CD133 as a Marker for Regulation and Potential for Targeted Therapies in Glioblastoma Multiforme

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KEYWORDS

• Cancer stem cell • CD133 • Glioblastoma • Glioma

KEY POINTS

- CD133 is a reliable and widely studied tumor marker for glioblastoma multiforme (GBM) cells with cancer stem cell (CSC)-associated phenotypes.
- Tumorigenic capacity is not limited in the $CD133^+$ subpopulation, and several models bridging the relationship between $CD133^+$ and $CD133^-$ tumorigenic cells in GBMS have been proposed.
- Many of the studies examining $CD133^+$ GBM cells as putative CSCs are complicated by the complex regulation of CD133, lack of a uniform protocol, and ability of in vivo and in vitro studies to replicate the true physiologic role of CD133 in patients.
- Associations between the presence of $CD133^+$ cells within resected tumors and clinical outcomes, including poorer prognosis and resistance to adjuvant therapies, have been demonstrated, and $CD133^+$ cells remain a promising target for future GBM therapies.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor found in adults. Despite standard treatment comprising surgical resection followed by concomitant radiotherapy and adjuvant temozolomide chemotherapy, the prognosis for GBM is poor, with a median survival of 14.6 months.[1] The major challenge for current treatment paradigms stems from the characteristically diffuse patterns of tumor infiltration throughout healthy brain parenchyma.[2] Most therapeutic efforts and glioma research to date have focused on the tumor in its entirety; however, recent findings have highlighted the incredible heterogeneity of GBM cells, denoted by the term *multiforme*, in terms of not only their immunogenic, histologic, and genetic profile but also their proliferative and tumorigenic potential. Thus, the characterization and identification of the different GBM cell types, particularly

Disclosure of interests: Marko Spasic was partially supported by an American Association of Neurological Surgeons Fellowship grant. Isaac Yang (senior author) was partially supported by a Visionary Fund Grant, an Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research UCLA Scholars in Translational Medicine Program Award, the Stein Oppenheimer Endowment Award, and the STOP CANCER Jason Dessel Memorial Seed Grant.

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Neurosurg Clin N Am 23 (2012) 391–405
doi:10.1016/j.nec.2012.04.011

those involved with driving tumor growth, are critical not only in the understanding of GBM formation and pathogenesis but also in the advancement of more effective cancer therapies.

Cancer Stem Cells

The cancer stem cell (CSC) model has provided a new paradigm in understanding what predominant cellular mechanisms drive tumor growth. Similar to the organization of growth processes within normal tissue such as bone marrow or intestinal epithelium, the CSC concept postulates the presence of a functional and cellular hierarchy within the heterogeneous tumor body.[3] Within this hierarchical model, the vast majority of the tumor bulk comprises rapidly dividing, partially or terminally differentiated cells with limited replicative potential, while neoplastic growth is driven by a small population of cancer cells with stemlike properties.[3] Similar to the properties of normal stem cells, CSCs represent a population of neoplastic cells that have the capacity to initiate and maintain tumors and are characterized by self-renewal, resistance to chemical insults and radiation, and ability to produce new tumors even after periods of dormancy.[3]

To be defined as CSCs, the cells must have the following characteristics: (1) self-renewal and proliferation, (2) multilineage differentiation into mature fates resembling tissue of origin, and (3) capacity to form new tumors resembling the original tumor. To date, studies have used similar procedures to identify and characterize the stemness of putative CSCs. A differentially expressed marker or set of markers is used to isolate a small subset of tumor cells within the tumor bulk.[3] The capacity to form nonadherent tumorspheres by limiting dilution in culture conditions permissive for stem cell proliferation is assessed to demonstrate properties of clonal expansion and self-renewal and is used to identify and expand potential CSCs. In vivo demonstration of CSC behavior is confirmed by the ability of putative CSCs transplanted into immunodeficient mice to form tumors resembling the original tumor.[4]

DISCOVERY OF CD133

CD133 was first identified by Yin and colleagues[5] in 1997 through generating a monoclonal antibody–recognizing AC133, a glycosylated CD133 epitope. AC133 expression was restricted to the $CD34^+$ subset of hematopoietic stem cells derived from human fetal liver, adult blood, and bone marrow, suggesting that AC133 is a novel marker for human hematopoietic stem cells and progenitor cells. Unlike CD34, the AC133 antigen is not found in other blood cells, endothelial cells (ECs), or fibroblasts and may be an important marker of more primitive progenitor cells.[5,6] In xenograft models, $AC133^+$ cells obtained from primary fetal sheep recipients demonstrated sustained proliferative and self-renewal potential when transplanted into secondary recipients.[5] Of note, a second glycosylated CD133 epitope, AC141, distinct from AC133, has also been characterized, and both AC141 and AC133 are commonly used to identify and purify $CD133^+$ cells.[7,8]

The gene for human CD133, located on chromosome 4p15.33, encodes a 120-kD protein with 865 amino acids and shares 60% homology with mouse prominin, which is localized to neuroepithelial stem cells.[9,10] Structurally, CD133 comprises 5 transmembrane domains, an extracellular N-terminus, a cytoplasmic C-terminus, 2 large extracellular loops containing 8 putative N-glycosylation sites, and 2 smaller cytoplasmic loops.[10] Although the precise function of CD133 is unclear, the localization of AC133 antigen expression on epithelial microvilli suggests a possible role in the organization of plasma membrane topography and the maintenance of apical-basal cell polarity.[6,11] Associations between CD133 and plasma membrane cholesterol have alluded to a role in the regulation of lipid composition within the cell membrane.[6,7] Loss of CD133 has been associated with degeneration of photoreceptors associated with improper retinal disk formation, suggesting a possible role in the regulation of phototransduction and neural retinal development.[12,13] In addition, $CD133^+$ progenitor cells obtained from human fetal aorta have also been shown to stimulate Wnt pathway–dependent angiogenesis during wound healing of ischemic diabetic ulcers.[14,15]

