Characteristics of tumor-associated endothelial cells derived from glioblastoma multiforme

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✓ Glioblastomas multiforme (GBMs) are highly vascular brain tumors characterized by abnormal vessel structures in vivo. This finding supports the theory that glioma-associated endothelial cells (ECs) have intrinsically different properties from ECs in normal human brain. Therefore, identification of the functional and phenotypic characteristics of tumor-associated ECs is essential for designing a rational antiangiogenic therapy. The GBM-associated ECs have a large, flat, and veil-like appearance, in contrast to normal ones, which are small and plump. Although the tumor ECs have the typical markers, they proliferate more slowly than these cell types in normal brain. The GBM-associated ECs are resistant to cytotoxic drugs, and they undergo less apoptosis than control cells. Also, GBM-associated ECs migrate faster than controls and constitutively produce high levels of growth factors such as endothelin-1, interleukin-8, and vascular endothelial growth factor. An understanding of these unique characteristics of glioma-associated ECs is important for the development of novel antiangiogenic agents that specifically target tumor-associated ECs in gliomas.

KEY WORDS • glioblastoma multiforme • angiogenesis • tumor • endothelium •
endothelial cell migration • proliferation rate

ANGIOGENESIS, the formation of new blood vessels from preexisting ones, is essential for efficient tumor growth and expansion.⁹ Glioblastomas multiforme are particularly hypervascular tumors characterized by extensive blood vessel growth and angiogenesis.⁷ A histological examination of glioma tissues demonstrates that the tumor blood vessels are usually structurally and functionally different from vessels in the adjacent normal brain tissue. Most tumor blood vessels, particularly GBM-associated ones, are disorganized, tortuous, dilated, leaky, and hemorrhagic, often displaying dead-end structures. Extensive literature exists in which investigators have demonstrated that blood vessels in a variety of tumors exhibit significant differences from normal vessels.^{1,4,11} There is little information, however, and few studies specifically concentrate on the tumor-derived ECs, and even fewer data are available on brain tumor ECs. Furthermore, there are conflicting reports regarding the expression of endothelial markers, adhesion molecules, and growth factors in the different studies in which tumor-associated ECs were used.^{1,4,5,11}

In recent publications,^{1,5,18,23} researchers have suggested that the tumor-associated ECs derived from GBM tissue

have different phenotypic and functional properties compared with normal ECs; these differences may result in less effective antiangiogenic therapy if the target molecules are only expressed on normal blood vessels.²⁵ Because antiangiogenic agents may target molecules expressed solely on normal ECs, and that are absent from tumor ECs, the properties of these tumor-associated cells must be thoroughly examined. In this review we will therefore provide an overview of the current state of knowledge regarding ECs derived from brain tumors, and we will discuss the morphology, phenotypic characteristics, and functions of these cells.

Origin of ECs

There are currently three hypotheses about the development and origin of tumor ECs. The first is that they develop from normal ECs recruited into the tumor site from adjacent normal tissue, and arise as a result of angiogenic growth factors derived from the tumor.⁴ This hypothesis is based on the fact that tumor ECs display characteristics of activated ECs.^{4,11} The second hypothesis is that tumor ECs develop from an endothelial progenitor cell, which has migrated to the tumor site and differentiated into vessels.⁴ This hypothesis is based on evidence that some tumor ECs express markers of endothelial progenitor cells, such as CD34.³² A third theory is that tumor-associated ECs develop from dedifferentiated tumor cells, and hence retain several of their properties.⁴ This is based on evidence that

Abbreviations used in this paper: EC = endothelial cell; ET-1 = endothelin-1; GBM = glioblastoma multiforme; IL-8 = interleukin-8; SMA = smooth-muscle actin; VE-cadherin = vascular endothelial cadherin; VEGF = vascular endothelial growth factor; VEGFR = VEGF receptor; vWF = von Willebrand factor.

certain tumor-derived ECs have the same chromosomal aberrations as the tumor cells with which they are associated.³⁰ At this time, none of these hypotheses has been confirmed, and it is likely that the tumor vasculature has multiple origins.

Morphological Findings

Endothelial cells were isolated according to published protocols.^{5,23} Different methods of EC isolation are currently in use; in our laboratory we use diacetylated low-density lipoprotein labeling for purification of these cells, whereas other researchers use magnetic beads.^{5,23} Glioma-derived ECs are positive for the typical markers (vWF, CD31, and CD105), thus confirming the purity of the EC population. Glioma cells, glia, or macrophages were not present, as judged from the lack of staining with antibodies to glial fibrillary acidic protein or CD11b, which are specific markers for astrocytes and microglia, respectively.

