

Review

Cancer Stem Cells in the Central Nervous System – A Critical Review

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Abstract

The cancer stem cell hypothesis postulates that tumors arise from, and are maintained by, a small subpopulation of cancer stem cells. This concept has recently become increasingly controversial, following a series of conflicting results. The cell-surface epitope CD133 has been proposed as a brain cancer stem cell marker, whereas a growing number of studies clearly show a tumorigenic potential among CD133⁺ cells as well. Diverging results suggest that assays for isolating cancer stem cells impose a selection bias on which cells are defined as cancer stem cells. Here, we highlight some recent developments, with an emphasis on reports that call for caution in the acceptance of the brain cancer stem cell hypothesis. *Cancer Res*; 70(21); 8255–8. ©2010 AACR.

Introduction

Numerous studies show that only a fraction of cancer cells are clonogenic *in vitro* (1), or tumorigenic *in vivo* (2). Moreover, tumors often mimic the histoarchitecture of the tissue that they originated from (3). From a developmental perspective, tumor-growth kinetics display a resemblance to the fast increase in biomass during embryogenesis orchestrated by stem and progenitor cells. Furthermore, cancers recapitulate fetal signaling pathways, and express markers of immature and multipotent cell types (4).

The cancer stem cell hypothesis postulates that tumors arise from, and are maintained by, a small subpopulation of cancer stem cells (CSC). They are defined by their ability to self-renew, producing tumorigenic daughter cells, and by their ability to give rise to different nontumorigenic cancer cell phenotypes. Collectively, these offspring reconstitute the cellular heterogeneity of the mother tumor. CSCs have reportedly been identified in several cancer types, including brain tumors, in which they have been isolated through sphere-formation assays (5), by their ability to avoid cell labeling through efflux of the marking dye (6), and with cell-sorting methods (7). Thus, experiments using different methodologies produce the same principal finding: that brain tumors contain multipotent, tumorigenic subpopulations expressing immature markers.

However, subpopulations isolated by different assays are phenotypically diverse (8, 9), and cells that are not selected with these methods may still initiate tumors, according to some studies (10, 11). Data also suggest that tumorigenicity depends

critically on the microenvironment, as well as properties inherent to the cancer cells. Furthermore, findings on the clinical implications of brain CSCs have been diverging. Similar to some other investigators, we have been unable to reconcile our experimental data with the CSC hypothesis. As such, we believe this concept has major limitations as a model to explain brain tumorigenesis. Thus, it should be stated that, in this review, we discuss data relating to these issues from a critical perspective, with an emphasis on observations that challenge the view that brain tumors arise from a small group of brain CSCs.

Assays for Isolating Cancer Stem Cells: Rationales and Limitations

The concept of brain CSCs emerged partly from reports that brain tumors contained sphere-forming cells expressing neuronal and glial markers when cultured in serum-free medium, supplemented with epidermal growth factor and fibroblast growth factor (stem cell medium; refs. 5, 8). Later studies also confirmed that such cells could be expanded in stem cell medium and produce tumors *in vivo* that could be serially passaged (12). However, although sphere formation is used to isolate CSCs, Singec and colleagues used time-lapse studies to show that spheres are highly motile, and can unite to create chimeric structures (13). Thus, sphere formation and growth may result from fusion events as well as proliferation. On the basis of these findings, they concluded that plating of single cells constitutes a more reliable method to assess clonogenic potential. Conversely, Bexell and colleagues show that sphere formation is not a prerequisite for accumulation of CSCs, and that glioma cells growing as monolayers were also tumorigenic (14). Thus, the sphere-formation assay has both a limited specificity and sensitivity for isolation of tumorigenic cells.