CD133 AS A STEM CELL MARKER

Following the initial characterization in $CD34^+$ hematopoietic stem cells, CD133 has been used to purify several progenitor and stemlike cell populations within both healthy and neoplastic tissues. $CD133^+$ neural progenitor cells have been identified in human fetal and postmortem brain tissue.[16–19] Neural stemlike cells have been isolated from human fetal brain tissue through flow cytometry. These $CD133^+$, $CD34^-$, $CD45^-$ cells demonstrated characteristic stem cell activity, including clonal expansion, serial neurosphere culture initiation, and differentiation.[17,18] On intracranial transplantation into neonatal nonobese diabetic, severe combined immunodeficient (NOD-SCID) mice, the sorted cells successfully engrafted, migrated, proliferated, and differentiated along neuronal and glial fates.[8,17]

Human epithelial-derived $CD133^+$ cells[20] and fetal liver-derived $CD133^+$, $CD34^+$, $CD3^-$ hematopoietic cells[21] have been shown to differentiate into neurons and astrocytes on transplantation into intracranial mouse and in culture with differentiation-promoting media. Vascular endothelial growth factor receptor 2–positive circulating human endothelial progenitor cells coexpress CD133, and plating of these nonadherent cells in vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 led to rapid differentiation, loss of CD133 expression, and formation of adherent colonies.[22] Similar downregulation of CD133 on differentiation was observed in the colon carcinoma–derived epithelial cell line Caco-2.[23]

Some studies have demonstrated the ability of these progenitor cells to reconstitute the original tissue.[16,24,25] $CD133^+$ human prostate basal cells showed increased proliferation in vitro and, on transplantation, formed fully differentiated prostate epithelium–expressing prostatic secretions.[24] CD133 expression either independently or in conjunction with other stem and progenitor cell markers has also been used to identify CSC subpopulations within a wide range of human cancers, including osteosarcomas,[26,27] laryngeal carcinomas,[26,28] melanomas,[26,29–32] breast cancers,[26,33] hepatocellular carcinomas,[26,34] prostate tumors,[26,35,36] retinoblastomas,[10] leukemias,[37–39] and non–small cell lung cancer.[40] In addition, CD133 expression has been used to purify CSC populations in a number of brain tumors, including medulloblastomas[5,26] and ependymomas.[26,41]

Gliomas

Initial evidence suggesting the presence of brain tumor cells with stemlike potential came from the isolation of a clonogenic subpopulation of cells from resected gliomas through neurosphere assays.[42,43] These neurospheres possessed the capacity for self-renewal and demonstrated intraclonal neuronal and glial cell lineage heterogeneity. Among the several markers associated with CSCs, CD133 is one of the most widely studied in brain tumors.[15] Singh and colleagues[44] first identified a subpopulation of brain tumor cells within medulloblastomas and gliomas coexpressing CD133 and nestin, a marker for undifferentiated neural stem cells. Unlike the $CD133^-$ population, $CD133^+$ cells, representing 0.3% to 25.1% of the tumor bulk, were capable of neurosphere formation in serum-free media following addition of the stem cell growth factors bFGF and EGF. These tumorspheres had greater proliferative capacity relative to neural stem cell controls and formed secondary tumorspheres with immunoreactivity for nestin and CD133. Tumorspheres resembled a more primitive state and were devoid of glial fibrillary acidic protein (GFAP) and β-tubulin III, markers of differentiated neural cells and glial cells, respectively. However, $CD133^+$ cells were capable of multilineage differentiation into oligodendrocytes, astrocytes, and neurons and expressed differentiated markers mirroring that of the parent tumor.[6] Although many xenograft assays require up to 10^6 tumor cells for successful engraftment,[45–47] as few as 100 purified $CD133^+$ tumor cells have been reported to be sufficient for intracranial tumor formation when transplanted into NOD-SCID mice. In contrast, injection of 10^5 $CD133^-$ cells failed to form tumors.[45] Engrafted $CD133^+$ GBM cells produced tumors with classic GBM features that were phenotypically identical to the patient's original tumor and could be serially transplanted.[45] In addition, engrafted tumors were widely infiltrating and comprised differential expression of CD133 and GFAP, suggesting that $CD133^+$ cells were capable of self-renewal and differentiation in vivo.[45]

Subsequently, 2 groups identified tumorsphere-forming subpopulations within tumor specimens obtained from patients with primary and secondary GBMs.[48,49] Spheres arising from less than 1% of all GBM cells coexpressed nestin and CD133. Unlike the progeny of normal neural stem cell (NSC)-derived neurospheres, differentiation of GBM tumorspheres led to progeny that closely mirrored the parental tumor phenotype, comprising 80% β-tubulin III^+ cells and 25% oligodendrocytes. In addition, tumorspheres demonstrated greater proliferative potential and the ability to retain stemlike features following differentiation, suggesting a role in the maintenance of the stem cell pool and production of differentiated cells within the tumor bulk.[49] Unlike the non–sphere-forming population, the sphere-forming GBM cells were able to form tumors following transplantation in nude mice, and the new tumors expressed both neural and glial markers.[49] Expression of CD133 is restricted to a subpopulation of tumor cells positive for the neural stem and progenitor cell marker nestin, which suggests that this subpopulation may represent a less-differentiated subset of tumor cells.[50] Consistent with CSC model, which proposes that CSCs represent a rare fraction of tumor cells, the extent of CD133 expression within gliomas is typically low or barely detectable, as demonstrated by quantitative analysis using flow cytometry.[7,45,51–54] However, some immunohistochemical reports have suggested that the $CD133^+$ glioma fraction comprises up to 25% of the tumor bulk.[50]

