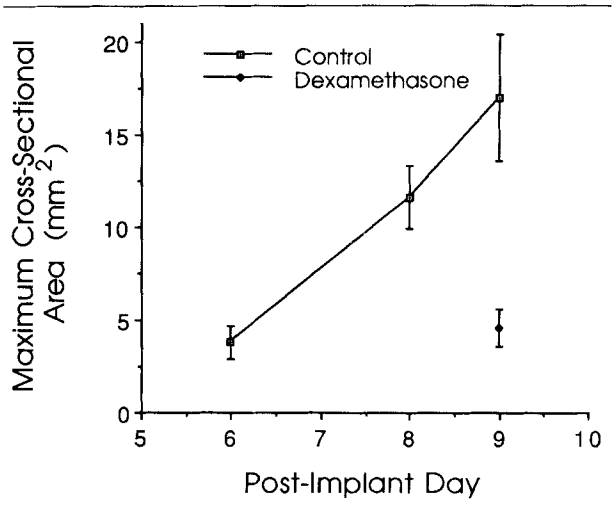

Fig 1. Growth curve of intracerebral 9L gliomas. Each point represents the mean of 6 tumors. Dexamethasone-treated tumors at 9 days were equivalent in size to 6-day control tumors. Error bars = standard error of the mean.

Vascular density did not vary significantly among control tumors of different postimplant days, having a mean of 282 ± 37 vessels/mm^2 (pooled mean ± 95% confidence interval) (Figs 2, 4). There was no correlation between individual tumor size and the density of microvessels ($r = 0.281$, $p > 0.2$). Dexamethasone-treated tumors, however, demonstrated a significantly lower vascular density than did control tumors of any size or postimplant day (102 ± 64 vessels/mm^2, $p < 0.005$ versus day 6 and 8 control tumors, $p < 0.05$ versus day 9) (see Figs 2, 4).

Glial Fibrillary Acidic Protein Expression

Since normal brain vessels are ensheathed by astrocytic processes thought to induce blood-brain barrier properties [26], we investigated whether the increased GLUT1 expression seen with dexamethasone therapy was associated with an induction of GFAP expression within the tumors. GFAP-positive processes were frequently seen surrounding normal brain vessels, but control and treated tumors demonstrated no staining by GFAP antiserum in tumor cells or in perivascular areas.

Discussion

We investigated changes in several vascular properties of intracerebral 9L gliomas in rats after systemic dexamethasone treatment. In control tumors, the percentage of vessels expressing GLUT1 and vascular density did not change significantly during tumor growth. A reduction of vascular permeability with dexamethasone was associated with an increased percentage of vessels expressing GLUT1, lower vascular density, and smaller tumor size.