Proposed CSCs are also isolated by their expression of ATP-binding cassette transporters (ABCT), which mediate resistance by pumping out drugs. These transporters also efflux Hoechst, a nuclear staining dye, making CSCs appear

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as an unlabeled side population (SP; refs. 6, 9, 10). Kondo and colleagues reported the presence of SP cells in the C6 glioma cell line, and that only the SP population was tumorigenic (6). However, Zheng and colleagues found that most single-sorted C6 glioma cells were clonogenic, although many were non-SP cells (10). Importantly, Hoechst staining reduced clonogenicity, suggesting that the method itself affects the tumorigenic potential. Yet, a major concern relates to the relevance of cancer cell lines that are widely used in many experiments. Because cancer cells change during repeated propagation *in vitro*, they may be of limited value when used for inferring about the properties about cancer cells *in situ*. Whether this bias has systematically shifted the interpretation in one direction is unknown. However, Bleau and colleagues identified SP cells in human biopsies from glioblastoma multiforme (GBM), but reported that both the SP and non-SP populations were tumorigenic in mice, although SP cells were associated with a shorter survival (9). Glioma cells have also been separated by expression of ABCG2, a major transporter system in gliomas. Interestingly, both ABCG2⁺ and ABCG2⁻ produced tumors (15). Furthermore, the ABCG2⁻ cells formed more clones and expressed higher levels of "stemness" genes.

Currently, cell sorting for the cell-surface protein CD133, known as prominin-1, is one of the most commonly used methods for isolating tumor-initiating cells. The CD133 epitope was initially identified as a neural stem cell marker, suggesting it could also have a role in brain tumorigenesis. Some pivotal studies reported that brain tumor-initiating cells in severe combined immunodeficient mice (SCID) were exclusively CD133⁺ (7), but others found that CD133⁻ cells may also initiate neurosphere growth *in vitro* and brain tumors *in vivo* (8, 11). Self-renewing CD133⁺ and CD133⁻ cell types can generate different clonal lines that again give rise to tumor xenografts with distinct histologic features (8). Moreover, these tumor-initiating cell types express numerous markers other than CD133, and gene expression analysis of tumorigenic CD133⁺ and CD133⁻ populations has not shown a unifying profile to suggest a shared genetic basis for CSCs (8, 16).

Concerns about the cell-sorting procedure have been raised by Clement and colleagues, who showed that anti-CD133-coupled microbeads were associated with nonspecific sorting of glioma cells irrespective of the protein expression (17). Even though the accuracy of fluorescence-activated cell sorting (FACS)-based cell sorting is steadily increasing, the negative cell populations will inevitably be contaminated with positive cells in numbers ranging up to 3% (18). One would, therefore, expect that negatively selected cell populations would be tumorigenic to a certain degree.

Data from other cancer types suggest that reported discrepancies can partly be explained by the use of different animal models to screen for tumorigenic cells. Xenograft models may be less efficient than syngeneic transplantations, due to a lack of species-specific growth stimulatory signals. In addition, even severely immunocompromised SCID mice can elicit efficient immune responses toward xenotransplants through natural killer activity (19). In this context, Kelly and colleagues showed, by using a syngeneic Eu-Myc mouse model for lymphoma and leukemia, that these malignancies can be maintained by a large proportion (>10%) of tumor cells, perhaps even the majority (20). Thus, some cells that do not form tumors in partly immunocompetent hosts may fail to do so because of their tendency to elicit an immune response, rather than their lack of ability to proliferate. These findings were substantiated by Quintana and colleagues who used the NOD-SCID-interleukin 2 receptor γ (IL2R γ null) mice, which lack both mature lymphocytes and natural killers, to investigate the impact of the immune system in xenograft models (21). Although previous studies showed that a small fraction of melanoma cancer cells produced tumors, they found that one in four cells were tumorigenic.

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Brain Cancer Stem Cells from a Clinical Perspective

The CSC concept implies that tumor-initiating cells organize a hierarchy in which they give rise to more committed and mature cells, with a limited proliferative potential. Because CSCs reside at the top level in this hierarchy that fuels tumor progression, the size of the CSC niche may be the factor that largely determines the disease course. Zeppenick and colleagues did immunohistochemistry (IHC) and reported that $\geq 1\%$ proportions of CD133⁺ cells and the presence of clusters with CD133⁺ cells predisposed glioma patients (grade II to IV) to a poorer survival outcome (22). However, subgroup analysis did not correlate overall survival with CD133 expression in grade IV gliomas, which constituted the biggest group. Conversely, Christensen and colleagues did not find that CD133 expression impacted survival, neither in a cohort of 72 GBM patients, nor in 42 other patients diagnosed with more low-grade astrocytomas (23). Interestingly, Joo and colleagues compared the clinical characteristics of GBMs grouped according to their levels of CD133⁺ cell ratios and detected differences both in growth pattern and gene expression profiles; tumors with a low CD133 expression grew more invasive and expressed genes typical of mesenchymal or proliferative subtypes, whereas high CD133 expressors grew as cortical, more demarcated tumors, expressing genes associated with a proneuronal phenotype (24).

