



## Research Report

# Endothelial differentiation in intracerebral and subcutaneous experimental gliomas

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### Abstract

Blood–brain barrier (BBB) properties of endothelial cells have an impact on brain tumor behavior, diagnosis, and response to therapy. Biochemical BBB properties are expressed by endothelial cells within intracerebral (IC) gliomas but little is known regarding the expression of BBB-associated proteins within gliomas established subcutaneously (SC), a site that is frequently used in experimental glioma models. We compared the expression of two BBB proteins, glucose transporter type-1 (Glut1) and endothelial barrier antigen (EBA), in IC and SC rat 9L and F98 gliomas. The percentage of microvessels with immunohistochemically-detectable Glut1 and EBA in IC 9L tumors (31–98%) contrasted with that found in SC 9L tumors (0–3.9%) ( $P < 0.0001$ ). Likewise, the percentage of immunohistochemically-positive vessels in IC F98 tumors (35–66%) differed markedly from that in SC F98 tumors (0%) ( $P < 0.0001$ ). These differences were not explained by effects of tumor location on vessel density or tumor histology. These findings demonstrate that the peritumoral environment influences endothelial differentiation within glial tumors and suggest that glioma cells maintain but do not induce the expression of barrier properties in vessels that infiltrate tumor from surrounding tissue.

**Key words:** Endothelium; Glioma; Differentiation; Microvessel; Blood–brain barrier

### 1. Introduction

Under normal conditions, blood vessels in the central nervous system (CNS) are quiescent and express a complex array of structural and biochemical properties that comprise the blood–brain barrier (BBB) [12,18, 23,28,41]. The abnormal growth and barrier properties of tumor blood vessels are critical determinants of brain tumor biology, diagnosis, and response to therapy [5,6,14,16,17]. Tumor diagnosis and drug delivery frequently rely upon structural abnormalities, such as fenestrations and diminished numbers of tight junctions, that have long been recognized to account for the increased permeability of brain tumor microvessels [25,35]. More recent studies have identified alterations in biochemical barrier properties of microvessels within human brain tumors [17,19,25,26,35]. Our laboratory

has found that microvessel GLUT1 expression diminishes with increasing tumor grade in human gliomas, and is absent in carcinoma metastatic to brain [17]. Subsequent studies using intracerebral 9L, F98, and C6 experimental gliomas, or the Walker 256 carcinoma, confirmed that a subset of microvessels within intracerebral tumors of glial origin, but not within tumors of nonglial origin, retain GLUT1 expression [15]. These results demonstrate biochemical heterogeneity amongst microvessels within intracerebral gliomas and are consistent with the generally held belief that the expression of endothelial BBB properties requires the presence of perivascular cells of astroglial origin [7,20,32,39]. These previous studies used intracerebral tumors that were vascularized by peritumoral BBB vessels and did not address the question of whether glioma vessels derived from non-BBB peritumoral vessels similarly express biochemical BBB characteristics. The influence of peritumoral environment on blood–tumor barrier expression is important because extracranial glioma models are frequently used in the

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preliminary assessment of diagnostic or chemotherapeutic agents [3,4,9,30,33,36,40,42].

This report examines the effect that the tissue site of experimental glioma implantation has on tumor vascularity, and the expression of BBB-specific endothelial antigens. Microvessel expression of two BBB-specific proteins, GLUT1 and EBA, was analyzed in experimental 9L and F98 gliomas grown both intracerebrally and subcutaneously in adult rats. Microvessels within tumors established outside of the CNS were found to express little or no GLUT1 and EBA in contrast to tumors grown within the brain. These and earlier findings demonstrate that endothelial expression of BBB-specific proteins within glial tumors is influenced by properties of both the glioma cells and the environment within which these cells reside. These findings have implications for the use of extracranial glioma models and for our understanding of the determinants of microvessel differentiation within the CNS.

## 2. Materials and methods

### 2.1. Cell lines

F98 and 9L cells were grown in Dulbecco's modified Eagles medium (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine (Mediatech), 200 U/ml of penicillin G (Mediatech), and 200 mg/ml of streptomycin sulfate (Mediatech) as previously described [15,21,34]. F98 cells were originally obtained from Joseph Goodman (Ohio State University School of Medicine) and 9L cells were originally obtained from Marvin Baker (University of California at San Francisco Brain Tumor Research Center).