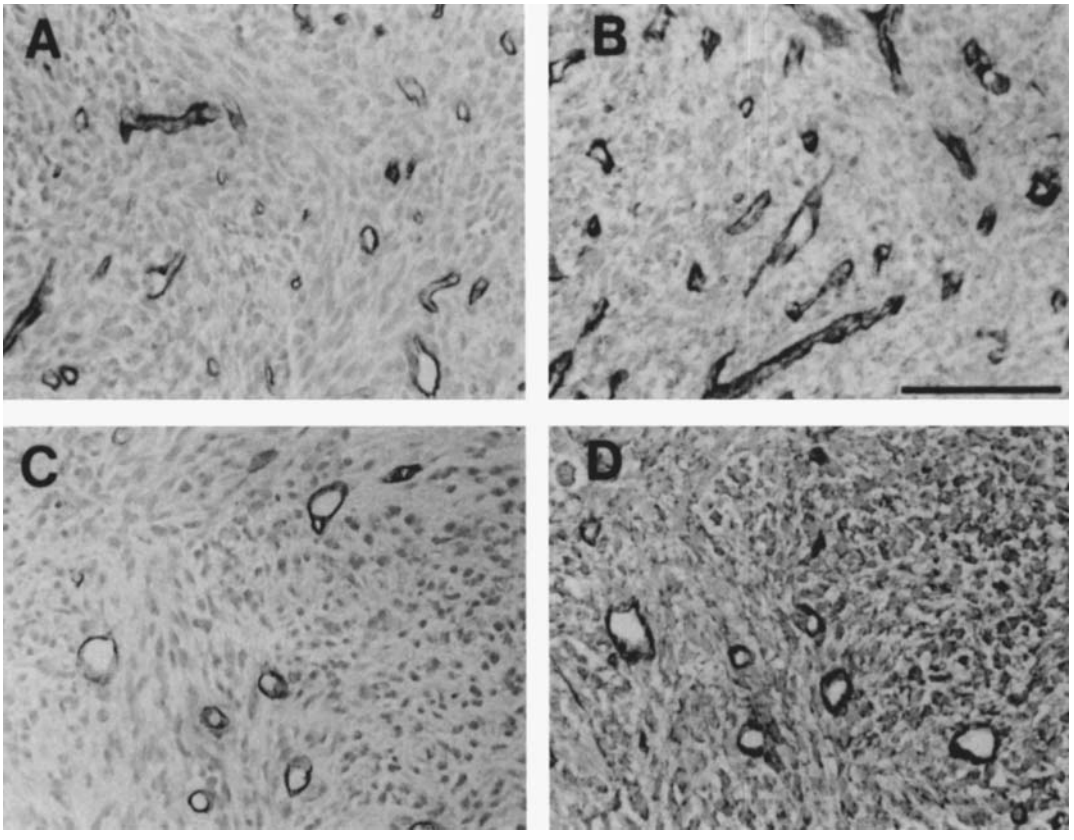

Fig 2. (A, B) Control and (C, D) dexamethasone-treated tumors stained with antisera to GLUT1 (A, C) or laminin (B, D). Treated tumors expressed GLUT1 in a greater percentage of vessels (essentially 100%) and had a lower vascular density than did control tumors. Scale bar = 100 microns. (Hematoxylin counterstain.)

In normal brain, endothelial GLUT1 expression is associated with the presence of a permeability barrier [10–12]. Since this barrier inhibits the parenchymal entry of polar compounds, high levels of GLUT1 expression are necessary to maintain adequate glucose supplies. The fact that endothelial barriers in the eye, peripheral nerve, and testis also express high GLUT1 levels, whereas nonbarrier endothelium in the brain and other peripheral tissues does not, has led to the proposal that GLUT1 may be a histological marker of barrier vessels [13, 14, 27]. Such a marker would be potentially useful in the evaluation and treatment of brain neoplasms, where the retention of barrier properties may adversely affect drug delivery.

In contrast to normal brain vessels, brain tumor vessels may express GLUT1 despite the loss of permeability restrictions. Harik and Roessmann [15] found essentially no human brain tumors containing GLUT1-positive vessels, but only a small number of specimens from each tumor type were evaluated and results were not correlated with vascular permeability assessments.

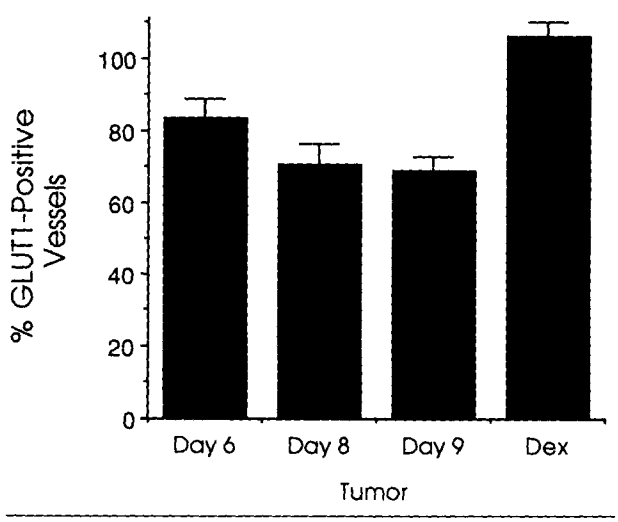

Fig 3. GLUT1 expression during growth of control tumors at postimplant days 6, 8, and 9 and dexamethasone-treated tumors (Dex) *at day 9 ($n = 6$ for each group). In the control tumors, GLUT1 expression during tumor growth did not vary significantly. However, dexamethasone-treated tumors had a higher percentage of GLUT1-positive vessels than did control tumors of the same size or same postimplant duration* ($p < 0.001$ *versus day 8 and 9 control tumors,* $p < 0.025$ *versus day 6; see Fig 1). Error bars = standard error of the mean.*