Recently, transcriptome profiling of purified CD133$^+$ and CD133$^-$ GBM cells identified a CD133 gene expression profile comprising 214 differentially expressed genes.[55] Computational comparison with established stem cell and cancer cell profiles demonstrated a close association of the CD133 gene expression signature with that of human embryonic stem cells. The CD133 gene signature distinctly differentiated between NSC-like GBM cells cultured in stem cell medium and GBM cells cultured in serum. Enrichment of the CD133 gene signature was closely associated with increasing glioma grade, with greatest resemblance in grade IV GBMs. The CD133 gene signature was associated with a more aggressive GBM subtype and significantly shorter median patient survival. In addition, the GBMs enriched for the CD133 gene signature were associated with a greatly increased number of genetic mutations. Overall, the CD133 gene signature, obtained from sorted CD133$^+$ populations, is characteristic of a stemlike cell population of tumor cells as well as more clinically aggressive and hypermutated subtypes of GBM cells.[55]

There exists a functional hierarchy within the heterogeneous GBM cell population consisting of a small CD133$^+$ fraction of GBM cells that formed tumorspheres in stem cell media, generated serial tumorspheres on dissociation, was enriched for several stem cell markers (nestin, Musashi 1, and SOX2), and gave rise to progeny-expressing neuronal, oligodendrocytic, and astrocytic markers on culturing under prodifferentiation conditions.[56] Consistent with in vitro features of stem cells, CD133$^+$ GBM cells demonstrated a heightened capacity for proliferation, self-renewal, and multilineage differentiation.[56] Transplantation led to the formation of highly invasive and angiogenic tumors that histologically and morphologically resembled the original patient GBM.[4,45] Altogether, these findings suggest that the CD133$^+$ GBM subpopulation contains putative stemlike cells that can initiate and maintain tumors that phenotypically resemble the original GBM and are capable of driving tumor growth even when challenged with serial transplantations.[56]

CD133$^-$ CELLS IN GLIOMAS

Although there has been mounting evidence for a selected population of cells with stemlike properties, the role of CD133 as a definitive marker for tumor-initiating cells is contentious. Studies have demonstrated that CD133$^+$ cells are absent from select GBM specimens and glioma cell lines that are capable of tumor formation in vivo.[7,52,57,58] Following initial studies characterizing the stemness of CD133$^+$ glioma cells and the lack of tumorigenicity in CD133$^-$ cells, several investigators demonstrated that CD133$^-$ glioma cells were also capable of in vivo tumor initiation and maintenance.[54,57] Beier and colleagues[57] demonstrated that both CD133$^+$ and CD133$^-$ populations obtained from primary GBM cell cultures formed tumorspheres in stem cell–permissive media. Although the CD133$^-$ sphere-forming population represented a smaller fraction than the CD133$^+$ sphere-forming cells, both subpopulations demonstrated stemlike features in vitro, including sustained proliferative ability and formation of tumorspheres containing cells expressing differentiated markers from all 3 neural lineages.[57] Transplantation of both CD133$^+$ and CD133$^-$ GBM cells formed GBM-resembling tumors within mice. Transplantation of CD133$^-$ cells derived from CD133$^+$ cell lines failed to produce tumors,[57] suggesting a functional heterogeneity between tumorigenic CD133$^-$ cells and CD133$^+$-derived CD133$^-$ cells.

Studies using marker-independent sorting methods have further demonstrated the presence of putative CD133$^-$ GBM CSCs. Using fluorescence-activated cell sorting (FACS) based on specific tumor cell autofluorescence, Clément and colleagues[51] isolated a subpopulation of glioma cells (FL1$^+$) with high nuclear:cytoplasmic ratios capable of multipassage tumorsphere formation. FL1$^+$ cells demonstrated marked capacity for self-renewal, differentiation associated with a loss of FL1 properties, and tumorigenicity when as few as 10^3 FL1$^+$ cells were transplanted into mice. FL1$^+$ cells displayed a heightened expression of several stemness-related genes, including POU5F1/OCT4, SOX2, NOTCH1, and NANOG. However, CD133 expression was not correlated with tumorigenicity, and differences in CD133 expression between FL1$^+$ cells and the remaining tumor block were not statistically significant. The side population (SP), sorted based on the ability to extrude Hoechst 33342 dye, represented a rare stemlike fraction of highly tumorigenic glioma cells that, unlike non-SP cells, can give rise to both SP and non-SP cells.[16,59] While studies examining the SP and CD133 expression have been scarce, a single murine glioma cell line (GL261) study found that sphere-forming and highly tumorigenic CD133$^+$ glioma cells demonstrating stemlike features did not reside within the SP.[16,60] Although this may suggest the presence of a tumorigenic CD133$^-$ SP cells, recent data from Broadley and colleagues[61] demonstrated that cells within the GBM SP could not self-renew and form tumors.

Son and colleagues[58] used stage-specific embryonic antigen 1 (SSEA-1), a marker for embryonic stem cells, to purify a subpopulation of patient-derived GBMs with high tumorigenic potential. Although CD133 expression was present in only half of the 24 GBM samples, SSEA-1 immunoreactivity was present in all but one. Relative to the SSEA-1$^-$ population, SSEA-1$^+$ cells expressed higher levels of established stem cell makers, including SOX2, Bmi2, L1CAM, Olig2, and Ezh2. Independent of CD133 expression, SSEA-1 allowed enrichment of a tumorsphere-forming population with increased clonogenic potential and the ability to differentiate down neuronal lineages. In addition, SSEA-1$^+$ cells could generate both SSEA-1$^-$ and SSEA-1$^+$ cells, suggesting a hierarchal organization underlying a heterogeneous population.[58] Unlike SSEA-1$^-$ cells, transplantation of as few as 10^4 SSEA-1$^+$ cells successfully formed tumors in mice that retained their tumorigenic capacity following serial transplantations.[58] Similarly, other investigators have successfully identified putative GBM CSCs expressing alternative markers, including integrin $\alpha 6$[62] and A2B5,[63] that displayed heightened tumorigenicity and distinct stemlike phenotypes that either lacked or were independent of CD133 immunoreactivity.[16,64] In addition, alternative methods and parameters have been used to identify putative CSCs within gliomas.[48,59,64–66]

