Title: Cancer stem cells and brain tumors.

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Abstract

Besides the role that normal stem cells play in organogenesis, cancer stem cells are thought to be crucial for tumorigenesis. Most current research on human tumors is focused on the molecular and cellular analysis of the bulk tumor mass. However, evidence in leukemia and, more recently, in solid tumors suggests that the tumor cell population is heterogeneous. During the last years, several groups have described the existence of a cancer stem cell population in different brain tumors. These neural cancer stem cells (NCSC) can be isolated by cell sorting of dissociated suspensions of tumor cells for the neural stem cell marker CD133. These $CD133^+$ cells, which also express nestin, an intermediate filament that is another neural stem cell marker, represent a minority fraction of the entire brain tumor population. The stem-like cancer cells appear to be solely responsible for propagating the disease in laboratory models. A promising new approach to treat glioblastoma proposes targeting cancer stem cells. Here, we summarize progress in delineating NCSC and the implications of the discovery of this cell population in human brain tumors.

Introduction

The cancer stem cell hypothesis proposes that cancers derive from a small fraction of cancer cells that constitute a reservoir of self-sustained cells with the exclusive ability to self-renew and maintain the tumor. There is increasing evidence that malignant tumors, such as leukemias, breast cancer and brain cancers, contain cells that maintain the characteristics of tissue-specific stem cells. These stem-like tumor

cells are bestowed with dysregulated potential for self-renewal, excessive proliferation, and aberrant differentiation into a heterogeneous progeny of cancer cells culminating in the intra-tumor heterogeneity. Rapidly accumulating evidence from various laboratories has shown that in several forms of human cancer, only a minority subpopulation of cancer cells are able to form new tumors when transplanted into immunodeficient mice. The population of cells selectively endowed with tumorigenic capacity can be purified from whole tumor tissues by virtue of a surface marker expression profile, and is currently defined as the "cancer stem cell" population. Cancer stem cells have been identified from various solid tumors including breast ($CD44^+$ and $CD24^{-/low}$), colorectal ($CD133^+$), ependymoma ($CD133^+$, $Nestin^+$ and $BLBP^+$) and glioblastoma ($CD133^+$) (Al-Hajj et al., 2003; Singh et al., 2004; Taylor et al., 2005; O'Brien et al., 2007). Currently, an intense debate is ongoing as to whether cancer stem cells originate from adult stem cells or from mature, committed progenitors and/or even terminally differentiated cells that have abnormally acquired self-renewal capacity(Singh et al., 2004). It is also suggested that niche cells could also be a primary target for the carcinogenic insult to adult stem cells, thereby inciting a tumorigenic response (Tlsty and Hein, 2001). Thus, the molecular mechanisms underlying the genesis of cancer stem cells are still obscure and identification of unique cell surface markers for cancer stem cell isolation could provide new tools to address these questions and allow for further molecular and functional characterizations.

High-grade gliomas, which include glioblastoma (GBM) and anaplastic astrocytoma, are among the most common intrinsic brain tumors in adults and are nearly uniformly fatal. While there has been progress in understanding the molecular genetics of these tumors(Kitange et al., 2003), the cell types of origin are still uncertain, and the molecular determinants of disease aggressiveness are not well understood. A better understanding of the cellular origin and molecular pathogenesis of these tumors may identify new targets for treatment of these neoplasms. Until recently, GBMs were presumed to arise from glial cells residing within the brain parenchyma However, recent evidence suggests that neural stem cells can be an alternate cellular origin of gliomas (Singh et al., 2003; Caussinus and Gonzalez, 2005; Zhu et al., 2005). In fact, recent evidence demonstrates that neural stem cells can give rise to neoplasms that recapitulates the histopathological hallmarks of human gliomas (Bachoo et al., 2002; Uhrbom et al., 2002).

