Glioma Stem Cell Research for the Development of Immunotherapy

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KEYWORDS

• Cancer stem cell • Glioma • CD133$^+$ • Immunotherapy

Human brain tumors are a diverse group of diseases characterized by the abnormal growth of brain cells contained within the skull, afflicting adults and children. According to the National Cancer Institute data, there are about 20,000 new cases of brain tumor and 13,000 deaths each year in the United States. In children, brain tumors are the leading cause of solid tumor cancer death; all forms of glioma make up about one-fifth of all childhood cancers (www.cancer.gov). In adults, the most common malignant brain tumor, glioblastoma multiforme (GBM), is also the most malignant primary tumor of the brain associated with one of the worst 5-year survival rates among all human cancers.[1,2] The median survival time is 14.6 months after first diagnosis.[3,4] Despite the advances in conventional treatments, composed of surgical resection, local radiotherapy, and systemic chemotherapy, the incidence and mortality rates for gliomas have changed little in the past decade. With greater understanding of the cellular and molecular mechanisms of cancer initiation and propagation, the cancer stem cell (CSC) hypothesis presents new insights for developing novel treatments that target this group of cells. In this article, the authors discuss the CSC hypothesis and its application to develop treatments for glioma.

CSC BRAIN TUMOR STEM CELL AND CD133 CSCS

The first conclusive evidence for CSCs came from the studies of acute myeloid leukemia (AML).[5,6]

Bonnet and Dick[6] isolated a subpopulation of AML cells that were capable of initiating AML in immunodeficient NOD/SCID (nonobese diabetic/severe combined immunodeficient) mice. These leukemia cells (leukemia stem cells [LSCs]) express cell-surface markers that are similar to normal hematopoietic stem cells (HSCs). The AML that is established from these LSCs recapitulates the morphologic and immunophenotypic heterogeneity of the original tumor. These seminal studies opened the door for CSC study. Besides the properties shared with normal stem cells (self-renewal and the ability to differentiate into other cells), candidate cells must present the following properties to be considered as CSCs: (1) the unique ability to engraft, (2) the ability to recapitulate the tumor of origin morphologically and immunophenotypically in xenografts, and (3) the ability to be serially transplanted.[7] These criteria are the standard to identify other CSCs not only in hematopoietic tumors but also in solid tumors.

The first solid tumor CSCs were identified from breast cancer by isolating CD44$^+$/CD24$^{-/low}$ cells from primary tumor cells.[8] The isolated CSCs can recapitulate the original breast cancer with the same morphologic and immunophenotypic features. CSCs could be isolated from these grafts and serially transplanted. For gliomas, several groups isolated brain tumor stem cells (BTSCs) from primary tumors based on the criteria mentioned earlier and the ability to form neurospheres as normal neural stem cells (NSCs)

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do.[9–16] In the authors' study, as few as 100 of these BTSCs could recapitulate the heterogeneity of GBM in immunocompromised rodents.[15] In addition to primary gliomas, the authors also isolated cancer stemlike cells from the commercial rat gliosarcoma cell line, 9L.[17] This cell line has been cultured in the laboratory over a long period under neurosphere conditions used for NSC expansion. Similar results were reported by Kondo and colleagues[18] for the rat GBM cell line, C6. These data indicate that glioma cell lines may retain the capacity for a stemlike phenotype even after years of in vitro culture. CSCs have also been identified in various other malignant primary tumors and cancer cell lines by using different cell-surface markers (**Table 1**).

Among the CSC-associated markers, CD133 (prominin-1) is one of the most important and well studied. It is a 120 kDa, 5-transmembrane-domain glycoprotein, with 2 cytoplasmic loops, 2 glycosylated extracellular domains, and a cytoplasmic C-terminal domain.[19–22] Despite mounting evidence that CD133 is an important marker for somatic stem cells and CSCs, its physiologic function is not known. Some studies suggested that CD133 is involved in neural-retinal development and phototransduction.[23,24] Due to its interaction with plasma membrane cholesterol and enrichment in cholesterol-based membrane microdomains, it may play some role in membrane topology.[25] Barcelos and colleagues[26] also demonstrated that $CD133^+$ progenitor cells could promote the healing of diabetic ischemic ulcer through stimulating angiogenesis and activating the Wnt pathway. This observation may suggest a role for $CD133^+$ CSCs in tumor angiogenesis and in related signaling pathways.

