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Endothelial cell implantation and survival within experimental gliomas

(gene therapy/blood–brain barrier/*lacZ*/angiogenesis)

BACHCHU LAL*†, RAVI R. INDURTI*†, PIERRE-OLIVIER COURAUD‡, GARY W. GOLDSTEIN*†§, AND JOHN LATERRA*†¶‖**

Departments of *Neurology, ¶Neuroscience, ‖Oncology, and §Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD 21205; †Kennedy Krieger Research Institute, Baltimore, MD 21205; and ‡Laboratoire d'Immunopharmacologie Moléculaire, Institut Cochin de Génétique Moléculaire, Centre National de la Recherche Scientifique, 415, Paris, France

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ABSTRACT **The delivery of therapeutic genes to primary brain neoplasms opens new opportunities for treating these frequently fatal tumors. Efficient gene delivery to tissues remains an important obstacle to therapy, and this problem has unique characteristics in brain tumors due to the blood–brain and blood–tumor barriers. The presence of endothelial mitogens and vessel proliferation within solid tumors suggests that genetically modified endothelial cells might efficiently transplant to brain tumors. Rat brain endothelial cells immortalized with the adenovirus E1A gene and further modified to express the β-galactosidase reporter were examined for their ability to survive implantation to experimental rat gliomas. Rats received 9L, F98, or C6 glioma cells in combination with endothelial cells intracranially to caudate/putamen or subcutaneously to flank. Implanted endothelial cells were identified by β-galactosidase histochemistry or by polymerase chain reaction in all tumors up to 35 days postimplantation, the latest time examined. Implanted endothelial cells appeared to cooperate in tumor vessel formation and expressed the brain-specific endothelial glucose transporter type 1 as identified by immunohistochemistry. The proliferation of implanted endothelial cells was supported by their increased number within tumors between postimplantation days 14 and 21 ($P = 0.015$) and by their expression of the proliferation antigen Ki67. These findings establish that genetically modified endothelial cells can be stably engrafted to growing gliomas and suggest that endothelial cell implantation may provide a means of delivering therapeutic genes to brain neoplasms and other solid tumors. In addition, endothelial implantation to brain may be useful for defining mechanisms of brain-specific endothelial differentiation.**

Gene therapy is receiving considerable attention as a method for targeting putative therapeutic biological substances directly to brain and brain tumors. The most efficient and least toxic methodologies for delivering functional genetic material to normal and neoplastic tissues within the central nervous system (CNS) are not established and will likely be tailored to specific diseases. Because the blood–brain and blood–tumor barriers limit entry of blood-borne compounds (1), innovative approaches for delivering genetic material to these sites are being explored. Among those presently receiving attention are (*i*) systemic administration of neurotropic viral vectors (2–4), (*ii*) direct interstitial administration of nucleic acids (5), and (*iii*) transplantation of cells after their genetic modification and characterization *ex vivo* (6, 7).

Endothelial cells are a particularly suitable platform for gene therapy (8–10). Their location at the parenchymal–blood interface is ideal for delivering transgene products either systemically or locally, and they are easily cultured from diverse tissue sources. Since endothelial cells proliferate *in vitro*, they accommodate a broad spectrum of vectors and to date have been successfully transduced to express numerous products with therapeutic potential (11–13). Various approaches to using endothelial cells as platforms for transgene expression *in vivo* have been successful, including direct gene transfer *in vivo* (14, 15) and the seeding of denuded host arteries (16), limb microvessels (17), and prosthetic vascular implants with endothelial cells genetically modified *in vitro* (18). These approaches hold promise for the systemic delivery of transgene products but do not specifically address their delivery across the blood–brain barrier.