### 2.2. Tumor models

**Intracerebral tumors.** Cell suspensions were implanted as previously reported [15,16]. Briefly, adult male Fischer 344 rats (Harlan, Indianapolis, IN) were anesthetized by intraperitoneal injection of 3 ml/kg of a solution containing 25 mg/ml ketamine (Parke-Davis, Morris Plains, NJ), 2.5 mg/ml xylazine (Mobay, Shawnee, KS), and 14% ethanol in normal saline solution. A midline skin incision was made, and a 1.5-mm-diameter hole was drilled in the skull 3 mm lateral to bregma. Animals were placed in a stereotactic frame, and 10  $\mu$ l of the cell suspension was injected 4 mm deep to the surface of the brain, into the left caudate-putamen. Wounds were closed with staples.

In certain experiments, 9L cells derived from s.c. tumors were transferred directly to brain. A 9L glioma grown subcutaneously was passed through a sterile tissue sieve into 10–15 ml normal saline solution. Two volumes of Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 200 U/ml of penicillin G, and 200 mg/ml of streptomycin sulfate, were added to this suspension, which was then passed sequentially through a 16-, 20-, 22-, and 26-gauge needle to disperse cells. Particles were counted with a Coulter counter (Coulter Electronics, Hialeah, FL), the suspension was centrifuged at 9,000  $\times g$ , and the supernatant with settled red blood cells was removed. The pellet, which consisted of single cells and multicellular clumps, was resuspended in medium to a concentration of  $10^5$  particles in 10  $\mu$ l. Ten  $\mu$ l of this suspension was injected into the brains of rats as described for the cultured

cells. Animals with intracerebral (i.c.) tumors were sacrificed at postoperative day (POD) 11 for F98, and POD 9 for 9L.

**Subcutaneous tumors.** Anesthetized rats were injected into their left flanks with 900  $\mu$ l of a 120,000 cell/ml suspension of 9L or F98 cells. Other rats were injected with 900  $\mu$ l of dissociated tissue obtained directly from intracerebral 9L tumors as described under 'Intracerebral tumors'. Animals with s.c. tumors were sacrificed on POD 14.

### 2.3. Immunohistochemical staining

Anesthetized animals were perfused transcardially with either 10% neutral buffered formalin (Fisher) or 4% neutral buffered paraformaldehyde at 60 ml/min. Tumors were removed and post-fixed overnight at 4°C in the same fixative. Specimens were processed to paraffin blocks, and serial 5–10-micron-thick sections were stained with hematoxylin and eosin, with rabbit antisera to the GLUT1 glucose transporter (kindly provided by Dr. Lester R. Drewes, Univ. of Minnesota, Duluth), laminin (GIBCO), or with SMI-71 mouse monoclonal antibody to rat endothelial barrier antigen (EBA) (Sternberger Monoclonals, Baltimore, MD). Antiserum to GLUT1 identifies the carboxyl terminus of the 'rat brain/human erythrocyte' glucose transporter, and 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 [10,15,16]. Laminin antisera stains vascular basal lamina and is a reliable marker of vessels in brain neoplasms and normal brain [8,11]. The antibody to EBA is supplied as a high-titer ascites fluid, and recognizes an antigen that is localized to endothelia that have a blood-brain barrier [31,37,38]. Both antibodies to GLUT1 and EBA localize to the luminal plasma membrane of brain vessels.