GLIOMA CSCS AND CLINICAL TREATMENT

CSCs are often resistant to conventional chemotherapy and radiation therapy. Glioma CSCs are resistant to radiotherapy and chemotherapy. $CD133^+$ glioma CSCs could preferentially activate the DNA damage checkpoint response under irradiation. The activation is Chk1 and Chk2 checkpoint kinase dependent.[27] Blazek and colleagues[28] also confirmed that $CD133^+$ glioma cells are more radiation resistant than $CD133^-$ cells. This study also reported that CD133 expression is upregulated 1.6 fold under 2% O_2 (hypoxic conditions). Similar results had been reported by other groups also.[29,30] Because hypoxic conditions exist in most solid tumors, including gliomas,

Table 1
Summary of identified cancer stem cells from different primary tumors and tumor cell lines

Tumor	Type	Isolation Markers	References
AML	Primary tumors	$CD34^+CD38^-$	5,6,78
Breast	Primary tumors	$CD44^+CD24^{-/LOW}$	8
Brain	Primary tumors	*CD133*+	9,10,13,27,32,79,80
	Cell lines	*CD133*+/sphere formation	17,28,81
	Cell lines	Side population (SP)	18
Colon	Primary tumors	*CD133*+	61,82,83
	Primary tumors	*CD133*+CD44+	84
	Cell lines	*CD133*+	85
Laryngeal	Cell lines	*CD133*+	86
Leukemia	Primary tumors	CD34+CD10-	87
Liver	Primary tumors/cell line/blood	CD90+CD44+	88
	Cell lines	*CD133*+	89–92
Lung	Primary tumors	ALDH1	93
	Primary tumors	*CD133*+	94
Melanoma	Primary tumors	ABCB5+	57
	Primary tumors	*CD133*+ABCG2+	95
Ovarian	Primary tumors	*CD133*+	96
Pancreas	Primary tumors	*CD133*+	97,98
	Cell lines	*CD133*+	99
Prostate	Primary tumors	*CD133*+	100

this upregulation of CD133 expression provides enhancement for specific targeting of glioma CSCs rather than NSCs.

An in vitro study showed that $CD133^+$ glioblastoma CSCs are more resistant to multiple chemotherapeutic agents than their $CD133^-$ counterparts.[31] The authors demonstrated that $CD133^+$ glioma CSCs express higher levels of the drug transporter gene, *BCRP*, combined with upregulation of the DNA repair protein, methylguanine DNA methyltransferase (MGMT) mRNA, and mRNAs of other genes that inhibit apoptosis, including *FLIP, Bcl-2, Bcl-X*, and some *IAP* family genes. These cells were significantly resistant to chemotherapeutic agents when compared with autologous $CD133^-$ cells.[32]

Glioma CSCs possess migration as an additional property to escape from conventional therapies. The authors and others reported that overexpression of chemokine receptors, such as CXCR4, is a common mechanism related to CSC migration.[32–34] As reviewed by Lefranc and colleagues,[35] glioma cell migration is a complex combination of multiple molecular processes, including the alteration of tumor cell adhesion to a modified extracellular matrix, the secretion of proteases by the cells, and modifications to the actin cytoskeleton. Intracellular signaling pathways involved in the acquisition of resistance to apoptosis by migrating glioma cells include PI3K, Akt, mTOR, NF-κB, and autophagy (programmed cell death type II).

TARGETING SIGNALING PATHWAYS IN CSCS

Signaling pathways, including Wnt, hedgehog, Notch, HOX family members, Bmi-1, phosphatase and tensin (PTEN) homolog, telomerase, and efflux transporters, are involved in balancing self-renewal and differentiation of NSCs and CSCs.[36–39] Newer studies also show that Notch, hedgehog, and bone morphogenic protein (BMP) pathways are involved in controlling $CD133^+$ CSC functions in glioma.[40–42] Bao and colleagues[43] showed that glioma CSCs generate vascular tumors through overexpression of vascular endothelial growth factor (VEGF). Because VEGF is a validated therapeutic target for glioma therapy,[44–46] this finding may indicate more favorable targeting of CSCs in glioma therapy.