Adult neural stem cells are cells in the adult nervous system that can self-renew and differentiate into all types of neural cells, including neurons, astrocytes, and oligodendrocytes (Gage, 2000). In the adult human forebrain, the majority of neurons are born by the early postnatal period, but it has been demonstrated that neurons continue to arise in two niches of the adult brain, the subventricular zone (SVZ) of the lateral ventricle and in the subgranular zone (SGZ) of the dentate gyrus (Luskin et al., 1997; Gage et al., 1998). In the hippocampus, granule neurons arise in the subgranular zone of the dentate gyrus. Progenitor cells in the SVZ migrate to the olfactory bulb (OB) through the rostral migratory stream (RMS) where they differentiate into granule and periglomerular neurons of the OB (Figure 1). Both cell-extrinsic and cell-intrinsic factors have been shown to influence the maintenance and regulation of the neurogenic system in vivo (Ostenfeld and Svendsen, 2003). A number of factors, including the brain-derived neurotrophic factor, insulin-like growth factor-1, epidermal growth factor (EGF) and the basic fibroblast growth factor (bFGF), have been shown to affect the proliferation and differentiation of precursor cell populations (Bartlett et al., 1995; Johe et al., 1996). This population of neural stem cells has distinct features such as the capacity of self-renewing, multipotency, asymmetric division, and express characteristics markers such as nestin, a cystoskeletal protein, CD133, a cell surface marker of normal neural ítem cells, and Notch. The demonstration that the adult human brain contains an abundant source of neural stem cells and that GBMs contain tumorigenic neural stem-like cells indicate that neural stem cells are a plausible origin of human gliomas and have given rise to speculation that more effective therapies will result from approaches aimed at targeting the stem cell-like component of GBM (Ignatova et al., 2002; Berger et al., 2004; Oliver and Wechsler-Reya, 2004; Fomchenko and Holland, 2005). Glioblastoma tumor stem cells possess the capacity to self-renewal leading to daughter cells with the same predisposition for replication as the parental cells, and the capacity to recapitulate the generation of a growing tumor (Clarke, 2004). These capacities have been shown for $CD133^+$ cells isolated from glioblastoma (Galli et al., 2004). A small number of $CD133^+$ cells are sufficient for the formation of glioblastoma in immunodeficient mice. The resulting primary xenograpgh consisting of a minority of $CD133^+$ cells and a majority of $CD133^-$ cells is a phenocopy of the patient's tumor (Singh et al., 2004).

In addition to possessing the fundamental stem cell properties of self-renewal and multi-potency, glioblastoma stem cells share other characteristics with neural stem cells. They were first isolated from tumors by virtue of the expression of CD133 that marks neural stem and progenitor cells (Singh et al., 2004). The similarities in gene expression suggest that common cell signaling systems might operate in normal and malignant neural stem cells. These phenotypic and functional similarities suggest that glioblastoma stem cells might arise from normal neural stem cells that retain self-renewal properties but acquire mutations necessary for tumorigenicity. Indeed, deletion of *Nf1* and *Trp53* from neural stem cells in mice initiates gliomagenesis in the subventricular zone (SVZ), where neural stem cells reside (Zhu et al., 2005). However, whether human glioblastoma stem cells arise from mutated neural stem cells or a more mature cell type that acquires self-renewal capacity remains to be determined.

The stem cell niche

It has been long recognized that normal stem cells of various tissues are tightly regulated by the immediate microenvironment or stem cell niche(Moore and Lemischka, 2006). Consequently, an important question is whether glioblastoma stem cells also depend on cues from the environment for survival. Stem cell niches are not merely repositories for stem cells, but are complex dynamic entities that actively control stem cell function (Scadden, 2006) regulating stem cell-renewal and fate. This microenvironment of stem cells is known to help maintain the cells in a "quiescent" state and preserve their potential to proliferate and differentiate (Fuchs et al., 2004; Ailles and Weissman, 2007). Direct genetic alterations or dysregulated crosstalk between signaling pathways of the CSCs and the cells of their microenvironment have been implicated as important determinants of functional tumor microenvironment preceding cancer development (Moinfar et al., 2000; Lopez-Otin and Matrisian, 2007). As commented in the Introduction, studies conducted during the last years have identified stem cells with regenerative capacity in the subventricular zone and the subgranular zone of the dentate gyrus. The central structural element of the neural stem cell niche is provided by capillaries within these zones. This organization places the stem cells in close proximity to endothelial and other vascular cells, facilitating communication among these cell types. The existence of niches extends to tumor cells as well. The tumor microenvironment, composed of nonepithelial stromal cells