Immunohistochemical staining for GLUT1 and laminin was performed as described by Guerin et al. [15,16]. Sections for laminin staining were digested for 15 min at 37°C with 0.2% pepsin (Calbiochem, LaJolla, CA) in 0.01N HCl prior to incubating with immunological reagents. For GLUT1 and laminin staining, sections were incubated sequentially with biotinylated goat anti-rabbit immunoglobulin (Vector) diluted 1:200 in PBS containing 1.5% normal goat serum, with avidin-biotin complex reagent (Vector) diluted 1:50 in PBS, and with 0.5 mg/ml 3,3'-diaminobenzidine (Sigma) in 50 mM tris-buffered normal saline containing 0.01% hydrogen peroxide. Immunohistochemical staining for EBA was similar, except that sections were not pepsin-digested, normal horse serum was substituted for normal goat serum, and the secondary antibody used was biotinylated horse anti-mouse immunoglobulin (Vector). Sections were counterstained with hematoxylin. Control slides in which non-immune goat, horse, or rabbit serum (Vector) was substituted for immune serum or ascites fluid were negative.

### 2.4. Assessment of staining

The total number of vessel profiles per high power field (0.19 mm<sup>2</sup>) was counted independently in adjacent serial sections stained for GLUT1, laminin, and EBA. Fifteen to forty-five fields per specimen were counted, spanning the total area of solid tumor and excluding infiltrating border zones where vessels may be surrounded by a mixture of normal and neoplastic cells. For each specimen, the percentage of GLUT1- or EBA-positive vessels was calculated as the average number of GLUT1- or EBA-positive profiles per field divided by the average number of laminin-positive profiles/field as previously described [15–17]. Vascular density was determined for each specimen as the average number of laminin-positive profiles per field divided by the area of the field. Normal brain assessments were made in the cortical gray of contralateral hemispheres.

### 3. Results

#### 3.1. Tumor histology

The proliferation and differentiation of tumor-associated blood vessels change with increasing tumor malignancy [5,6,17,22,25,27,35]. Therefore, we closely examined tumors grown subcutaneously and intracerebrally for any changes in histology or differences in mitotic indices that might suggest differences in tumor behavior or malignancy.

The 9L gliosarcoma formed well-defined, encapsulated tumor masses with microscopic extensions invading normal tissue as previously described [15,16]. Subcutaneous 9L tumors were substantially larger than i.c. 9L tumors. Most of the 9L i.c. tumors had central necrotic areas, which were absent in the s.c. tumors. Palisading of cells, while present in both i.c. and s.c. tumors, was more subtle in the s.c. tumors (Fig. 1). Cellular atypia, nuclear pleiomorphism, and the presence of a large number of tumor giant cells was characteristic of both i.c. and s.c. tumors. The number of mitotic figures quantified in 9L i.c. tumors established with cultured cells ( $11.1 \pm 0.7$  mitotic figures/hpf, mean  $\pm$  S.E.M.) and 9L i.c. tumors established by transferring dissociated cells derived from s.c. tumors ( $9.6 \pm 1.3$  hpf), did not differ significantly ( $P = 0.2521$ ). The mitotic indices for the s.c. tumors established with cultured cells ( $10.5 \pm 0.8$  hpf) also did not

differ significantly from that of s.c. tumors established with cells dissociated from i.c. tumors ( $11.5 \pm 1.0$  hpf,  $P = 0.4579$ ). Mitotic indices for i.c. and s.c. tumors did not differ significantly ( $P = 0.7989$ ).

The F98 glioma formed connected islands of tumor cells. Subcutaneous F98 tumors were substantially smaller than their i.c. counterparts. Palisading was notable in both the i.c. and s.c. tumors, as was cellular atypia. The number of mitotic figures within the i.c. tumors ( $8.0 \pm 0.9$  hpf) differed significantly from that of the s.c. tumors ( $3.1 \pm 0.5$  hpf,  $P < 0.0001$ ).

#### 3.2. Vascular densities

Vascular densities were determined for each tumor type using immunostaining for laminin, a vascular marker in normal brain and brain neoplasms [8,11,15]. Tumor parenchyma occasionally stained with antisera to laminin, but with a pattern clearly different from that of the vascular basal lamina.