Limitations

The variable protocols used in the purification of CD133$^+$ cells and the assessment of stemness make comparing study results difficult.[67] Tumor samples have been obtained from a variety of sources (eg, fresh resections from patients,[50] glioma cell lines[60]) and cultured in variable conditions, including serum-free media[16,44] and in the presence of fetal calf serum.[16,68] Because serum has been shown to induce differentiation, culture media should be taken into account when assessing results from studies examining CD133 expression.[16,67,69]

Furthermore, many of the CD133 studies use AC133 monoclonal antibodies, which recognize glycosylated CD133 epitopes. Accurate detection of CD133 may be limited because of the unknown specificity of AC133 binding to differential glycosylation patterns of the 8 potential glycosylation sites on CD133.[5,16] Furthermore, studies have characterized several different tissue-dependent isoform tissue produced by alternative splicing of the CD133 transcript within human[37] and murine models.[8,70] It is possible that AC133 monoclonal antibodies, unable to uniformly recognize different isoforms, would provide an underestimate of true CD133 protein expression levels within gliomas.[8,71] Recently, Osmond and colleagues[71] reported that GBM cells found to be AC133$^-$ contained a truncated and possibly functional CD133 variant that was localized within the cytoplasm. It is unclear whether the isoform type, the extent of protein expression, or the specific glycosylation status is the more biologically relevant marker of CD133 expression.[8] Tissue distribution of CD133 messenger RNA is much more prevalent than expression of AC133,[10] as the downregulation of the AC133 epitope is independent of intracellular levels of CD133 messenger RNA.[8,23,72] Thus, the presence of AC133 epitopes is not necessarily equivalent to CD133 expression.

Although transplantation of sorted cells into immunodeficient mice remains the gold standard of assessing tumorigenicity, it has several limitations. The process of sorting and transplanting tumor cells into mice subjects the cells to several procedural insults and places them in a foreign environment vastly different from the original tumor niche.[3] Inherent differences between the patient and mice microenvironment due to various species barriers (eg, differences in cytokines and growth factors important in tumor growth) may drastically alter proliferation potential.[3] In addition, the xenographic immune response in mice receiving transplants is significantly more robust than the native immune response within the patient.[73] Fundamentally, the issue is the applicability of tumor xenograft assays in immunocompromised mice in studying the clinical behavior of human cancers.

LINKING CD133$^-$ AND CD133$^+$ SUBPOPULATIONS

Despite the limitations, there is still strong support for the role of both CD133$^+$ and CD133$^-$ cells in driving GBM tumorigenesis and maintenance. Progress in understanding the multifaceted mechanisms regulating CD133$^+$ expression has led to several models proposed to bridge the functional relationship between these 2 cell types.

The Multiple CSC Model

Recent data have suggested that CD133$^+$ and CD133$^-$ tumorigenic glioma cells may represent distinct CSC populations associated with different GBM subtypes.[65,74] Following the initial characterization of CD133$^-$ glioma cells with stemlike properties,[57] Lottaz and colleagues[74] compared gene expression profiles in various GBM CSC lines and identified a 24-gene signature that faithfully distinguished 2 distinct subgroups of GBM CSC

cells. Compared with a previously established gene signature differentiating different GBM subtypes,[74,75] type I CSCs showed a proneural transcriptional profile, whereas type II CSCs displayed a mesenchymal profile. Type I CSCs resembled fetal NSCs and were strongly CD133 positive, whereas type II CSC lines resembled adult NSCs and were mainly CD133 negative. In accord with previous studies reporting different growth capacities and extent of stemlike phenotypes between the 2 CSC types,[57,65] $CD133^+$ type I CSCs formed tumorspheres in culture, whereas type II cells displayed semiadherent growth. Key molecular differences among the 2 types included differences in TGF-β/BMP pathway activation and expression of extracellular matrix and adhesion molecules.[65,74] Compared with their presumed cells of origin, both CSC types demonstrated heightened metabolic and proliferative activity as well as greatly impaired differentiation capacity. As suggested in previous studies of genetically engineered medulloblastoma and glioma models,[76–81] tumorigenic CSCs are derived through distinct mechanisms from cells that have either preserved or gained (eg, trough dedifferentiation) features reminiscent of fetal NSCs and adult NSCs.[16,74] The findings, corroborated by an earlier array-based classification system by Gunther and colleagues,[65] suggest that $CD133^+$ and $CD133^-$ CSCs represents at least 2 different GBM subtypes with distinct molecular and phenotypic characteristics.