Tumor EC cultures are clearly distinguishable from normal ECs morphologically. Data from our and other laboratories reveal that ECs derived from GBMs, meningiomas, and neuroblastomas have a flat appearance, with large nuclei, abundant cytoplasm, multiple nucleoli, and veil-like structures.^{1,5} Furthermore, these tumor-associated cells do not acquire the cobblestone-like appearance that is typical of normal ECs. In contrast, normal brain ECs are smaller, with limited cytoplasm.⁵ However, others report that GBM-associated ECs maintain a cobblestone-like morphology in culture;²³ these differences may be attributed to the region of tumor from which the ECs were obtained. The specific characteristics of GBM-derived ECs are summarized in Table 1.

Endothelial Marker Expression

There are several reports in which the hypothesis has been advanced that ECs derived from a variety of tumors express different markers compared with normal ECs. Tumor EC lines derived from renal cell carcinomas, ovarian carcinomas, and brain tumors express the typical markers such as vWF, CD105, CD31, and VE-cadherin, similarly to control ECs.^{1,2,4,5,23} Our studies of ECs derived from GBMs confirm these findings.⁵ Furthermore, we observed that approximately 50% of the glioma-associated ECs express α -SMA, a protein that is totally absent from control ECs obtained in the brain.

We know that SMA is a cytoskeletal protein present mostly in mural cells, such as smooth-muscle cells and pericytes.³ This protein is involved in initiation of cell contraction by stimulation of the contractile apparatus, a function directly related to cell migration.³³ Consequently, expression of SMA by tumor ECs may be directly related to their enhanced migratory ability. Contradictory reports exist regarding the expression of CD34 and CD105 in tumor ECs. The expression of CD34, a marker for endothelial progenitor cells, was reported to be elevated in some tumor blood vessels (supporting the suggestion that tumor ECs might derive from endothelial progenitor cells), and it was lowered in others, whereas the expression of CD105 (endoglin) was downregulated.^{12,13,32,34} Endoglin is a co-re-

TABLE 1
Summary of functional and phenotypic characteristics of GBM-associated compared with normal brain ECs

Property	ECs	
	GBM	Normal Brain
morphology	large, flat cells	small, plump cells
proliferation	low	high
drug sensitivity	resistant	sensitive
migration	high	low
growth factor production	VEGF, ET-1, & IL-8 production	low growth factor production
endothelial marker expression	vWF, CD105, & CD31	vWF, CD105, & CD31
VE-cadherin	low expression	high expression
CD31	cytoplasmic localization	localized at plasma membrane
α -SMA	~50% positive	negative

ceptor mediating signaling through the transforming growth factor- β receptor; therefore, increased expression of endoglin correlates with increased angiogenesis. In addition, CD105 expression correlates with highly proliferative vessels.

In our studies, we detected no differences in the expression of CD34 and CD105 between glioma-associated and control ECs. The differences in CD105 expression between different types of tumor ECs may reflect the heterogeneity in their proliferation rates. Consequently, the highly proliferating angiogenic vessels in the periphery of a tumor may be more positive for endoglin than the slowly proliferating blood vessels located close to the necrotic region of the tumor.²² Also, it is likely that the tumor origin and grade affect EC proliferation and CD105 expression.

Our studies demonstrated that GBM-derived ECs have altered expressions of CD31 and CD144 (or VE-cadherins). The VE-cadherin CD144 is a tight junction protein that plays an important role in the integrity of the blood-brain barrier. Therefore, the reduced expression of this junctional protein may contribute to leakiness in tumor blood vessels.⁷ The presence of VE-cadherin is also critical for angiogenesis; blocking CD144 leads to abnormal capillary junctions and the formation of aberrant tubules.^{11,34} The loss of VE-cadherin expression in gliomas was recently confirmed by other investigators.²³ In fact, it has been demonstrated that in GBMs, tumor microvessels lose the expression of several tight junction proteins such as claudin-1, claudin-5, and occludin.^{20,26} Consequently, this leads to opening of the microvessel junctions and leaking of fluid into the brain.⁷

In stained slides of tumor-associated and normal brain ECs, we observed that the intensity of the CD31 staining was similar in both; however, the distribution of CD31 (also known as platelet endothelial cell adhesion molecule-1) was different. In glioma-associated ECs, CD31 was present in the cytoplasm, whereas in control cells this receptor was predominantly expressed on the cell surface. The VE-cadherin CD31 is a receptor that is present on both ECs and leukocytes, and it is involved in leukocyte transmigration, angiogenesis, and apoptosis.¹⁶ In the past it has been reported¹¹ that there are low numbers of infiltrating, cytotoxic T cells in gliomas, compared with the number of

T cells involved in an inflammatory response in the brain, although in both cases high levels of angiogenic factors are produced. This diminished leukocyte infiltration may be caused by the inability of lymphocytes to interact with ECs, and the consequently reduced transmigration of leukocytes into the tissue. Studies of leukocyte–endothelial interactions in tumors are worthy of investigation.