There were slight differences in vascular density between 9L i.c. tumors established with either cultured cells ( $170.9 \pm 13.8$  vessels/mm<sup>2</sup>) and with cells dissociated from s.c. tumors ( $127.6 \pm 14.2$  vessels/mm<sup>2</sup>,  $P = 0.0430$ ). Vascular densities in 9L s.c. tumors established with cultured cells ( $170.2 \pm 24.0$ ) were not significantly different from those established with cells dissociated from i.c. tumors ( $206.3 \pm 17.2$  vessels/mm<sup>2</sup>,  $P = 0.2334$ ). There was no significant difference in vascu-

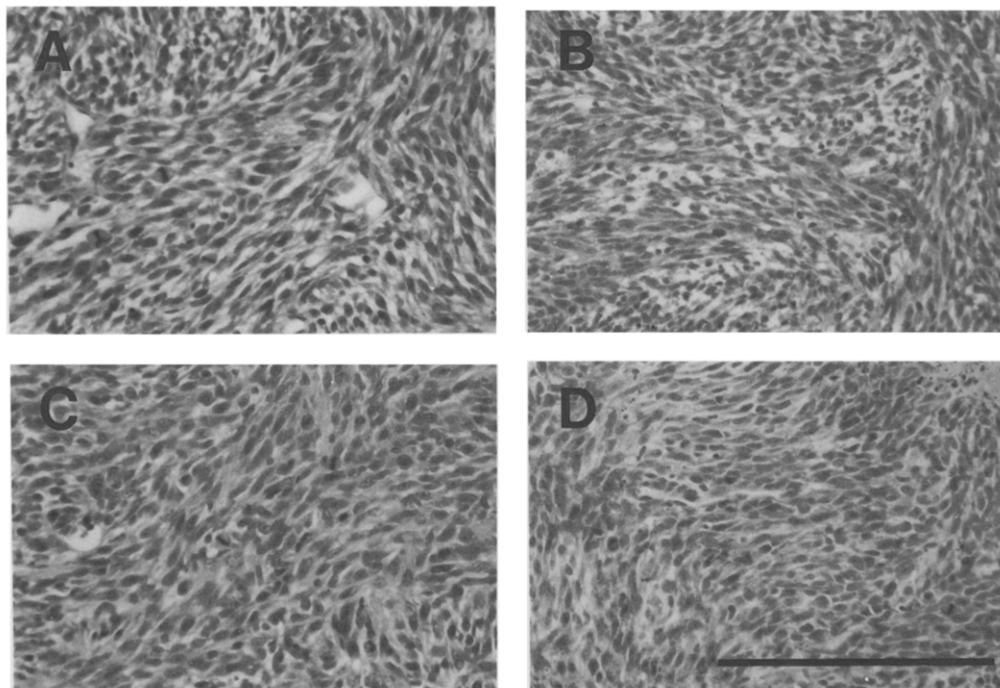


Fig. 1. Photomicrographs of 9L intracerebral (i.c.) (A) and subcutaneous (s.c.) (B) gliosarcomas, and of F98 i.c. (C) and s.c. (D) gliomas stained with hematoxylin and eosin to demonstrate tumor histology. Histological characteristics are similar for each tumor type regardless of implantation site. Bar = 500 microns.

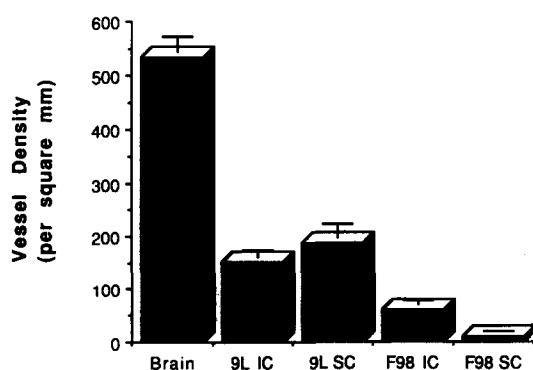


Fig. 2. Vascular densities in normal brain, and in 9L and F98 intracranial (i.c.) and subcutaneous (s.c.) gliomas. Data represents mean  $\pm$  S.D.

lar density between the i.c. and s.c. 9L tumors ( $P = 0.0504$ ). F98 s.c. tumors had consistently lower vascular densities than their i.c. counterparts. Consistent with our earlier findings, each tumor type had lower vascu-

lar density than that present in normal brain ( $534.9 \pm 127.2$  vessels/mm $^2$ ,  $P < 0.0001$ ) (Fig. 2).