increasingly is shown to be essential for tumor growth (Figure 2) (Kenny et al., 2007). Neural cancer stem cells have been shown to lie within a vascular microenvironment. In this regard, Calabrese et al recently provided convincing evidence that stem cells from various brain tumors, including glioblastoma, are indeed maintained within vascular niches that mimic the neural stem cell niche (Calabrese et al., 2007). Using co-immunofluorescence and multi-photon laser scanning microscopy, they showed first that $CD133^{+}$/$Nestin^{+}$ tumor cells are closely associated with vasculature. Furthermore, increasing the number of endothelial cells and blood vessels in xenografts augmented the NCSC population and the rate of tumor growth. Clinical trials of the anti-angiogenic drug bevacizumab (Vredenburgh et al., 2007) have demonstrated a potent anti-tumor effect in patients with glioblastoma. This effect could be the result of a depletion of the tumor blood supply. However, the presence of a glioblastoma stem cell niche would imply that this drug might also function to disrupt stem cell maintenance. In this regard, Calabrese and colleagues showed that treating glioblastoma-bearing mice with bevacizumab depleted tumor blood vessels and caused a significant reduction both in the NCSC population and tumor growth rate. Interestingly, this treatment did not alter the proliferation or survival of most of the cells in the tumor, suggesting that the drug was specifically acting on the cancer stem cells. The notion that cancer stem cells exist in aberrant cell niches is an attractive one. A recent study of human gliomas suggests that bone morphogenic proteins, which are niche-derived regulators of neural stem cell fate might also regulate the differentiation status of cancer stem cells (Piccirillo et al., 2006). Therefore, the tumor microenvironment offers a novel approach to treatment through the targeting of cells inside the tumor niche.

Identifying Glioblastoma Stem Cells

Correctly identifying stem cells in vivo remains the biggest obstacle to progress in understanding stem cell biology. The identification of reliable markers will allow prospective isolation and characterization of a pure population of CSCs, not just a population of cells containing CSCs. Normal stem cells and their neighboring cells within tissues can rarely be located by histological methods. Some properties that have been widely assumed to mark stem cells, such as preferential bromodeoxyuridine (BrdU) label retention (caused by an expected tendency of stem cells to divide more slowly than many of their progeny) have frequently proven to be unreliable without the

use of other markers (Crittenden et al., 2006; Barker et al., 2007; Kiel et al., 2007). The "side population" (SP) is defined by Hoechst dye exclusion in flow cytometry and has been commonly used as one of the methods of enriching for cancer stem cells in glioblastomas (Kondo et al., 2004) and also in other types of tumor cells. Goodell *et al.* have demonstrated that the exclusion of Hoechst 33342 dye by SP cells is a dynamic process involving the multidrug resistance transporter 1 (MDR1), a member of the ABC transporter transmembrane proteins (Goodell et al., 1996; Hirschmann-Jax et al., 2004). However, MDR1 cannot be taken as a single marker to identify and isolate SP cells, and additional transporters should be analyzed.