The endothelial mitogens and prominent vessel proliferation within solid tumors suggested to us that endothelial cells might efficiently be transplanted to brain tumors (19, 20). This report demonstrates that genetically modified endothelial cells successfully implant, proliferate, and survive for extended periods of time in three experimental rat gliomas. Our findings establish the potential for using genetically modified endothelial cells as platforms for delivering therapeutic gene products to brain tumors. They also suggest that endothelial transplantation to the CNS may be a useful tool for studying mechanisms of CNS vascularization, endothelial differentiation, and blood–brain barrier formation in brain and brain tumors.

METHODS

Endothelial Cells. Endothelial cells to be used for implantation were isolated from rat brain; after one passage, they were immortalized by transfection with pE1A-neo, which contains the adenovirus 2 E1A gene and a neomycin-resistance gene. One clone, designated RBE4, has been further characterized; it exhibits contact inhibition and growth factor- and anchorage-dependent proliferation and is labeled with the endothelial-specific markers factor VIIIrAg and acetylated low density lipoprotein (21). RBE4 cells were subsequently exposed to the replication-defective MFG-NB retroviral vector containing a modified *lacZ* gene (nls–*lacZ*) (22), which codes for the *Escherichia coli* β-galactosidase (β-gal) fused to a 21-amino-acid nuclear localization sequence (nls) from simian virus 40 large tumor antigen (kindly provided by Anne Weber, Institut Cochin de Génétique Moléculaire, Paris) (23). Rat brain endothelial cells expressing the nls–*lacZ* transgene (RBEZ) cells were labeled with the fluorescent β-gal substrate fluorescein di-β-D-galactopyranoside

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Abbreviations: β-gal, β-galactosidase; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; RBEZ cells, rat brain endothelial cells expressing the nls–*lacZ* transgene; Glut-1, glucose transporter isotype 1; CNS, central nervous system.

**To whom reprint requests should be addressed at: Kennedy Krieger Research Institute, 707 North Broadway, Baltimore, MD 21205.

and selected by fluorescence-activated cell sorting (24). RBEZ cells were cultured on fibronectin-coated substrata in α-MEM/F10 medium supplemented with 10% (vol/vol) fetal calf serum, basic fibroblast growth factor at 1 ng/ml, and Geneticin (G418; GIBCO) at 300 μg/ml.

Tumor Cells. 9L and F98 glioma cells (25) were grown in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Washington, DC) supplemented with 10% (vol/vol) fetal bovine serum (HyClone). C6 cells (26) were grown in DMEM containing 10% bovine calf serum. All growth media contained 2 mM L-glutamine and gentamycin at 50 μg/ml. All cells were grown at 37°C in 5% CO_2/95% air.

Tumor and Endothelial Cell Implantation. Confluent cells were trypsinized and resuspended in DMEM immediately before implantation to host animals. For intracranial implantation, glioma cells alone (10^5 cells) or a mixture of glioma and RBEZ cells (10^5 and 5×10^5 cells, respectively) in DMEM were injected stereotactically with a 26-gauge, beveled-tip Hamilton syringe to the caudate/putamen of anesthetized 200- to 250-g Fisher 344 rats (27). With bregma as a landmark, injection site coordinates were L 3.0 mm, at a depth of 4.5 mm from dura. Cells were injected in 5-μl volumes, and the needle was left in place for 2 min after injection to limit leakage.

For subcutaneous implantation, anesthetized Fisher 344 rats received 100 μl of DMEM containing 10^6 9L cells alone or together with 10^6 RBEZ cells by subcutaneous injection with a 22-gauge syringe (28).

Tissue Preparation. Rats were anesthetized with ether, and after thoracotomy the right atrium was incised and a cannula was inserted into the left ventricle, which was then perfused sequentially with 120 mM NaCl/2.7 mM KCl in 10 mM phosphate buffer at pH 7.4 (1 ml per g of body weight), and lastly with 3.7% (wt/vol) paraformaldehyde. Brains were placed in the same fixative for 30 min, cryoprotected in 30% (wt/vol) sucrose in phosphate-buffered saline (PBS), and frozen on dry ice. Tissue sections were cut 12 μm thick and mounted on chrome alum/gelatin-coated slides.