### 3.3. GLUT1 and EBA expression in normal brain

As previously reported, essentially all cerebral hemispheric vessels in normal brain expressed GLUT1 ( $111.5 \pm 9.1$ , not significantly different from 100%) [2,10,15,16]. Consistent with the findings of Sternberger et al., all cerebral hemispheric vessels in normal brain expressed EBA ( $121.5 \pm 5.7\%$ , not significantly different from 100%) [37]. Vessels outside of the CNS (e.g., subcutaneous tissue and muscle) lacked EBA and GLUT1 staining (not shown).

### 3.4. GLUT1 and EBA expression in 9L and F98 tumors

Figs. 3 and 4 demonstrate substantial differences in i.c. and s.c. glioma expression of these barrier-specific

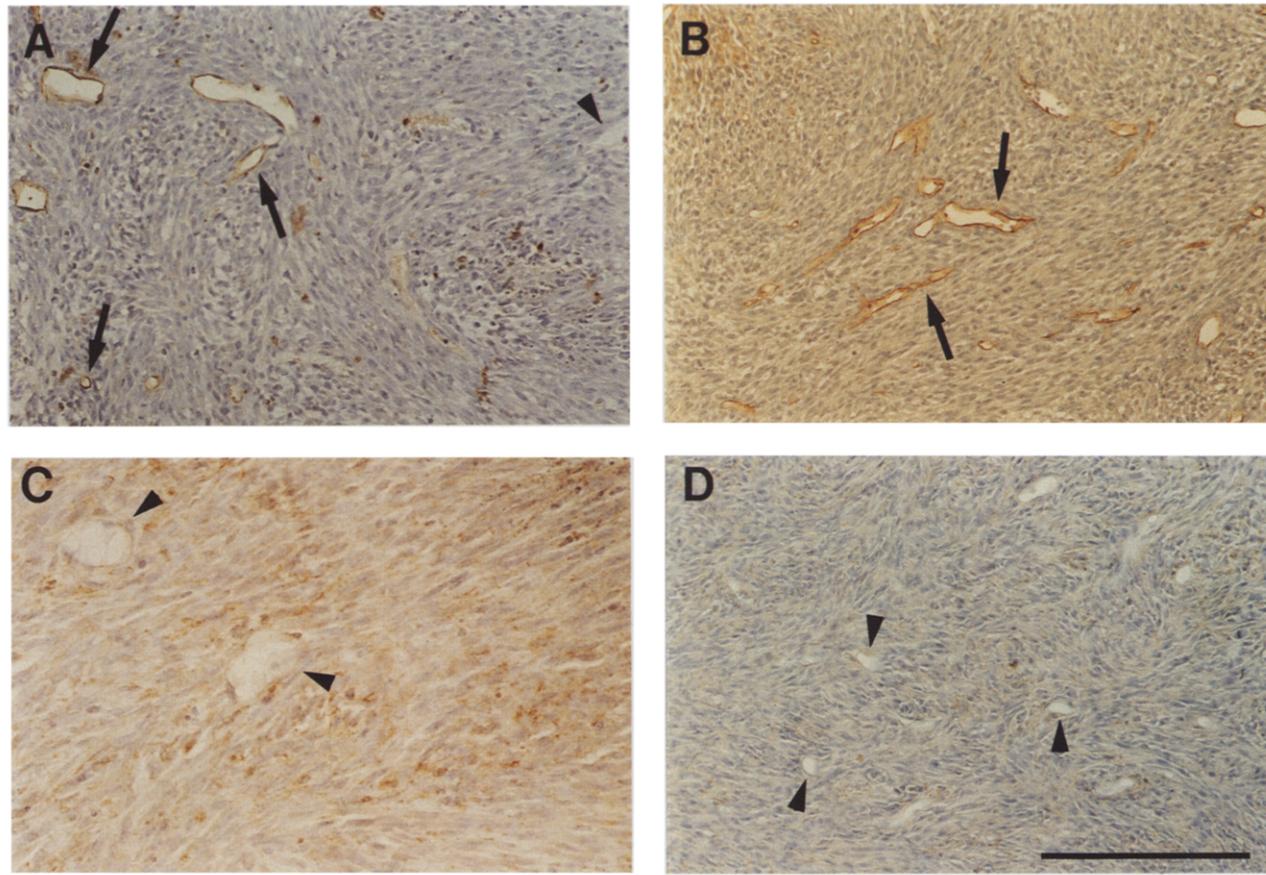


Fig. 3. Photomicrographs of microvessels in intracranial (A) and subcutaneous (C) F98 gliomas immunohistochemically stained for EBA, and intracranial (B) and subcutaneous (D) 9L gliomas immunohistochemically stained for GLUT1. Arrows indicate antigen-positive vessel profiles and arrow heads indicate antigen-negative vessel profiles. Vessels in subcutaneous tumors (arrows) lack GLUT1 and EBA staining. Bar = 300 microns in panels A, B and D, and 150 microns in panel C.

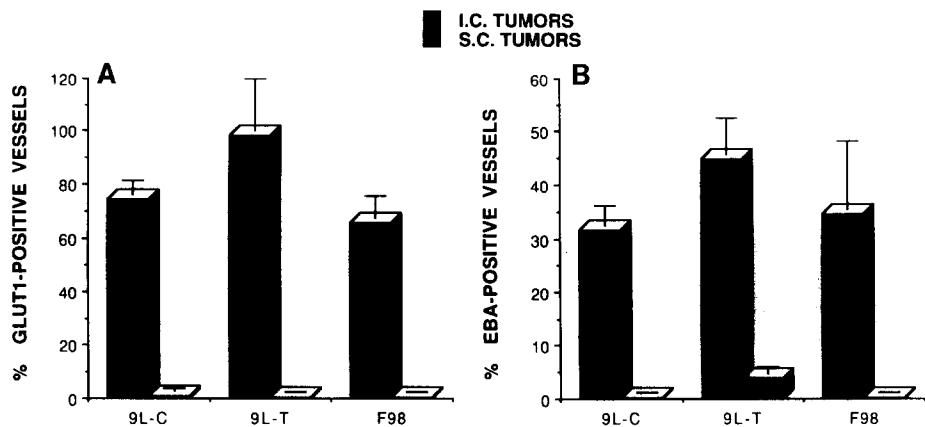


Fig. 4. Percentages of vessels with immunohistochemically-detectable GLUT1 (A) and EBA (B) in 9L and F98 gliomas. 9L gliomas were established either by implantation of cultured cells (9L-C), or by transfer of tumor fragments from intracranial (i.c.) and subcutaneous (s.c.) tumors (9L-T). F98 tumors were established by implantation of cultured cells only. Data represents mean  $\pm$  S.D.