Due to the common pathways and cell-surface markers shared by NSCs and CSCs, it is important to develop CSC-specific therapies that avoid potential toxicities to NSCs. Selective targeting of AML CSCs by Guzman and colleagues[47] demonstrated the possibility of such selectivity. They showed that LSCs, but not normal HSCs, were susceptible to the apoptotic effects of the proteasome inhibitor MG-132 combined with the anthracycline idarubicin through NF-κB activity. NF-κB inhibitors could induce LSC apoptosis but spare normal HSCs.[48] In a subsequent study, the same group also showed that 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) treatment could induce oxidative stress and selectively kill LSCs in vitro but not HSCs.[49] Other studies demonstrated that AML is PTEN pathway dependent. Rapamycin, a PI3K/PTEN signaling pathway inhibitor, could dramatically decrease leukemia burden.[50] In addition, this treatment appeared to be specific for the LSCs because normal HSCs were unaffected.

When selective targeting of CSCs becomes possible, another strategy to target CSCs is by forcing them to differentiate and become more sensitive to conventional chemo-radiotherapies. Differentiation therapy is based on this concept, and several agents had been tested in recent years.[51,52] All-trans retinoic acid (ATRA) is the most studied differentiation therapy molecule. Sell[53] reported that about 90% of newly diagnosed patients with acute promyelocytic leukemia achieved complete remission and more than 70% were cured by ATRA therapy. Differentiation with ATRA was also reported in early-stage mouse embryonic stem cells,[54] rat C6 glioma cells,[55] and human embryonic NSCs.[56] These studies opened up the possibility of using ATRA to induce differentiation of glioma CSCs as a therapeutic technique. Besides ATRA, other agents have also been tested for this approach of differentiation therapy. Piccirillo and colleagues[42] have shown that treating CSCs with differentiation factors can effectively deplete CSCs in human glioma. In this study, researchers reported that BMPs, especially BMP4, activate BMP receptors and trigger the Smad signaling cascade in cells isolated from human glioblastomas. This activated signaling pathway leads to a reduction in proliferation and increased expression of differentiated neural markers in both $CD133^+$ CSCs and normal glioma cells. When xenotransplanted BMP4–pretreated glioma CSCs were transplanted into mice, invasive glioma was not detected. These data provided evidence that differentiation therapy is a promising noncytotoxic strategy to deplete CSCs.

TARGETING CSCS USING PASSIVE IMMUNOTHERAPY

Antibody therapy (passive immunotherapy) directed against CSCs has resulted in several experimental therapeutic successes. Schatton

and colleagues[57] identified melanoma CSCs with the expression of the chemoresistance mediator ABCB5+ (ATP binding cassette B5+). Treatment with anti-ABCB5 antibody for xenografted melanomas resulted in significant reduction of tumor size. Moreover, this direct targeting of the CSC antigen induced tumor cell death through antibody-dependent cell-mediated cytotoxicity. Another encouraging result of antibody therapy was reported by Jin and colleagues.[58] In their study, CD44 had been identified as an AML CSC surface marker. Although the same marker is expressed on normal bone marrow HSCs at a lower level, treatment with anti-CD44 antibody before transplant can selectively block engraftment of AML LSCs but not normal HSCs. Treatment of previously engrafted AML LSCs with the same antibody led to a significant reduction in disease burden by 83% to 100%. In vivo treated AML CSCs resulted in lower engraftment, suggesting that anti-CD44 antibody treatment directly altered the fate of CSCs either by inducing differentiation or by inhibiting their repopulation ability. This study provided evidence that passive immunotherapy with antibodies targeting CSC antigens could be effective even when the same antigen is shared with NSCs. Krause and colleagues[59] also reported that the expression of CD44 is required on leukemic cells that initiate chronic myeloid leukemia (CML). Anti-CD44 antibody treatment attenuated the induction of CML-like leukemia in recipients, suggesting that CD44 blockade may be beneficial in autologous transplantation in CML.

Passive immunotherapy targeting solid tumor CSCs has also been reported. Smith and colleagues[60] demonstrated that antibody-drug conjugates (ADCs) could be used for both hepatocellular and gastric cancers. When an anti-CD133 antibody was conjugated to a potent cytotoxic drug, monomethyl auristatin F, this conjugate effectively inhibited the growth of Hep3B hepatocellular and KATO III gastric cancer cells in vitro by inducing apoptosis in $CD133^+$ CSCs. In vivo administration of this ADC also resulted in significant delay of tumor growth in SCID mice.

In addition to directly targeting CSC surface antigens, antibody therapy has also been used as sensitizing agents combined with chemotherapy. Todaro and colleagues[61] showed that treatment of $CD133^+$ colon CSCs with anti-IL-4 antibody before treatment with oxaliplatin, 5-FU (fluorouracil), or TRAIL (tumor necrosis factor–related apoptosis-inducing ligand) resulted in increased cell death. In vivo injection of IL-4 neutralizing antibodies followed by oxiplatin effectively reduced tumor burden.