Histochemistry and Immunohistochemistry. For the detection of RBEZ cells, which express the nls–*lacZ* reporter gene, mounted sections were rinsed three times with PBS and then incubated at 37°C for 1–2 h in PBS containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) at 0.5 mg/ml, 20 mM potassium ferricyanide, 20 mM potassium ferrocyanide, and 2 mM $MgCl_2$ (29). Sections incubated in the absence of X-Gal substrate were used as negative controls. Sections were then rinsed in PBS and mounted in 90% (vol/vol) glycerol in PBS containing 0.02% sodium azide. Under these reaction conditions, no staining was detected in control animals that did not receive RBEZ cells (30).

Some sections were immunohistochemically stained for laminin (27), the nuclear proliferation antigen Ki67 (31), or the brain-specific endothelial glucose transporter type 1 (Glut-1) (27) after staining with X-Gal. Sections for laminin staining were digested for 15 min at 37°C with 0.2% pepsin in 0.01 M HCl prior to incubation with immunological reagents. Sections were sequentially incubated with 1% normal goat serum and either rabbit anti-laminin (Sigma), rabbit anti-Glut-1 (kindly provided by Lester Drewes, University of Minnesota, Duluth), or rabbit anti-Ki67 (Dakopatts, Glostrup, Denmark). Sections incubated with anti-laminin and anti-Glut-1 were then incubated with biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories) and then avidin–biotin complex reagent (Vector Laboratories) followed by 3,3'-diaminobenzidine (0.5 mg/ml; Sigma) in 50 mM Tris-buffered normal saline containing 0.01% hydrogen peroxide. Sections incubated with anti-Ki67 were incubated with fluorescein-labeled goat anti-rabbit IgG (Sigma). Control slides were incubated with normal rabbit serum in place of immune serum. Sections were mounted in 90% glycerol in PBS.

Histologic Quantification of Implanted Cells. Tumor cross-sectional areas and the number of implanted cells per tumor section were quantitated by computer-assisted image analysis by use of the Microcomputer Imaging Device (MCID) software package of Imaging Research Incorporated (Brock University, St. Catherines, ON Canada), a Hamamatsu high-resolution charge-coupled device camera, and a Compaq DeskPro 486/33 computer.

Estimates of the total number of RBEZ cells per tumor were derived from the number of RBEZ cells per tumor volume (12-μm section), and estimates of total tumor volume were derived from maximum cross-sectional tumor areas (32).

PCR. Synthetic oligonucleotides complementary to the DNA sequences located in the nls–*lacZ* gene (5'-CGACTCCTGGAGCCCGTCAGTATC-3') and in the vector upstream of the 3' long terminal repeat (5'-GACCACTGATATCCTGTCTTTAAC-3') were used as primers (22). PCR reactions were performed on genomic DNAs isolated from control tumors, experimental tumors, and positive and negative control cell lines. Thirty-five cycles of amplification with *Thermus aquaticus* (*Taq*) polymerase (Cetus) were performed at temperatures of 95°C for denaturation, 60°C for hybridization, and 72°C for elongation.