The Hierarchical Model

Other studies have demonstrated the ability of $CD133^-$ glioma cells to give rise to both $CD133^+$ and $CD133^-$ progeny. Intracranial transplanted glioma spheroids obtained from human GBM biopsies in nude rats gave rise to invasive CD133-negative tumors with minor signs of angiogenesis.[54,82] Successive serial transplantation of these initial tumors gave rise to tumors with increasing CD133 immunoreactivity, which was closely correlated with an increased angiogenic phenotype and decreased survival in the host rats. Transplantation of $CD133^-$ cells, purified via FACS of the serially transplanted tumors, into nude rat brains gave rise to tumors with both $CD133^+$ and $CD133^-$ cells. Tumors derived from $CD133^-$ GBM cells contained up to 5% $CD133^+$ cells, suggesting that a subset of $CD133^-$ cells can not only initiate and support tumor growth but also recapitulate the initial tumor heterogeneity.[54]

Similarly, Chen and colleagues[76] demonstrated that both $CD133^+$ and $CD133^-$ GBM cell fractions are capable of neurosphere formation and also demonstrate varying degrees of tumorigenicity in vivo. Accounting for all clonogenic GBM cells within neurosphere cultures, the investigators proposed 3 categories of GBM CSCs organized within a lineage hierarchy representing different stages of differentiation. $CD133^-$ type I cells give rise to a mixture of $CD133^+$ and $CD133^-$ cells, $CD133^+$ type II cells also give rise to a mixture of $CD133^+$ and $CD133^-$ cells, but $CD133^-$ type III cells only give rise to a population of self-renewing $CD133^-$ cells. All 3 cell types expressed the NSC marker nestin, were capable of multilineage differentiation, and formed tumors following serial transplantation in mice. Much of the histologic and molecular differences between the 3 types place types I and III at 2 extremes, whereas type II cells possessed intermediate features. Although $CD133^-$ type III cells were restricted to only producing $CD133^-$ tumors, type I cells could produce type I, II, and III cells. Tumors derived from type III cells grew the slowest and gave rise to well-circumscribed tumors, whereas type I cells were more elongated and generated more aggressive and invasive tumors with diffuse borders. Consistent with the observed histologic characteristics, type I and II clones expressed significantly higher levels of the radial glial marker, FABP7, among several other NSC markers in comparison with type III clones. FABP expression has been established as a GBM marker for increased invasion and shorter survival[76,83,84] and is associated with maintaining the stem cell features during neural development.[76,85] In contrast, type III grafts displayed higher levels of intermediate progenitor markers such as TBR2, DLX1, DLX2, and CUTL2.[76] Within this model, type I and III $CD133^-$ cells represent the most primitive and differentiated states along a spectrum. Taken together, $CD133^-$ progenitor cells are capable of forming both $CD133^+$ and $CD133^-$ cells, supporting the presence of a lineal hierarchy of self-renewing cells that support GBM growth.

Enrichment of neurosphere-forming GBM cells have identified 2 lineally related but distinct populations of $CD133^-$ cells (type I and III) with unique gene signatures and in vivo phenotypes representing differing stages of differentiation. These differences within the $CD133^-$ population, comprising both type I and type III $CD133^-$ cells, may account for the discordant results of previous studies examining the ability of $CD133^-$ cells to demonstrate in vitro or in vivo stem cell properties.[76]

The Dynamic Model

In addition, the cancer stem–like phenotype associated with CD133 expression may actually

represent a dynamic and plastic trait that responds to the changing signals and stresses. Oxygen has been the well-characterized signaling molecule involved with mediating various signaling pathways and regulating gene expression,[86,87] and availability of oxygen within the tumor microenvironment has been thought to influence the proliferative phase underlying neoplastic growth.[88] Although oxygen tensions within the normal brain range from 5% to 10% and likely lower within the tumor bulk, in vitro studies typically culture glioma cells under normoxic conditions of 20% O_2.[86,89,90] Platet and colleagues[91] demonstrated that culturing of GBM resection specimens at 3% O_2, representing a more physiologically relevant concentration, was associated with a significant increase in CD133 expression in comparison with GBM cells cultured at atmospheric concentrations of 20% O_2. McCord and colleagues[86] demonstrated similar results with more mild reductions of oxygen concentration and that disaggregated GBM spheres derived from surgical resection samples had a 2-fold increase in percentage of $CD133^+$ cells when recultured and allowed to form neurospheres in 7% O_2 compared with normoxic 20% O_2 conditions. $CD133^+$ cells cultured in 7% O_2 had a higher frequency of colony formation, shorter doubling time, and enhanced ability to differentiate along glial and neuronal lines, suggesting that hypoxic conditions not only increase the $CD133^+$ cell composition but also modify and enhance the associated tumorigenic and stemlike phenotype. $CD133^+$ neurospheres cultured in hypoxic conditions demonstrated increased expression of other stem cell markers, including nestin, Oct4, and SOX2. Levels of HIF-2α were increased in $CD133^+$ cells cultured in 7% O_2, and consequent small interfering RNA silencing of HIF-2α led to decreased Oct4 and SOX2.[86] Consistent with the induction of the stemlike phenotype, growth in 7% O_2 induced alterations in global gene expression patterns with the upregulation of critical stem cell–associated genes including those involved with the notch and frizzled-2 signaling pathway, angiogenesis, and transforming growth factor β.[86] When $CD133^+$ cells were moved from 7% to 20% O_2, rates of colony formation and expression of HIF-2α and stem cell markers reversed to levels originally observed in normoxic conditions. Oxygen-induced stemlike features, partially mediated by HIF-2α, are reversible and mediated by epigenetic changes and are not the result of the hypoxic selection of a $CD133^+$ subpopulation of tumorigenic cells.[86]

