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Minireview

Molecules and Cells

Biology of Glioma Cancer Stem Cells

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Gliomas, much like other cancers, are composed of a heterogeneous mix of neoplastic and non-neoplastic cells that include both native and recruited cells. There is extensive diversity among the tumor cells, with differing capacity for *in vitro* and *in vivo* growth, a property intimately linked to the cell's differentiation status. Those cells that are undifferentiated, self-renewing, with the capacity for developing tumors (tumorigenic) cells are designated by some as cancer stem cells, because of the stem-like properties. These cells may be a critical therapeutic target. However the exact identity and cell(s) of origin of the so-called glioma cancer stem cell remain elusive. Here we review the current understanding of glioma cancer stem cell biology.

INTRODUCTION

Glioma, the most common type of primary brain tumor, is classified by the World Health Organization into 4 distinct grades based on histological features of cellularity, nuclear morphology, mitotic activity, necrosis, and vascular proliferation (Louis et al., 2007). A higher histologic grade corresponds to a less differentiated phenotype and to increasing malignancy. The most common form, a grade IV glioma, called glioblastoma multiforme (GBM) has a median survival of 14-15 months in spite of aggressive multimodality treatment by surgery, external beam radiation therapy, and chemotherapy (Stupp et al., 2005). As asserted nearly seventy years ago by Hans Joachim Scherer (Peiffer and Kleihues, 1999) GBM can either develop by dedifferentiation from a lower grade tumor ("secondary GBM") or can arise "de novo" ("primary GBM"). The differences in clinical and molecular features of the two types of GBM hint at a distinct pathogenesis.

Although gliomas are a relatively rare form of cancer, they account for disproportionately high morbidity and mortality because their location in the brain prevents adequate surgery and other therapies are largely ineffective. Gliomas rarely metastasize outside of the brain, but instead, infiltrate extensively into surrounding normal brain. Thus, surgery is not curative but can establish the diagnosis and relieve symptoms by decompressing the brain located in a poorly compliant intracranial cavity. Radiation therapy and chemotherapy increase survival; however, disease recurrence is virtually inevitable.

Both the invasive nature of the tumor and its heterogeneity probably contribute to the poor response to currently available treatment regimens. Heterogeneity is traditionally believed to result from regional variations in the tumor microenvironment and the diversity of cancer cell subpopulations that results from progressive stochastic genetic alterations. The recent reports describing the presence of cancer-initiating stem-like cells or cancer stem cells (CSC) may help to explain cellular heterogeneity (stem cells have an indefinite lifetime and reproduce over long periods of time making them likely to accumulate mutations that could lead to genetic instability) and explain resistance to therapy (Reya et al., 2001). The observations that cancer cells and stem cells share the common defining features of incompletely differentiated state and unlimited self-renewal capacity, have led to the cancer stem cell hypothesis. In this brief review, we will discuss the current evidence for CSC in glioma pathogenesis.

Cancer stem cells in tumors

Cancers generally retain histological and gene expression programmatic resemblance to the tissue of origin. Usually, surgical pathologist can identify the organ for which a neoplasm arose. When anatomic review alone is insufficient, molecular studies such as genomic profiling and gene expression analysis are often successful in determining the tissue of origin. Also, close examination of cancer histopathology often demonstrates an attempt to recapitulate the organ-specific functional morphology such as formation of follicles in thyroid cancer. Such tissue-specific growth patterns exhibited by cancers suggest a pathologic regenerative process. The observation that stem cells and some cancers cells share the common defining features of incompletely differentiated state and self-renewal capacity, in conjunction with technical developments for comparative studies, have led to the cancer stem cell hypothesis supported by numerous compelling studies.

The recent attention to cancer stem cells as the source of various malignancies represents a rebirth of an old idea. The German physician considered by many to be the father of pathology, Rudolf Virchow, suggested as early as 1858 that cancers arise from embryonic-like tissue (Virchow, 1858). Virchow's assertion, driven by observations of histologic similari-

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ties between developing normal tissues and poorly differentiated cancers, was further extended by subsequent investigators who proposed that cancer results from "embryonal rests" due to disrupted developmental programs in which tissues fail to appropriately differentiate to instructive specification (Conheim, 1875; Harris, 2005). The histopathologic terms "poorly differentiated," and "de-differentiated" used to describe some cancers invoke early developmental processes. The link between cancer and primordial cells is also suggested by analysis of germ cell tumors where multipotent teratomas exhibit differentiation into tissues of all three germ layers. This finding strongly suggests that certain cancers likely of monoclonal origin, retain pluripotency. Thus, the belief that cancer may be initiated and maintained by uncommitted self-renewing cells was not common, even though definitive proof was lacking.