RESULTS

Implantation of Endothelial Cells to Intracranial and Subcutaneous Experimental Gliomas. The immortalized microvascular endothelial cell line from rat brain (33) modified to express the nls–*lacZ* reporter gene (RBEZ) (22) was used to explore endothelial implantation to 9L, F98, and C6 rat gliomas *in vivo*. In these studies, glioma cells alone or a mixture of RBEZ cells and glioma cells were implanted to rat caudate/putamen. Because intracranial tumor-related mortality begins during postimplantation week 3, tumors generated by implanting a mixture of 9L glioma cells and RBEZ cells to rat flanks were used to evaluate longer periods of RBEZ cell survival. After staining with the X-Gal chromogenic substrate, blue nuclear β-gal reaction product was identified in histological sections of all 7- to 12-day postimplantation intracranial tumors and all 14- to 35-day postimplantation subcutaneous tumors as shown in Fig. 1 and Tables 1 and 2. Interestingly, engrafted endothelial cells were distributed throughout the intracranial tumors, including their finger-like infiltrating margins, but did not appear to migrate into normal tissues unassociated with glioma cells (data not shown). No obvious differences were found in the distribution of RBEZ cells implanted with the different tumor cell types, and no blue X-Gal reaction product was detected in tumors implanted without RBEZ cells (30). In addition to these histological endpoints, PCR was used to verify the presence of MFG-NB proviral vector DNA in high molecular weight nuclear DNA isolated from tumors implanted with RBEZ cells. Vector-specific DNA sequences were found in DNA isolated from tumors engrafted with RBEZ cells but were not found in control tumors (Fig. 2).

Location and Differentiation of Implanted Endothelial Cells. We next asked whether implanted endothelial cells associate with the host-derived tumor vessels or reside in tumor parenchyma independent of tumor vessels. Frozen sections from postimplantation day 12 tumors were histochemically stained with X-Gal to detect implanted RBEZ cells and then immunohistochemically stained with either anti-Glut-1 or anti-laminin to detect tumor microvessels (27, 34). Interestingly, in all tumor types examined numerous engrafted RBEZ cells were found to be associated with microvascular profiles (Fig. 1*B*). In addition, capillary-like profiles consisting of