In their studies with the established human glioma cell line U251MG, Griguer and colleagues[92] further proposed that mechanisms underlying hypoxia-induced CD133 upregulation and functional changes partly involved loss of mitochondrial function. In accord with previous studies, U251MG glioma cells, containing undetectable levels of CD133 above background in 21% O_2 culture conditions, became strongly CD133 positive when changed to 1% O_2. Following exposure, 20% of U251MG cells were $CD133^+$ within 24 hours and up to 60% were $CD133^+$ within 72 hours. On return to 21% O_2, levels of CD133 expression decreased to original normoxic levels. In addition, treatment of U251MG glioma cells with rotenone, an electron transport chain blocker, resulted in a significant and dose-dependent increase in $CD133^+$ expression, reminiscent of exposure to hypoxic conditions. Depletion of mitochondrial DNA similarly led to constitutive and substantial increases in CD133 expression that persisted through multiple cell passages. Relative to controls, mitochondrial DNA–depleted glioma cells demonstrated a more aggressive phenotype of increased anchorage-independent growth and invasiveness. These cells readily initiated and expanded as tumorspheres in serum-free media that expressed the stem cell markers nestin and CD133 and had multilineage differentiation potential. In addition, rescue of mitochondrial function by transfer of parental mitochondrial DNA reversed the elevated CD133 expression levels. In their proposed model of glioma progression, Griguer and colleagues[92] suggest that tumor growth is driven by a dynamic and adaptive biological response to oxygen and metabolic demands in reaction to a changing tumor microenvironment. Stringent nutrient and oxygen barriers selectively signal a switch within glioma cells in hypoxic regions to gain phenotypic changes associated with increased survival and migration.

Overall, the data support a critical role of reduced oxygen tension and disruption of mitochondrial function in mediating an in vitro response within glioma cells characterized by upregulated CD133 expression.[16,86,91–95] These hypoxia-induced changes are associated with global alterations in the expression of stem cell–associated genes and promote CSC phenotypes, including increased clonogenicity, proliferation, invasiveness, capacity for multilineage differentiation, and tumorigenicity in xenograft models. While it is unclear if the extent of fluidity is present in vivo, the sensitivity of glioma cells toward changes in oxygen levels and the reversibility of hypoxia-induced characteristics suggest a dynamic regulatory component of CD133 expression and stemlike features. In addition, the low frequency of $CD133^+$ glioma cells reported in literature may be linked to

normoxic culture oxygen concentrations that do not reflect physiologic levels.

Recent studies have begun to provide further insight into the complex mechanisms regulating the expression of CD133 and its associated phenotype in gliomas. Differential methylation patterns have been identified as transcriptional regulators of CD133 in several cancers.[96,97] CD133 promoter methylation, not present within normal brain tissue, represents an abnormal epigenetic regulator of differential CD133 expression in glioma cells.[97] Yi and colleagues[97] reported a high frequency of CD133 promoter hypermethylation in both GBM and colon cancer cells lines and primary tumor samples; however, such methylation patterns were absent in $CD133^+$ cells. Upregulation of CD133 expression was strongly associated with genetic and drug-induced inhibition of DNA methyltransferase activity. This mode of regulation is unique to CD133, as hypermethylation profiles of other genes do not vary between $CD133^+$ and $CD133^-$ populations.[97] In addition, Jaksch and colleagues[98] demonstrated that the extent of AC133 immunoreactivity is cycle dependent in embryonic stem cells, colon cancer, and melanomas. Specifically, AC133 immunoreactivity was highest in the actively dividing cell subpopulation with 4N DNA content and lowest in cells in the G_0 and G_1 phases with 2N DNA content. MELK protein expression, which has been demonstrated to be cell cycle dependent,[98,99] mirrored AC133 immunoreactivity with respect to cell cycle status. Prolonged culturing of purified $CD133^-$ and purified $CD133^+$ cells produced similarly heterogeneous cell populations comprising both $CD133^+$ and $CD133^-$ cells. The ability of cells at either extremes of CD133 immunoreactivity to produce CD133-heterogeneous populations suggests that CD133 immunoreactivity may not be unique to a discrete and stable population.[98] Overall, although it does appear that CD133 expression may be used to enrich a glioma population with increased tumorigenicity, it is possible that CD133 expression and the associated phenotype are fluid traits responding to dynamic extracellular and intracellular processes.

CONTRIBUTION TO ANGIOGENESIS

The extensive tumor vascularity, high endothelial proliferation rates, and elevated VEGF production within GBMs have provided promise for antiangiogenic therapies.[100] Indeed, in clinical trials using the anti-VEGF antibody, Avastin, roughly half of the patients responded to treatment.[101,102] However, the effect was transient, and antiangiogenic resistance developed in most patients.[102,103] Bao and colleagues[56] reported the formation of highly vascular and hemorrhagic tumors following mice xenograft of purified $CD133^+$ GBM cells relative to $CD133^-$ cells. $CD133^+$ cells demonstrated a marked elevation of VEGF expression in comparison with $CD133^-$ cells in both normoxic and hypoxic conditions. $CD133^+$ promoted EC migration and formation of vasculature in vitro, and these proangiogenic effects were blocked by treatment with anti-VEGF antibodies (bevacizumab). In addition, bevacizumab reduced the growth and vascularity of tumors derived from mice transplantation.

Recently, $CD133^+$ GBM cells have demonstrated to contribute to tumor neoangiogenesis through direct transdifferentiation down endothelial lineages to give rise to tumor-derived ECs (TDECs).[104–106] Wang and colleagues[105] identified a subpopulation within the $CD133^+$ fraction of GBM cells that coexpressed vascular endothelial cadherin (CD144) that were capable of giving rise to ECs. These TDECs harbored the same mutations found within the parent tumor, namely, gains in chromosome 7 and epidermal growth factor receptor.[105] He and colleagues[104] further demonstrated that $CD133^+$ GBM CSCs are capable of forming TDECs and were localized within niches that were in close proximity to blood vessels. Blood vessels surrounding $CD133^+$ tumor cell niches expressed tumor-specific markers, further suggesting that these ECs are of neoplastic origin and arise from the differentiation of GBM stem cells.[104]