Much work has been carried out on brain tumor stem cells enriched by the cell surface marker, CD133. It is unclear at this time whether the SP overlaps with the CD133 population, but both markers have been shown to be highly enriched in neurosphere-forming capacity (Yuan et al., 2004; Beier et al., 2007), one of the defining characteristics of neural stem cells and progenitors (Figure 3**)**. Recently, several groups have isolated NCSC from glioblastomas (Singh et al., 2003). They cultured dissociated tumor samples and expanded the cells on a defined, serum-free medium containing fibroblast growth factor and epidermal growth factor. These cells form floating aggregates (neurospheres) just as normal neural stem cells do in the same conditions. These neurospheres retain the self-renewing capacity and expressed neural stem cell markers, such as nestin, CD133, and Notch. Such aggregates, highly enriched in long-term, self-renewing multipotent cells in vitro, formed malignant tumors when transplanted in vivo in immunodeficient mice. These findings indicate that glioblastomas contain cancer initiating neural stem-like cells, which can be identified by their staining with CD133 (Singh et al., 2003; Singh et al., 2004). Furthermore, it has been recently shown that CD133 expression correlates with patient survival in gliomas, lending support to the current cancer stem cell hypothesis (Zeppernick et al., 2008). These authors have found, using a large panel of human glioma samples, frequencies of $CD133^+$ cells to increase with tumor grade, with many glioblastomas containing > 25% positive cells. In contrast, tissue sections of many WHO grade tumors were devoid of immunoreactive cells, probably indicating a low frequency of CSCs in these less malignant tumors. These findings provide strong valuable evidence for the CSC hypothesis and the clinical relevance of the CD133-positive cell population in glioblastomas.

Another important trait of brain tumor stem cells is the signaling trough Notch receptor. Notch signaling is strongly activated in primary human gliomas and in several glioma cell lines (Pahlman et al., 2004) where its depletion reduces tumor proliferation (Purow et al., 2005). Moreover, transfection of downstream mediators of the Notch pathway results in an increase in the growth and sphere formation of human glioma $CD133^+$ cells, indicating that Notch signaling is essential for the maintenance and proliferation of the tumor stem cell population (Zhang et al., 2008). A similar effect has been observed in medulloblastoma stem cells, where loss of tumor forming capacity is attributed to depletion of cancer stem cells in response to Notch signalling blockade (Fan et al., 2006). This data are in agreement with the studies in non-neoplasic stem cells that attribute Notch signalling the role of inhibiting neuronal differentiation and maintaining the neural progenitor pool (Yoon and Gaiano, 2005).

Neural cancer stem cells as therapeutic targets

Malignant primary brain tumors are characterized by a short median survival and almost 100% tumor-related mortality. Therefore, this brain tumor remains one of the most lethal forms of human cancer. Glial neoplasms are the most frequent primary intracranial neoplasms in man accounting for more than 60% of all primary brain tumors. Although glioblastomas, the most malignant of these, rarely spread outside the nervous system, they infiltrate crucial structures in the brain, preventing curative surgical resection. Radiation and chemotherapy offer only modest benefits and remain essentially palliative (Stupp et al., 2005). Conventional chemotherapy and/or radiation therapies are not usually designed to target a specific cell subpopulation, and their clinical efficacy is measured by their capacity to induce regression of bulk tumor lesions. It is therefore difficult to know whether traditional anti-tumor treatments are able to target cancer stem cells, which are thought to be resistant to such treatments (Guzman et al., 2002; Bao et al., 2006). If glioblastomas are maintained by NCSCs, cells that are characterized by low rates of division and proliferation, it is clear that therapies such as chemotherapy or radiation, which target actively cycling cells, are doubtful to be effective, therefore it is unlikely that existing treatments will ever cure most patients with glioblastoma. Thus, the concept of cancer stem cells provides an interesting conceptual framework to interpret the phenomenon of tumor relapse as well

as the heterogeneity found inside tumors in terms of aberrant cell proliferation and differentiation (Reya et al., 2001). Cancer treatment has traditionally been based on the implicit assumption that human cancer populations are homogeneous. Cancer is resilient to treatment because malignant cells survive chemotherapy and radiation or avoid immune surveillance of endogenous cytotoxic T cells and natural killer cells. Since cancer stem cells have a capacity for unlimited self-renewal, as well as the ability to initiate and drive tumor progression in an animal model (Singh et al., 2004; O'Brien et al., 2007) they would seem the most probable candidates responsible for tumor chemoresistance and recurrence.