CSCs have also been implicated in clinical settings like treatment failure and tumor recurrences, because of their resistance toward chemo- and radiotherapy. According to this view, relapsing tumors evolve from expansion of surviving CSC clones. For instance, Liu and colleagues found increased CD133 expression after radio- and chemotherapy in patient biopsies from GBM recurrences, compared with biopsies taken prior to treatment (25), whereas others reported an increased fraction of SP cells in experimental gliomas following temozolomide treatment. However, Beier and colleagues found that temozolomide preferentially depleted CSCs in glioblastomas (26).

Most likely, different treatments may select for the outgrowth of different subclones preexisting in the GBM. Whether all these clones display characteristics of CSCs apart from their treatment resistance is not known. Alternatively, Hunter and colleagues sequenced kinase domains in GBM biopsies harvested before and after treatment, and found that the

relapsing tumors had evolved from a new inactivating mutation in the mismatch repair gene *MSH6* (27). Thus, chemoresistance may be acquired through clonal evolution as well as by expansion of preexisting, treatment-resistant subclones with or without CSC properties.

Microenvironment Versus Inherent Cancer Cell Programs

Early observations to suggest a role for the host tissue in cancer comprised patient autopsy data, which revealed a nonrandom pattern of spread, affecting only certain organ systems. From his findings, Stephen Paget proposed the seed-and-soil hypothesis, stating that cancer cells, like seeds, require a favorable environment (soil) to grow. Since then, it has been shown that many cancer types display an organ-specific pattern of metastasis (28), implying that a cancer cell that is tumorigenic within one niche may not be tumorigenic in others. Experimental studies also indicate that the biological effects of genetic alterations are setting dependent. For instance, *c-myc* expression in organized mammary structures does not induce cell-cycle progression, whereas disruption of their histoarchitecture causes the cells to enter a hyperproliferative state (29). Furthermore, a compelling demonstration of the influence of the microenvironment on tumorigenesis was provided by Dolberg and colleagues who showed that Rous sarcoma virus induced tumors in newly hatched chicks but not in chick embryos (30). Collectively, these studies show that the microenvironment can override inherent cancer cell programs and determine whether a transformed cell will grow to become a tumor. This observation again has direct bearings on the interpretation of studies involving CSCs: Disruption of the cancer cell niche through tissue dissociation, and subsequent manipulation such as cell sorting, involves exposure to new external cues. Thus, cells

that were tumorigenic *in situ*, may not initiate tumors under experimental conditions.