Endothelial Cell Adhesion Molecules

Endothelial cells derived from a variety of tumors have differences in the expression of leukocyte adhesion molecules; for example, intercellular adhesion molecule–1 expression on lymphoma-derived ECs is upregulated, whereas its expression on ECs derived from colon, ovarian, and renal cell carcinomas is downregulated.^{1,11} Similar discrepancies have been reported for vascular cell adhesion molecule–1 expression.^{1,19,24,27} An activation marker for ECs, E-selectin, was consistently reported to be absent from normal endothelium and present on the tumor vascular endothelium.³⁵ The presence of E-selectin was also found in blood vessels in high-grade glioma, but it was absent from blood vessels in low-grade glioma and in normal tissue.²³ Nevertheless, one group reported E-selectin expression in both normal and tumor-derived ECs.²³ Interestingly, GBM-derived human ECs were shown to have high levels of intercellular adhesion molecule–1, vascular cell adhesion molecule–1, and E-selectin.^{1,4}

Proliferation

Based on the highly vascular nature of glial tumors, proliferation rates of tumor ECs were analyzed. Several studies showed that these cells proliferate faster than normal ECs,^{4,8,23} whereas we demonstrated a significantly slower rate of replication of GBM-associated compared with normal human brain ECs.⁵ This discrepancy may be due to the fact that in previously published studies the vasculature examined was derived from mouse tumor explants *in vitro*⁸ or EC lines,⁴ whereas in our studies we used human primary cultures of GBM-derived ECs. Furthermore, there is evidence that the tumor vasculature is not homogeneous throughout the lesion.²² Thus, differences could be due to the fact that some groups examined ECs derived from the tumor periphery, where active angiogenesis and EC proliferation takes place, whereas other groups examined ECs derived from the internal part of the tumor, where hypoxia and extensive necrosis do not favor EC proliferation.²² In addition, our results are consistent with the observation that there may be a temporal dichotomy between migration and proliferation, because glioma cells with decreased motility demonstrate increased proliferation.¹⁰

Cell Survival

Rates of cell death and survival are critical for tumor growth. Therefore, the level of apoptosis in tumor ECs was studied. In renal carcinoma–derived ECs, increased survival and upregulation of the antiapoptotic protein Bcl-2 and proteins of the inhibitor of apoptosis family were demonstrated; these cells were resistant to serum starva-

tion and to vincristine-induced apoptosis.⁴ Murine tumor EC cultures were shown to be resistant to etoposide.¹⁴ These observations were similar to those in GBM-derived ECs, which underwent less apoptosis after serum starvation *in vitro*.⁵ Our data showing that tumor ECs have enhanced survival properties were consistent with complementary DNA microarray studies demonstrating that migrating glioma cells exhibit a downregulation of proapoptotic and upregulation of antiapoptotic genes.²¹ The presence of abnormal centromeres in tumor ECs may result in this genetic instability, which may be the cause of the intrinsic chemoresistance of tumor ECs.¹⁴

Differences in the signaling pathways responsible for regulation of cell survival were observed in tumor ECs. Renal tumor–derived ECs exhibited increased levels of Akt phosphorylation and activation.⁴ The phosphatidylinositol 3-kinase/Akt signaling pathway is known to be one of the major cellular pathways controlling cell survival.¹⁷ In addition, the expression of PTEN, an inhibitor of phosphatidylinositol 3-kinase, was downregulated in human renal carcinoma ECs compared with normal cells.⁴

Migration and Tubule Formation

Tumor EC migration was investigated, because these cells participate in tumor angiogenesis. Glioblastoma-derived ECs were found to migrate more than those in normal brain, based on results of modified Boyden chamber migration assays.^{5,6} In addition, these cells were unresponsive to stimulation by such growth factors as VEGF, ET-1, and IL-8. The tubule-formation assay was performed on tumor-associated ECs derived from different organs. Bussolati and coworkers⁴ demonstrated that renal tumor ECs form tubules both *in vivo* and *in vitro*, even in the absence of serum; this is in contrast to normal ECs, which formed tubules *in vitro* but not *in vivo*. Similarly, Alessandri and colleagues¹ reported that ECs derived from GBMs, meningiomas, neuroblastomas, and ovarian and kidney carcinomas were all able to form tubules *in vitro*. These data demonstrate that tumor ECs have angiogenic properties and therefore can actively participate in tumor angiogenesis.

