Characteristics of tumor-associated endothelial cells derived from glioblastoma multiforme

CHRISTIANA CHARALAMBOUS, PH.D., THOMAS C. CHEN, M.D., PH.D., AND FLORENCE M. HOFMAN, PH.D.

Departments of Molecular Microbiology, Immunology, Pathology, and Neurosurgery, University of Southern California Keck School of Medicine, Los Angeles, California

Angiogenesis, the formation of new blood vessels from preexisting ones, is essential for efficient tumor growth and expansion. Glioblastomas multiforme are particularly hypervascular tumors characterized by extensive blood vessel growth and angiogenesis. 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. 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.

In recent publications, 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. 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. This hypothesis is based on the fact that tumor ECs display characteristics of activated ECs. The second hypothesis is that tumor ECs develop from an endothelial progenitor cell, which has migrated to the tumor site and differentiated into vessels. This hypothesis is based on evidence that some tumor ECs express markers of endothelial progenitor cells, such as CD34. A third theory is that tumor-associated ECs develop from dedifferentiated tumor cells, and hence retain several of their properties. 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.
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. 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 laboratories use magnetic beads. 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. 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. However, others report that GBM-associated ECs maintain a cobblestone-like morphology in culture; 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. Our studies of ECs derived from GBMs confirm these findings. 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. This protein is involved in initiation of cell contraction by stimulation of the contractile apparatus, a function directly related to cell migration. 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. Endoglin is a co-

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

C. Charalambous, T. C. Chen, and F. M. Hofman

---

**TABLE 1**

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

<table>
<thead>
<tr>
<th>Property</th>
<th>GBM</th>
<th>Normal Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>morphology</td>
<td>large, flat cells</td>
<td>small, plump cells</td>
</tr>
<tr>
<td>proliferation</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>drug sensitivity</td>
<td>resistant</td>
<td>sensitive</td>
</tr>
<tr>
<td>migration</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>growth factor production</td>
<td>VEGF, ET-1, &amp; IL-8</td>
<td>low growth factor</td>
</tr>
<tr>
<td>endothelial marker expression</td>
<td>vWF, CD105, &amp; CD31</td>
<td>vWF, CD105, &amp; CD31</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>low expression</td>
<td>high expression</td>
</tr>
<tr>
<td>CD31</td>
<td>cytoplasmic localization</td>
<td>localized at plasma membrane</td>
</tr>
<tr>
<td>α-SMA</td>
<td>~50% positive</td>
<td>negative</td>
</tr>
</tbody>
</table>
Characteristics of tumor-associated endothelial cells from GBMs

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.11 Similar discrepancies have been reported for vascular cell adhesion molecule–1 expression.10,24,27 An activation marker for ECs, E-selectin, was consistently reported to be absent from normal endothelium and present on the tumor vascular endothelium.22 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.24 Interestingly, GBM-derived human ECs were shown to have high levels of intercellular adhesion molecule–1, vascular cell adhesion molecule–1, and E-selectin.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.3 This discrepancy may be due to the fact that in previously published studies the vasculature examined was derived from mouse tumor explants in vitro6 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.25 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.20

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 starvation 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.3 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.27 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

Neurosurg. Focus / Volume 20 / April, 2006
Our studies demonstrated that GBM-derived ECs have increased expression of VEGF, ET-1, and IL-8, compared with their normal counterparts. 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. 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. Furthermore, it was demonstrated that transforming growth factor–β1, a major negative regulator of IL-8 production in normal brain ECs produced by gliomas, stimulates IL-8 production in GBM-associated ECs. 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 tumors compared with normal endothelium, and that most of these transcripts were EC surface molecules. 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. 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. 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 specifically 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. These approaches involve targeting VEGFRs, endoglin, and vascular integrins, such as αvβ3 which is overexpressed in tumor endothelium. 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.

**References**

Characteristics of tumor-associated endothelial cells from GBMs


Manuscript received January 17, 2006. Accepted in final form March 1, 2006. This work was supported by grants from the Connell and Kriegel Foundations to Dr. Chen, and from the James H. Zumbarge Faculty Research and Innovation Fund and the California Breast Cancer Research Program to Dr. Hofman.

Address reprint requests to: Florence M. Hofman, Ph.D., Department of Pathology, University of Southern California Keck School of Medicine, 2011 Zonal Avenue, HMR 315, Los Angeles, California 90033. email: hofman@usc.edu.