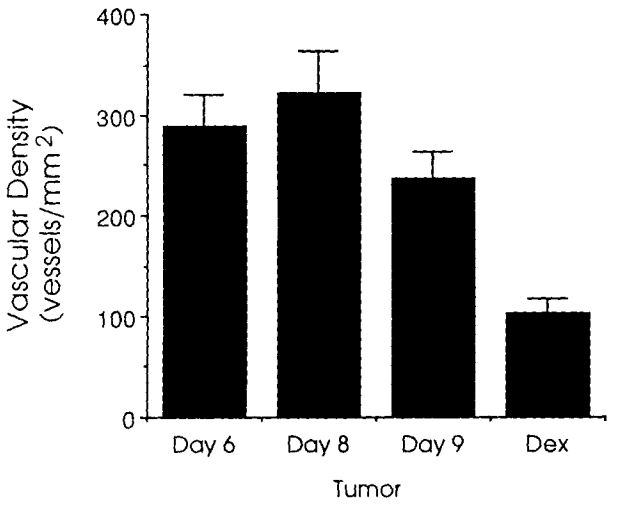

Fig 4. Vascular density during growth of control tumors at postimplant days 6, 8, and 9 and dexamethasone-treated tumors (Dex) *at day 9 (n = 6 for each group). In the control tumors, vascular density during tumor growth did not vary significantly. However, dexamethasone-treated tumors had a lower vascular density than did control tumors of the same size or postimplant duration* ($p < 0.005$ *versus day 6 and 8 control tumors,* $p < 0.05$ *versus day 9; see Fig 1). Error bars = standard error of the mean.*

In an earlier report, we likewise found that vessels in most contrast-enhancing (i.e., permeable) human glioblastomas and metastases are GLUT1-negative [5]. However, most human anaplastic astrocytomas did express GLUT1 in all their vessels despite having demonstrated high vascular permeability [5]. Similar findings were noted in the highly permeable, rat F98 glioma model in which essentially all vessels express GLUT1 [16]. Thus, we concluded that the relationship between vascular permeability and endothelial GLUT1 expression in brain neoplasms is different from that found in normal tissue. Consequently, GLUT1 cannot be used as a barrier marker in brain neoplasms.

The present study extends our understanding of GLUT1 expression in gliomas. Intracerebral 9L gliomas normally express GLUT1 in a significant proportion of vessels despite the presence of high permeability. When permeability is lowered by dexamethasone, all tumor vessels become GLUT1-positive, thus resembling differentiated normal brain vessels. Additional observations in dexamethasone-treated tumors are also consistent with the presence of a more differentiated state. Studies of human neoplasms suggested that increased malignancy is associated with increased vascular density [28, 29]. In our study, treated tumors had a significantly lower vascular density than did control tumors. These tumors were also consistently smaller than were control tumors of the same age. Therefore, the increase in vascular differentiation appears to be associated with a decrease in the biological aggressiveness of treated tumors.

The mechanisms by which these vascular properties are altered remain unclear. The observed effects may result from a direct action of dexamethasone on endothelial cells. In vitro studies demonstrated the direct effects of dexamethasone on GLUT1 protein and messenger RNA levels [30–32]. However, none of these studies were done in endothelial cells. Corticosteroids also play a role in the inhibition of tumor-induced neovascularization, which may lower vascular density [33, 34]. However, it is unlikely that selective inhibition of the growth of GLUT1-negative vessels accounts for increased GLUT1 expression, since the observed vascular density of treated tumors is half that predicted by such an effect. Dexamethasone may also act via perivascular intermediaries. Inflammatory cells release cytokines that affect GLUT1 expression and vascular properties [35–37]. Since glial cells induce several blood-brain barrier properties in endothelial cells [26, 38–40] and influence vascular morphogenesis [41], dexamethasone may also act through 9L cells.

These findings are relevant to the biology of malignant gliomas and of the blood-brain barrier in general. The multiple endothelial specializations comprising the blood-brain barrier do not appear to be regulated as a single entity. The range of GLUT1 expression seen in brain neoplasms suggests that permeability-restricting components of the barrier can be downregulated without affecting the expression of biochemical components like the glucose transporter. Furthermore, increased GLUT1 expression by glioma vessels in association with the appearance of other signs of differentiation suggests that immunostaining for GLUT1 may identify neoplasms that are biologically less aggressive, and thus may be of potential prognostic significance.

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