endothelial antigens. A large percentage of the microvessels in i.c. 9L tumors expressed immunohistochemically detectable GLUT1, consistent with previous findings from our laboratory [15,16]. The percentage of vessels that expressed GLUT1 in i.c. tumors established with cultured cells ( $74.0 \pm 5.4\%$ ) was not significantly different from that in i.c. tumors established by transferring dissociated cells derived from s.c. tumors ( $98.2 \pm 20.1\%$ ) ( $P = 0.2130$ ). In contrast, GLUT1 expression in i.c. tumors was significantly different from that in s.c. tumors established with either cultured cells ( $1.2 \pm 0.7\%$ ) or cells dissociated from i.c. tumors ( $0.0 \pm 0.0\%$ ) ( $P < 0.0001$ ).

A substantial percentage of vessels in i.c. 9L tumors expressed EBA. This was true for tumors established with either cultured cells ( $31.4 \pm 3.6\%$ ) or with cells dissociated from s.c. tumors ( $44.9 \pm 6.6\%$ ) ( $P = 0.0691$ ). Similar to that found for GLUT1 expression, 9L s.c. tumors established from either cultured cells or from cells dissociated from i.c. tumors contained few EBA-positive vessels ( $0.2 \pm 0.2\%$ , and  $3.9 \pm 1.1\%$ , respectively). EBA expression in i.c. tumors was significantly different from that in s.c. tumors ( $P < 0.0001$ ).