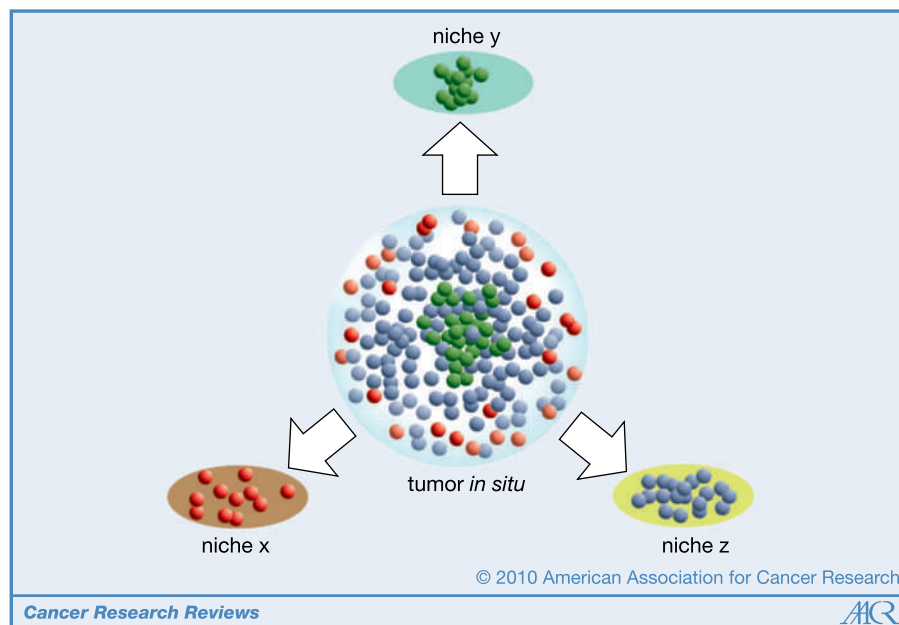
The heterogeneous microenvironment that is present in human glioblastomas requires that glioma cell types adapt a range of different niches. Piccirillo and colleagues investigated the spatial distribution of tumor-cell subpopulations in human GBMs and reported that glioma cells obtained from the core and periphery differed both in their cytogenetic profiles as well as functional properties (31). Importantly, only the core cells were tumorigenic in SCID recipients. In patients, however, gliomas invariably recur after surgical debulking with removal of the tumor core. Thus, a tumorigenic potential must also be retained among cells at the tumor periphery.

Others have investigated the niche for CD133⁺ cells and reported that they reside in perivascular niches (23). Similarly, Calabrese and colleagues found that nestin-positive glioma cells associated with the tumor vasculature (32), and that more CD133⁺ than CD133⁻ cells associated with endothelial cells in a matrigel assay. Moreover, coimplantation of tumor cells with endothelial cells increased the fraction of CSC in the xenograft tumors, whereas anti-angiogenic therapies eradicated these cells. From these data, they concluded that the CSC pool is maintained in perivascular niches. Yet, anti-vascular endothelial growth factor (VEGF) therapy has not prolonged survival in glioma patients, although the treatment may induce a more infiltrative phenotype. Furthermore, CD133⁺ cells are also contained within pseudopalisade formations delineating necrotic regions (23), and others have reported that hypoxia promotes self-renewal and neurosphere formation in glioma cells (33).

Concluding Remarks

The CSC concept has become a subject of controversy because of recent findings in different malignancies, suggesting

Figure 1. Different niches promote different glioma cell types. Assays for isolating tumor-initiating cells, whether *in vitro* or *in vivo*, represent various combinations of growth conditions that constitute a range of different niches. Malignant gliomas contain a multitude of tumor cell types. Cancer cells preexisting in the tumor, which meet the growth requirements within a specific niche, will be selected if this niche is applied as a CSC assay. In addition, other cellular phenotypes within the tumor may successfully adapt this niche and establish colonies.



that tumorigenicity may not be restricted to small subpopulations of cancer cells. In order to determine whether this concept has relevance only to some cancer types rather than to cancer biology in general, future research will need to address these controversies.

In brain tumors, currently used assays for isolating CSCs have undoubtedly identified tumor-initiating cells. However, glioma cells identified with different methods display variable phenotypes, and some cells not identified with these methods also have a tumorigenic potential. This cellular heterogeneity makes it unlikely that the ability to initiate tumors is confined to one subpopulation of glioma cells. Notably, data on the clinical relevance of CSCs in brain tumors are inconclusive, whereas data documenting the significance of the tumor microenvironment are substantial.

We believe that the characteristics required for a cancer cell to be tumorigenic are not a set entity of properties but a dynamic one, reflecting the changing tumor microenvironment. Thus, different niches select for different glioma subtypes (Fig. 1). In

addition, glioma cells exhibit a high degree of plasticity to acquire whatever features are needed to optimize their chance of survival and continuous growth, given the niche that they are in. As such, the concept of CSCs, in which cells are dichotomized into tumorigenic and nontumorigenic subpopulations on the basis of a specific marker or other static features, may not be helpful to advance our understanding of glioma biology. After all, it is their lack of a single defining trait to target that constitutes the major therapeutic challenge.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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