TARGETING CSCS USING ACTIVE IMMUNOTHERAPY

Active immunotherapy is designed to generate vaccines that could stimulate the host's intrinsic immune response to the tumor. Early-stage active immunotherapy vaccines for glioma treatment used irradiated whole tumor cells for inoculation, cells that are either engineered to secrete cytokines[62] or combined with cytokine secreting cells[63] or cytokine itself.[64] Although promising data have been obtained from those tumor cell–based vaccination strategies, the success of this approach was limited by the poor inherent antigen-presenting capacity of glioma cells. The use of professional antigen-presenting cells, such as dendritic cells (DCs), to initiate tumor-specific T-cell responses may be a more promising strategy for cancer vaccination. Emerging evidence showed that DC-mediated antigen presentation might be more effective than using irradiated tumor cells because DCs abundantly express many of the costimulatory molecules that are essential for appropriate activation of naive T cells. Also, they have the ability to efficiently process and present antigenic peptides in combination with cell-surface MHC (major histocompatibility complex).[65–70] For glioma immunotherapy with DC vaccines, different tumor-associated antigens (TAAs), including specific tumor-associated peptides, tumor RNA and cDNA, tumor cell lysate, or apoptotic tumor cells, have been tested in various studies.[71]

In the authors' phase I study using DC vaccines in patients with newly diagnosed high-grade glioma,[72] DC vaccine was generated with the patients' peripheral blood mononuclear cell–derived DCs that are pulsed ex vivo with autologous tumor cell–surface peptides isolated by means of acid elution. After surgical resection and external beam radiotherapy, 9 patients were given DC vaccination intradermally every other week over a 6-week period. Four patients, who showed disease progression, underwent surgery again after receiving the third DC vaccination. The harvested tumor tissue samples from 2 of the 4 patients showed robust infiltration with $CD8^+$ and $CD45RO^+$ T cells, which was not apparent in the same patients' tumor specimens before the vaccination. The median survival period for the study group was 455 days, which was longer than the 257 days for the matched control population. As the results were promising and without any observed destructive autoimmune responses, this study was expanded into a phase II trial.

In another phase I study using DCs pulsed with tumor lysate as antigen,[73] 14 patients with malignant glioma were given 3 vaccinations over a 6-week period and were followed with immunomonitor assay using an HLA-restricted tetramer staining protocol. Results in 4 patients showed that at least 1 or more TAA-specific cytotoxic lymphocytes (CTL) were activated against specific glioma antigens, including melanoma antigen-encoding gene-1, gp-100, and human epidermal growth factor receptor-2. The median survival period of the study group was significantly longer than the control group of recurrent glioblastoma patients, 133 weeks versus 30 weeks.

In a study by Liau and colleagues,[74] 12 glioma patients were treated with DC vaccination by using autologous DCs pulsed with acid-eluted autologous tumor peptides. Results showed that 6 patients generated peripheral tumor-specific CTL postvaccination, without major adverse events and autoimmune reactions. The patients who developed systemic antitumor cytotoxicity had longer survival times than patients with a negative response. All the patients who had stable disease generated a positive CTL response, whereas those with active progressive disease did not show statistically significant CTL response.

With encouraging data generated from these DC vaccine clinical trials, current studies are attempting to further improve the efficacy of this strategy not only by inducing glioma specific CTL but also by depleting inhibitory T_{reg} (regulatory T) cells.[75,76] Two European group studies showed that depletion of T_{reg} cells before DC vaccination could boost antiglioma immune response, leading to tumor rejection and long-term immunity. The 2 studies thus suggested that combination of T_{reg} depletion and DC vaccination is a more effective option to generate antiglioma immunity.

SUMMARY

With emerging evidence that glioma CSCs play an important role in tumor initiation, one can escape from conventional surgical and chemotherapies and target glioma CSCs with different therapeutic strategies, providing new hope for treatment of glioma. Studies that used immunotherapy to target glioma have achieved promising results. But because of the complex and divergent mechanisms with which glioma evades immune surveillance and the genetic instability of CSCs,[77] a combination of therapies with 2 or more immunotherapy strategies may be more effective in eliminating gliomas. With a better understanding of stem cell biology (especially CSC biology), glioma CSC-specific immunotherapy (based on the new discovery) combined with other therapeutic strategies may eventually provide new approaches to treat gliomas.

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