These TDEC comprised up to 90% of ECs in the tumors and appeared to contribute to glioma angiogenesis.[105,106] Although $CD133^+/CD144^-$ cells were capable of sphere formation and differentiation along neuronal lineages, cocultures of tumor cells and purified $CD133^+/CD144^-$ cells generate ECs through intermediate $CD133^+/CD144^+$ progenitor cells. When cultured in EC media, $CD133^+/CD144^+$ cells lose CD144 expression and display features associated with an epithelial phenotype (eg, CD105 and CD31 expression, and DiI-AcLDL uptake). Endothelium derived from $CD133^+/CD144^+$ GBM cells formed vessels with glomeruloid features morphologically reminiscent of abnormal tumor vasculature. Unlike $CD133^-/CD144^+$ cells, transplantation of $CD133^+/CD144^+$ and $CD133^+/CD144^-$ populations gave rise to highly invasive and aggressive tumors, with $CD133^+/CD144^+$-derived tumors displaying significantly increased levels of angiogenesis. In addition, $CD133^+/CD144^-$ fractions were capable of maintaining its multilineage potential following serial transplantations. Elevated levels of HIF-1α in tumors exposed to hypoxic conditions lead to increased angiogenesis through VEGF

production.[103,107,108] Similarly, hypoxia seems to induce the transdifferentiation of select glioma cells into ECs through elevation of HIF-1α levels in vitro.[103] However, unlike normal ECs, most TDECs lacked VEGF-R1, VEGF-R2, and VEGF-R3 expression.[103] Accordingly, treatment with anti-VEGF did not inhibit in vivo TDEC tube formation and did not produce improved survival in murine GBM models.[103] TDEC transdifferentiation is VEGF independent and may explain GBM resistance to anti-VEGF therapies.[103] Furthermore, selective targeting of TDEC in mouse xenografts led to marked tumor regression, indicating that TDECs play a critical role in maintaining tumor viability.[106]

Although tumor angiogenesis is typically thought to be driven by bone marrow–derived circulating endothelial precursors,[103,109] tumor cells appear to be closely involved with tumor angiogenesis and TDECs have been identified in other cancers, including myeloma, lymphoma, and chronic myelocytic leukemia.[103,110–112] Taken together, the data suggest that $CD133^+$ GBM cells not only are capable of giving rise to the tumor bulk but also can contribute to tumor angiogenesis partly through transdifferentiation through a $CD133^+$/$CD144^+$ intermediate to generate ECs of neoplastic origin that can form functional vessels.

The Stem Cell Niche

Normal stem cells have been demonstrated to exist within a stem cell niche comprising differentiated cell types that help regulate and maintain the stem cell trait.[113–116] Coculture studies have demonstrated a role of ECs within the stem cell niche in modulating and maintaining NSCs.[117] ECs secrete pigment epithelium–derived factor, which modulates capacity for self-renewal in adult neural stem cells within the subventricular zone.[118] Similarly, other diffusible factors released by ECs within the stem cell niche, including brain-derived neurotrophic factor and leukemia inhibitory factor, have been characterized to regulate NSC proliferation and differentiation.[119,120] In addition, recent studies have suggested that a parallel interaction exists between glioma stem cells and tumor ECs within the vascular niches critical in maintaining CSCs.[113]

$CD133^+$ brain tumor cells demonstrated a high affinity for ECs and formed close interactions along vascular tubes formed by primary human ECs (PHECs) in culture. When $CD133^+$ tumorspheres were cocultured with PHECs, the tumorspheres demonstrated heightened capacity for proliferation and self-renewal.[113] ECs similarly enhanced the CSC phenotype in vivo, as transplantation of tumor cells into mice in the presence of PHECs was associated with increased expansion of $CD133^+$ cells and quicker tumor initiation and growth. Increased number of blood vessels in tumor xenografts, and consequently an increase in the release of endothelial derived factors, led to a significant expansion of self-renewing tumorigenic cells while treatment with antiangiogenic drugs blocked tumor growth and depleted the self-renewing $CD133^+$/$nestin^+$ tumor population.[113] A recent study of 87 resected grade II–IV glioma samples identified CSC niches characterized by $CD133^+$ blood vessels surrounding and infiltrating $CD133^+$ glioma cell clusters.[121] Prevalence of $CD133^+$ niches, ranging from 11.57% to 24.81%, was correlated with increasing tumor grade and extent of $CD133^+$ blood vessels. In accordance with previous reports of glioma CSC–derived EC cells, $CD133^+$ cells were localized around $CD31^+$ blood vessels that contained cells that coexpressed CD31 and CD133. In contrast to NSC maintained in predominantly quiescent states by NSC niches, a fraction of $CD133^+$ cells within the neoplastic perivascular niche were proliferating cell nuclear antigen positive and actively proliferating.[121] Thus, ECs and the vascular niche are critical for not only nutrient supply but also providing a tumor microenvironment that supports and promotes the proliferation of CSCs.[113]

Recently endothelium-derived nitric oxide (NO) was demonstrated to regulate stemlike features of glioma cells within the perivascular niche in platelet-derived growth factor–induced mouse glioma models.[122] Charles and Holland[122] demonstrated a close correlation between endothelial NO synthase (eNOS) expression, which was limited to ECs, and Notch1 expression in adjacent $nestin^+$ glioma stem cells within the perivascular niche. NO activation of the Notch signaling pathway heightened the stemlike features of glioma cells in vitro and enhanced tumorigenicity and tumor growth in tumor xenografts. In addition, mice lacking eNOS exhibited impaired notch pathway activation and tumor growth and improved survival. These results complement the established role of NO in mediating glioma angiogenesis and highlight the critical importance of the tumor perivascular niche in maintaining and supporting resident tumor stem cells.[122] Thus, the capacity for $CD133^+$ CSCs to transdifferentiate and directly contribute to tumor vasculature, to modulate regulated angiogenesis, and to respond to endothelium-derived signals, among other mechanisms, represents an intricate and bidirectional cross talk between glioma CSCs and their microenvironment niche.