In fact, recent investigations in the field of brain and breast cancers implicate cancer stem cells in radiation resistance (Bao et al., 2006; Phillips et al., 2006; Woodward et al., 2007). Bao *et al.* demonstrated that radiation resistance in highly malignant gliomas (GBM) is most likely mediated by tumor stem cells (Bao et al., 2006). This work show that radiation treatment fails in the long run because it cannot kill the subpopulation of $CD133^+$ tumor-initiating cells. They showed that $CD133^+$ cancer stem cells contributed to glioma resistance through preferential activation of DNA damage checkpoint response and an increase in DNA repair capacity compared with $CD133^-$ tumor cells. The radioresistance of CD133 glioma stem cells could be reversed with a specific inhibitor of Chk1 and Chk2 checkpoint kinases, which are closely associated with cellular resistance to radiation, thereby providing a therapeutic advantage to reducing brain tumor occurrence. As the cell cycle of a normal stem cell is tightly controlled by the checkpoint to maintain genomic stability and integrity, the defective checkpoint responses associated with early cancer development (Bartkova et al., 2005; Gorgoulis et al., 2005) point to an abnormal checkpoint control as a potential contributor to the transformation of normal cells into cancer stem cells. Therefore, targeting the checkpoint response in $CD133^+$ glioblastoma cells may help to overcome the radioresistance of this tumor. Further studies may confirm a rate-limiting role of DNA repair for the functionality of glioblastoma cells.

Also, Liu *et al* demonstrated an increased resistance of CD133-positive brain tumor stem cells in response to treatment with chemotherapeutic agents such as carboplatin, paclitaxel, and etoposide, compared to CD133-negative cells (Liu et al., 2006). These studies revealed a higher expression of the multidrug resistance gene

BCRP1 and genes that inhibited apoptosis in the CD133-expressing cancer stem cells. The work also showed that CD133 expression was significantly higher in recurrent glioblastomas compared to their respective newly diagnosed tumors. These results suggest that, although chemotherapy kills most of the cells in a tumor, NCSCs remain viable and can then reappear due to their enhanced chemoresitance.

Regarding the clinical implications of cancer stem cells, Piccirillo *et al.* first showed that human glioblastoma cells expressed bone morphogenetic proteins (BMPs) and their cell surface receptors, BMPs being the soluble factors that normally induce neural precursor cells to differentiate into mature astrocytes, a subtype of brain cells called glial cells (Piccirillo et al., 2006). These authors showed that BMPs could also promote the differentiation of $CD133^+$ brain tumor stem cells, seriously weakening their tumor-forming ability. The results further imply that tumor populations at least partially retain a developmental hierarchy based on stem cells, and remain able to respond to the normal signals that induce them to mature. These findings should lead to renewed interest in devising therapies that promote the differentiation of cancer cells.

Future directions

In conclusion, although major questions remain unanswered concerning the origin and function of neural cancer stem cells, their existence in glioblastomas is a widely accepted hypothesis. How these NCSC control cell growth and cell-cycle progression of glioblastomas, however, is not yet clear. It is not clear whether exists a stem cell-specific machinery that controls growth and proliferation in a variety of stem cell lineages and why does stem cell proliferation get out of control when asymmetric cell division is compromised. Furthermore, what are the molecular events that occur when such a compromised stem cell becomes unresponsive to growth control signals? The striking discovery of stem cell lineages in many tumors, including glioblastomas, might lead to the identification of entirely new mechanisms for stem cell control. All the data obtained so far suggest that in the coming years, NCSCs will be identified as a powerful new potential therapeutic target, and knowledge of the detailed biology and clinical significance of this noticeably defined population will provide further support for the NCSC hypothesis. Additionally, current efforts focus on the evaluation of target

expression profiles in neural cancer stem cells in glioblastomas and on the potential of these cells to escape therapy. Ultimately, focusing research efforts on the NCSC may drive important advances in our understanding of glioblastoma biology and developing potential cures for this devastating disease.

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