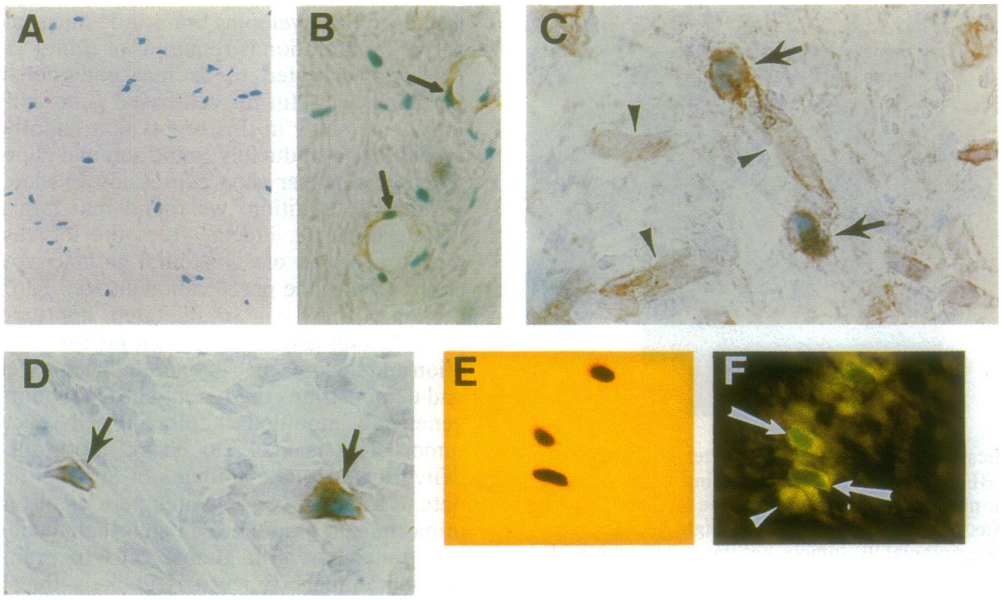

FIG. 1. Histological examination of RBEZ cells implanted to rat intracranial gliomas. Glioma cells were implanted concurrent with RBEZ cells to rat caudate/putamen, and brains were perfusion-fixed 12 days later. Frozen sections (12 μm) through tumors were incubated with X-Gal under conditions that specifically stain the nuclei of RBEZ cells expressing β-gal blue. Sections were then stained using immunohistochemistry for Glut-1 (*B* and *D*) and laminin (*C*) or using fluorescein immunofluorescence for the proliferation antigen Ki67 (*F*) as described in *Methods*. (*A*) Photomicrograph of 9L tumor showing numerous blue RBEZ cells interspersed with glioma cells. (*B*) F98 tumor containing blue RBEZ cells (arrows) that are associated with microvessels expressing Glut-1. (*C*) Immunohistochemistry with anti-laminin labels numerous microvascular profiles within the 9L tumor (arrowheads), one of which contains two blue RBEZ cells (arrows). (*D*) Immunohistochemistry with anti-Glut-1 demonstrates that RBEZ cells (arrows) implanted to 9L tumor express this brain-specific endothelial transporter. (*E* and *F*) Same field visualized under either bright-field (*E*) or epifluorescent illumination (*F*). RBEZ cells expressing β-gal (*E*) also label with anti-Ki67 (*F*; arrows). Proliferating glioma cells not labeled with X-Gal also label with anti-Ki67 (*F*; arrowheads). (*A*, ×200; *B*, ×470; *C* and *D*, ×1200; *E* and *F*, ×600.)

linearly associated RBEZ cells were also observed (Fig. 1*C*). These findings suggest that RBEZ cells implanted in this fashion have the potential to engraft in an anatomically correct fashion in tumors.

One of the most striking features of brain endothelial cells is their high level expression of the blood–brain barrier glucose transporter Glut-1 (35). This is consistent with the prominent transendothelial transport of D-glucose, the primary energy substrate of brain. We have previously established that endothelial cells within human and rat intracranial gliomas also express this transporter (27). In contrast, brain endothelial cells rapidly down-regulate Glut-1 expression in culture (ref. 36 and unpublished data). To determine if RBEZ cells express this brain-specific endothelial transporter after engraftment to gliomas, tissue sections from intracranial tumors containing implanted RBEZ cells were stained with X-Gal and then immunohistochemically stained with antiserum to Glut-1 (27). Numerous β-gal-positive cells within tumors were found to stain prominently for Glut-1 (Fig. 1*D*).

Table 1. Endothelial cell implantation to 9L gliomas

No. tumors examined	No. tumors containing β-gal-positive cells	Days after RBEZ implantation
4	4	7
10	10	12
4	4	14
3	3	21
3	3	28
4	4	35

Immortalized RBEZ cells were implanted concurrent with 9L glioma cells to either rat caudate/putamen or flank as described in *Methods*. At the indicated postimplantation times, animals were sacrificed by perfusion fixation, and tumor sections were stained with the X-Gal substrate to detect reporter gene expression. Control tumors completely lacked staining.

Proliferation of Implanted Endothelial Cells. The effectiveness of endothelial-based gene delivery to tumors is likely to depend, in part, upon the ability of the implanted genetically modified endothelial cells to proliferate in growing tumor. To address this, we examined the expression of the proliferation antigen Ki67 (31) by RBEZ cells implanted to intracranial 9L tumors. Tissue sections obtained from 12-day postimplantation tumors were stained sequentially with X-Gal and anti-Ki67. Cells expressing both nuclear β-gal and Ki67 antigen were found (Fig. 1 *E* and *F*). This indicates that a substantial number of implanted RBEZ cells are proliferating outside of G_0 (31).

Table 2. Quantification of implanted endothelial cells

Tumor type	No. RBEZ cells per tumor, mean ± SEM
Intracranial	
9L	166,440 ± 19,550
C6	145,840 ± 42,160
F98	232,560 ± 69,070
Subcutaneous	
9L (14 days)	494,560 ± 422,500*
9L (21 days)	5,252,160 ± 611,380

RBEZ cells were implanted concurrent with glioma cells to intracranial or subcutaneous sites in rat as described in *Methods*. At postimplantation day 12 for intracranial tumors and at the indicated times for subcutaneous tumors, animals were sacrificed by perfusion fixation, and tumor sections were stained with X-Gal to detect surviving RBEZ cells. Tumor cross-sectional areas and the number of RBEZ cells per tumor section were quantitated by image analysis. Total tumor volumes were estimated from cross-sectional areas (32) and used to calculate total tumor RBEZ cells as described in *Methods*.