GLUT1 and EBA expression in F98 tumors was similar to that found in 9L tumors. The percentages of vessels in i.c. F98 tumors that expressed GLUT1 or EBA were  $65.5 \pm 8.0\%$ , and  $34.5 \pm 12.8\%$ , respectively. F98 tumors grown subcutaneously contained no GLUT1-positive ( $0.0 \pm 0.0\%$ ) or EBA-positive ( $0.0 \pm 0.0\%$ ) vessels. There was no significant correlation between vascular density and the percentage of GLUT1- or EBA-positive vessels in either 9L or F98 tumors ( $F = 105.092$ ,  $P < 0.0001$ ).

#### 4. Discussion

In this report we examine the expression of two blood-brain barrier endothelial proteins, GLUT1 and

EBA, by the microvessels of experimental gliomas grown intracerebrally and subcutaneously. This comparison was made in order to determine if the peritumoral environment influences the expression of these proteins by endothelial cells within glial tumors. We confirm our previous studies that found GLUT1 expression in the vasculature of intracerebral human and experimental rat gliomas [15,17], and extend those findings by demonstrating the expression of the rat endothelial barrier antigen, EBA, within the intracerebral tumors. Although significant heterogeneity in microvascular GLUT1 and EBA expression was found, all intracerebral gliomas, regardless of whether they were established from cultured cells or from tumor fragments, contained a large percentage of antigen-positive vessels. In contrast, vessels within subcutaneous tumors essentially lacked histochemically-detectable EBA and GLUT1. The absence of these endothelial barrier antigens in subcutaneous gliomas could not be simply explained by greater tumor malignancy within subcutaneous sites since histologic markers of tumor malignancy (vessel density and mitotic index) were either the same or less than those found in intracerebral tumors. Our findings demonstrate that the expression of barrier-specific endothelial antigens within gliomas is dependent upon properties of the peritumoral environment, and are consistent with those of Groothuis et al., demonstrating that blood vessels within subcutaneous gliomas are more uniformly permeable to horseradish peroxidase than are vessels within intracerebral tumors [13]. The higher percentage of intracerebral tumor vessels expressing GLUT1 than EBA suggests that the expression of these two barrier proteins is independently regulated and that changes in EBA expression is a more sensitive marker of rat vessel dedifferentiation [31].

The factors that determine endothelial barrier expression within normal brain and brain tumors are poorly defined. Evidence from cell culture and tissue

transplantation studies points to a principal role for perivascular parenchymal cells [39] and more specifically astroglia [7,20,32], in the induction or maintenance of blood–brain barrier endothelial properties. The near or complete absence of endothelial barrier within central nervous system metastases contrasts with the retention of barrier properties by vessels within intracerebral tumors of glial origin [15,17]. This supports a role for glioma cell-derived signals in blood–glioma barrier expression. Our present findings are consistent with the ability of glioma cells to maintain endothelial barrier expression but not induce the expression of barrier properties in endothelial cells derived from nonbarrier vessels. This, in conjunction with the production by gliomas of substances such as vascular permeability factor [1,24,29], indicate that barrier expression by vessels within gliomas is determined by the combined effects of glioma-derived factors, and the phenotype of the peritumoral vessels from which the tumor vessels originate.

Subcutaneous experimental gliomas are frequently used as an alternative to intracerebral tumors in the preliminary assessment of tumor imaging characteristics, tumor metabolic properties, and new therapeutic modalities [3,4,9,30,33,36,40]. Differences in endothelial properties such as permeability and the expression of barrier-specific proteins may result in subcutaneous tumors that differ from their intracerebral counterparts. The likelihood for increased drug delivery to subcutaneous tumors due to increased vascular permeability provides a rationale for using this extracranial site for the screening of water-soluble chemotherapeutic drugs [3,13]. However, caution must be used in extrapolating biochemical characteristics of subcutaneous tumors to their intracranial counterparts or vice versa. Further comparisons of subcutaneous and intracranial gliomas may yield new insight into the functional interrelationship between the glioma cells and the microvasculature of glial tumors.

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