THERAPEUTIC POTENTIAL

The glycosylated CD133 epitope has been identified as a reliable tumor marker for the purification of a subpopulation of GBM cells demonstrating CSC phenotypes. Isolated tumorsphere-forming $CD133^+$ GBM cells demonstrated heightened in vitro proliferation, self-renewal, and invasive capacity. When cultured in prodifferentiation conditions, $CD133^+$ GBM cells were capable of differentiating along neuronal, oligodendrocytic, and astrocytic lineages. Orthotopic transplantation of $CD133^+$ cells led to the formation of heterogeneous tumors that were phenocopies of the original patient tumor. Thus, $CD133^+$ cells are highly tumorigenic and demonstrate extensive capacity for self-renewal even when challenged with serial transplantation. Given these characteristic stemlike properties of the $CD133^+$ population, the CSC model has been extended to GBMs. The application of the CSC model may account for the highly heterogeneous, invasive, and therapy-resistant nature of GBMs[67] and has directed investigators to identify specific cellular elements for targeted therapies.

Later CSC studies using CD133-independent means to isolate putative CSCs in GBMs have demonstrated that these in vitro and in vivo CSC features are not present in all $CD133^+$ GBM cells nor are they unique to the $CD133^+$ subpopulation. The identification and characterization of tumorigenic $CD133^-$ cells has led to not only a greater appreciation of the multifaceted regulation of CD133 in GBMs but also a more comprehensive understanding of the role of CD133 in GBM tumorigenesis. These findings have suggested several models that are not necessarily mutually exclusive: (1) $CD133^+$ and $CD133^-$ GBM cells comprise unique and separate CSC populations responsible for driving the growth of different GBM subtypes, (2) $CD133^-$ GBM cells are functionally heterogeneous and may comprise more primitive cells capable of giving rise to tumorigenic $CD133^+$ cells, and (3) the $CD133^+$ stemlike phenotype is transient and fluid, and CD133expression depends on temporally and spatially dynamic intracellular and extracellular cues.

Most of the studies analyzing CD133 expression are faced with technical challenges from accounting for the complex genetic and epigenetic regulation of CD133 to the ability of the in vitro and in vivo assays to reflect the true physiologic role of CD133 in patients' tumors. However, a growing number of studies have linked clinical outcomes with the presence of $CD133^+$ cells within resected tumors.[50,55,123,124] Analysis of 95 resected gliomas found that increased CD133 expression and presence of $CD133^+$ clusters were significant prognostic predictors of worse overall survival and progression-free survival independent of glioma grade, patient age, or extent of resection.[50] In addition, greater extent of CD133 expression is correlated with increasing glioma grade and is typically found in advanced-stage gliomas.[125] The greater reliability of CD133 expression relative to histologic analysis in predicting patient outcomes has suggested that the gain of CD133 expression may be a key step in the progression to secondary GBM.[124] Furthermore, $CD133^+$ cells have been found to play a critical role in mediating GBM resistance to radiation and chemotherapy. Standard cancer treatments, which mainly target rapidly dividing cells of the tumor bulk, are not effective in eradicating CSCs, which are slowly dividing and often quiescent cells. GBM CSCs preferentially activate DNA checkpoint proteins to ensure proper repair of DNA damage resulting from treatment.[126] $CD133^+$ glioma cells persist in greater fractions after treatment with ionizing radiation through preferentially activating Chk1 and Chk2 checkpoint kinases, and this radioresistance is lost following Chk1 and Chk2 inhibition.[126] Chemoresistance of $CD133^+$ glioma cells is mediated through several mechanisms, including the upregulation of adenosine triphosphate–binding cassette transporters to facilitate drug efflux,[127] elevated expression of multidrug-resistant associated proteins 1 and 3,[128] and the enrichment of the "side population" that is resistant to cytotoxic drugs.[60] In addition, $CD133^+$ glioma cells upregulate the DNA repair protein, *O*-methylguanine-DNA methyltransferase, as well as other antiapoptotic genes, including Bcl-2, Bcl-X, and FLIP.[129] Adjacent ECs within the perivascular niche, along with the activation of a number of developmental pathways, have been suggested to contribute to $CD133^+$ GBM resistance to radiotherapy and chemotherapy.[26,130]

While CD133's status as a stable, obvious CSC is still not established and its biological functions are unclear, CD133 remains a promising marker for targeted and personalized therapeutic intervention. Although several putative models exist, CD133 consistently identifies cells with not only stemlike properties intimately involved with tumor growth and angiogenesis but also profound clinical consequences in dictating patient outcomes and resistance to chemotherapy and radiotherapy. Thus, targeting the $CD133^+$ subpopulation represents a promising adjuvant in conjunction with standard therapies. Several exciting and novel approaches have been proposed, including treatment with prodifferentiation agents, drugs that target aberrant CSC signaling pathways, disruption of the perivascular niche, and the use of

CSC-targeted immunotherapies. Research into therapies targeting CSCs is still in nascent stages and must consider several challenges: (1) several different and heterogeneous glioma CSCs may exist, (2) the CSC phenotype is regulated by several complex extracellular and intracellular mechanisms, and (3) treatment of glioma CSCs may also affect healthy neural stem cells that share similar markers and phenotypes.[26] Indeed, the optimal treatment strategies likely include a combinatorial approach and rely on continued exploration of the complex regulation of GBM CSCs and their underlying biology.

ACKNOWLEDGMENTS

Marko Spasic was partially supported by an American Association of Neurological Surgeons Fellowship. Daniel Nagasawa was supported by an American Brain Tumor Association Medical Student Summer Fellowship in Honor of Connie Finc. Isaac Yang (senior author) was partially supported by a Visionary Fund Grant, an Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research UCLA Scholars in Translational Medicine Program Award, and the STOP CANCER Jason Dessel Memorial Seed Grant.

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