*The difference between the number of implanted cells found in 9L (14 days) and 9L (21 days) was statistically significant at $P = 0.015$.

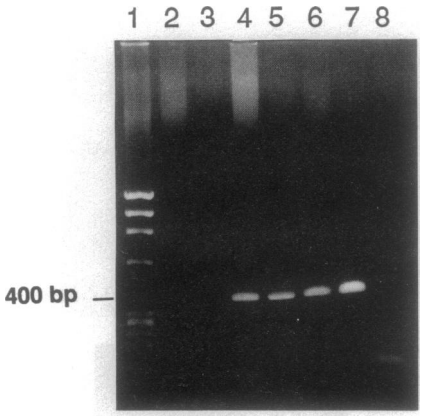

FIG. 2. Identification of the nls–*lacZ* reporter gene in tumors implanted with RBEZ cells by PCR. PCR using oligonucleotide primers specific for nls–*lacZ* sequences within the MFG-NB expression vector (22) was run on high molecular weight genomic DNA isolated from two intracranial 9L tumors not implanted with RBEZ cells (lanes 2 and 3), three tumors implanted with RBEZ cells 14 days prior to DNA isolation (lanes 4–6), cultured RBEZ cells (lane 7), and endothelial cells lacking the proviral vector (lane 8). The expected 400-bp PCR product is present in samples derived from tumors implanted with RBEZ cells.

Temporal changes in the number of implanted RBEZ cells were determined as an independent way of assessing whether RBEZ cells proliferated. Animals bearing RBEZ-containing subcutaneous 9L tumors were sacrificed at postimplantation days 14 and 21, a time interval during which tumor volumes increased about 14-fold. Tumor sections were histochemically stained with X-Gal to detect implanted RBEZ cells. Tumor cross-sectional areas and the numbers of implanted cells per tumor volume (e.g., per 12 μm tumor section) were quantitated by computer-assisted image analysis. The numbers of RBEZ cells per whole tumor were then calculated by using total tumor volumes estimated from their cross-sectional areas (32). These data indicate that the total number of RBEZ cells per tumor increased about 10-fold over this time interval (Table 2). These immunofluorescent and morphometric data are consistent with endothelial proliferation within the growing tumors.

DISCUSSION

The delivery of foreign genes to specific tissues remains an important obstacle to gene therapy. One approach is to implant genetically modified "bystander cells" that deliver either transgene protein products (6) or replication-defective viral vectors (37) to neighboring target cells. While it is theoretically possible that nonimmunogenic universal donor cells might someday be available for cell-based gene delivery, autologous implantation is presently optimal to avoid graft rejection. Consequently, primary emphasis has been placed on fibroblasts (6, 38), myoblasts (7), and endothelial cells (16–18), since they are easily obtained from patient biopsy specimens and are readily expandable in culture. Genetically modified fibroblasts and myoblasts produce therapeutic effects after their implantation to experimental animals bearing brain tumors (37) or after brain injury (7). While genetically modified endothelial cells can be implanted to the vasculature of systemic tissue (16–18), to our knowledge, their potential to serve as platforms for delivering genes to either the CNS or to solid tumors has not been previously demonstrated.