Growth Factor Production

Angiogenic growth factor production is an essential characteristic of tumor cells. It has been demonstrated in recent studies that tumor ECs are also able to produce high amounts of growth factors. Human renal carcinoma–derived EC lines were shown to have increased levels of angiopoietin, angiogenin, VEGF-D, and VEGFR-2 (Flk/KDR) expression. In addition, other studies have shown an upregulation of IL-8 expression in ECs derived from GBMs and ovarian and kidney carcinomas.^{1,6} These studies also demonstrated expression of the VEGFRs Flk-1/KDR and Flt-1, and the IL-8 receptors CXCR1 and CXCR2, on ECs derived from GBMs and from ovarian and kidney carcinomas. However, no difference was detected in the level of receptor expression when this was compared with adrenal cortex microvascular ECs, but higher levels of VEGFR expression were detected when compared with ECs obtained from human umbilical vein.¹

Our studies demonstrated that GBM-derived ECs have increased expression of VEGF, ET-1, and IL-8, compared with their normal counterparts.^{5,6} The fact that glioma-associated ECs are actively producing functional growth factors is consistent with the high migration rate of these cells and their enhanced survival in serum-deprived media.^{1,4,5} Treatment of glioma-associated ECs with neutralizing antibody to VEGF significantly reduced their IL-8 production, indicating the existence of autocrine loops, in which specific growth factors induce the production of other growth factors.⁶ Furthermore, it was demonstrated that transforming growth factor- β 1, a major negative regulator of IL-8 production in normal brain ECs produced by gliomas,^{15,29} stimulates IL-8 production in GBM-associated ECs.⁶ From these data we infer that both paracrine and autocrine activations are taking place within the tumor EC microenvironment.

Gene Expression and Cytogenetic Studies

The distinct functional and phenotypic properties of ECs derived from a variety of tumors have led to the investigation of their gene expression and cytogenetic studies. Gene expression analysis of colorectal tumor ECs demonstrated that 46 transcripts were increased in tumorous compared with normal endothelium, and that most of these transcripts were EC surface molecules.³¹ Furthermore, fluorescence in situ hybridization analysis performed in mouse ECs isolated from human tumor xenografts demonstrated cytogenetic abnormalities, such as aneuploidy and abnormal centrosomes, in tumor ECs.¹⁴ Similarly, other studies have demonstrated that B-cell lymphoma-derived ECs exhibited the same chromosomal aberrations that were observed in the lymphoma from which they were derived.³⁰ These cytogenetic abnormalities were attributed to the exposure of ECs to the tumor microenvironment.

Conclusions

Current research has demonstrated that tumor ECs have different functional and phenotypic properties compared with control cells. Tumor ECs derived from a variety of lesions were found to differ from normal ones morphologically and in the expression of specific markers. In addition, glioma-associated ECs exhibited different proliferation, migration, adhesion, and growth factor production properties compared with normal brain ECs. These properties may be particularly important in understanding the survival mechanism of glioma-associated ECs and providing information leading to the treatment of gliomas with antiangiogenic therapy.

The evidence that tumor ECs found in gliomas may actually be proliferating at a much lower rate than normal ECs is particularly important in terms of antitumor therapy, because most antiangiogenic drugs currently used for tumor therapy target rapidly proliferating cells. Therefore, conventional antiangiogenic drugs may be less effective in targeting the tumor blood vessels. Consequently, antiangiogenic therapy needs to be focused on the different characteristics of tumor ECs.

The study of glioma-associated ECs is important for the development of novel antiangiogenic therapies that spe-

cifically target the tumor vasculature. An approach using multiple drugs targeting tumor cells, the rapidly proliferating ECs, and the tumor-associated ECs should be considered. Combination therapies are currently being used in many kinds of cancers, including gliomas.²⁸ These approaches involve targeting VEGFRs, endoglin, and vascular integrins, such as $\alpha\beta$ 3 which is overexpressed in tumor endothelium.²⁵ Most of these approaches, however, present problems such as partial patient response and/or toxicity. Successful antiangiogenic therapy should target proteins or growth factors that are specifically overexpressed in tumor vasculature and absent in normal vessels. Such an approach would increase the drug's specificity and selectivity, and reduce its toxicity. Thus, understanding the characteristics of glioma-associated ECs and the interactions between normal ECs and the tumor microenvironment is essential for the development of more selective antiangiogenic therapies.

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