Endothelial cells are distinguished from other candidate cell types (e.g., muscle and fibroblast) since (*i*) they are normal components of brain and brain tumors (20), (*ii*) they proliferate in response to the endothelial-specific mitogens found within developing brain and brain tumors, and (*iii*) their active proliferation is required for tumor growth (39). These features suggested to us that endothelial cells might be particularly useful for cell-based gene delivery to brain tumors. We show in this report that endothelial cells can be implanted reproducibly to intracranial gliomas and that they maintain reporter gene expression in tumors for prolonged periods. In addition, we found that implanted endothelial cells proliferate, judging from the increased cell numbers during a period of substantial glioma growth and on their expression of the proliferation antigen Ki67 (31). The proliferation of these contact-inhibited, nontumorigenic endothelial cells (40) is consistent with host-derived vessel proliferation and the presence of endothelial mitogens within gliomas and demonstrates the potential to maintain large numbers of genetically modified endothelial cells even within growing tumor. This is likely to be an important requirement for delivering adequate amounts of therapeutic transgene products to tumors over extended periods of time. In this study, immortalized brain-derived endothelial cells were implanted simultaneously with glioma cells to brains of non-tumor-bearing animals. Since it is unlikely that brain endothelial cells autologous to patients bearing brain tumors will be readily obtainable, it will be important to determine if endothelial cells derived from a peripheral source similarly survive implantation to established tumors.

Messina *et al.* (17) described the engraftment of endothelial cells to the rat hindlimb microvasculature. In their report, endothelial cells were delivered to hindlimb microvessels via femoral artery perfusion. We examined the localization of implanted endothelial cells relative to host-derived tumor vessels to determine whether endothelial cells implanted to tumors interstitially have the capacity to organize into vascular forms. Regardless of their localization within tumors, essentially all implanted β-gal-positive cells stained prominently with anti-laminin, consistent with the endothelial phenotype. In addition, numerous cells were associated with small- and medium-sized vessels, suggesting an ability to form vascular structures and to integrate functionally with the host-derived tumor microvasculature. Thus, interstitially implanted endothelial cells appear to undergo vasculogenesis (the coalescence of endothelial cells into vessels) within tumors that are normally vascularized exclusively by angiogenesis (the ingrowth of elongating vessels from surrounding tissues). In light of the blood–tumor barrier, this finding suggests that genetically modified endothelial cells might be a particularly versatile cell-based platform for gene therapy. In addition to the direct delivery of secreted transgene products to tumor cells, implanted endothelial cells engineered to express novel transport systems might enhance the transvascular delivery of blood-borne drugs to tumor (1). More extensive light and electron microscopic studies will be required to quantify the number of implanted cells that associate with tumor vessels and to determine if they intercalate with host-derived endothelial cells at the blood–tumor interface.

Our finding that brain-derived endothelial cells were able to express the brain-specific endothelial glucose transporter Glut-1 after implantation is consistent with the expression of Glut-1 by a substantial percentage of host-derived endothelial cells within these tumors (27). That brain endothelial cells including RBEZ cells rapidly stop expressing Glut-1 in culture (ref. 36 and unpublished data) suggests that intracranial gliomas contain factors that induce the expression of this transporter in endothelial cells. Additional experiments are required to determine if these implanted cells express other biochemical or anatomic blood–brain barrier properties. Stewart and Wiley showed in the early 1980s that embryonic avian brain contains factors that induce endothelial cells to express anatomic blood–brain barrier features (41) and sub-

sequent studies have implicated perivascular glial cells as the source of this inductive influence (42). These findings have not yet been replicated in intact adult mammalian tissues and the role of perivascular signals in inducing endothelial expression of barrier-specific enzymes and transporter remains unclear. Answers to these questions might be achieved through the implantation of peripheral endothelial cells to brain.

The results of this study establish the feasibility of engrafting endothelial cells that have been genetically modified *ex vivo* to intracranial gliomas. The prospect of endothelial engraftment to the blood–brain interface suggests that these cells might be particularly sensitive to blood-borne regulators of transgene expression and that they might also be used for selective modification of blood–brain barrier function. Additional studies will be required to determine if endothelial implantation can be used to deliver therapeutically effective genes to brain neoplasms and other selected sites with the CNS.

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