Evidence for the above concept came from technical advances in *in vitro* cell propagation and from improved understanding of normal developmental processes. Refinements of *in vitro* cell culture methods, and identification and cloning of key growth factors permitted isolated studies of cancer cells. Fidler and Kripke observed striking heterogeneity of dissociated tumor cells with respect to the clonogenic ability to form metastasis (Fidler and Kripke, 1977; Kripke et al., 1978). Clonogenicity may in some cases, serves as a surrogate marker of self-renewal capacity. Recognition of clonogenic cells in cancers combined with the enhanced understanding of lineage development of normal cells allowed subsequent investigators to examine cancers with the cancer stem cell hypothesis in mind. Perhaps in part due to easy access to normal stem cells, the hematopoietic developmental patterns were first characterized (Spangrude et al., 1988). Relying on established markers of normal hematopoietic development, John Dick identified a leukemic cancer stem cell capable of recapitulating the disease in an immunodeficient mouse (Lapidot et al., 1994). This was a seminal paper in the development of the cancer stem cell field. Subsequently, the existence of "cancer stem cells" or "cancer initiating cells" have been identified in a variety of solid tumors including gliomas, medulloblastomas, breast cancer, lung cancer, prostate cancer, and colon cancer (Al-Hajj et al., 2003; Bonnet and Dick, 1997; Collins et al., 2005; Hemmati et al., 2003; Kim et al., 2005; O'Brien et al., 2007; Singh et al., 2003).

Cancer stem cells in gliomas

Aggressive brain tumors are well known to contain poorly differentiated cells, reflected in the use of the term "blastoma" in glioblastoma, pineoblastoma, neuroblastoma, and medulloblastoma. Percival Bailey and Harvey Cushing suggested an embryonic origin for medulloblastoma as early as 1926. They hypothesized that medulloblastomas may originate within the embryonal rests in the roof and the ependymal lining of the fourth ventricle. Ronald McKay's group eventually provided experimental evidence (Valtz et al., 1991). Expression of nestin, a marker of neural stem-progenitor cells was subsequently demonstrated in a variety of neuroepithelial brain tumors (Dahlstrand et al., 1992; Tohyama et al., 1992). The renewed interest in applying developmental biology to cancers, perhaps fueled by John Dick's work on leukemia, led to the identification and *in vitro* propagation of malignant cells with stem cell-like properties of undifferentiated state, self-renewal capacity, and multipotency in clinical neuroepithelial tumor specimens (Galli et al., 2004; Ignatova et al., 2002; Singh et al., 2003; Yuan et al., 2004). Singh et al. demonstrated that the expression of a putative neural stem cell marker, CD133, in malignant tumor cells was both sufficient and necessary to initiate and recapitulate the tumor upon transplantation into immunodeficient mice (Singh et al., 2004; Uchida et al., 2000). Since these initial observations, numerous laboratories have joined the effort to further investigate and clarify the field of CSC in brain tumors. However, the suggestion that only CD133 positive cells are capable of recapitulating the parental tumor in immunodeficient animals has been disputed (Beier et al., 2007; Joo et al., 2008; Ogden et al., 2008; Wang et al., 2008). A clear separation of the CD133 positive and negative populations is technically difficult (Rich and Eyler, 2008). Therefore, in working with impure populations of cells, a definitive conclusion concerning respective subpopulations is murky at best. In addition, a host of surrogate markers such as BMI-1, Nestin, Sox2, Musashi, SSEA-1 (CD15), and activated Notch pathway have also been suggested to identify the glioma CSC. Some of these findings have created confusion and disagreements rather than adding clarity, possibly because of a lack of uniform definition of exactly what constitutes a glioma cancer stem cell.

In the murine system, the criteria defining the hematopoietic stem cell are clear. A single cell must be able to rescue and establish long-term reconstitution of the lymphohematopoietic system in the recipient (Osawa et al., 1996). Such a simple and precise assay does not exist for brain tumor models. Instead, the model relies on recapitulation of the original tumor upon transplantation into immunodeficient mice. The xenograft model is an imperfect assay in determining the identity of "a CSC" because formation of brain tumor from single cell injection is not yet feasible. At best, one can demonstrate that a pool of tumor cells as few as 100, if tumorigenic, is enriched with glioma cancer stem cells (Singh et al., 2004). Also, because a stem cell is capable of generating the heterogeneous cellular constituents of a tissue or an organ, the CSC must demonstrate recapitulation of the parental tumor in addition to mere tumorigenicity. Additional technical refinements are likely to provide methodological tools to define the identity of glioma cancer stem cells with greater resolution. For instance, Sean Morrison's group has recently shown that single cell injections of melanoma cells can be tumorigenic at an unexpectedly low frequency of 1 in 4 (Quintana et al., 2008). They used a highly immunocompromised mouse strain consisting of combination of subacute combined immunodeficiency disease and interleukin-2 receptor gamma-null features. Because the authors did not observe a relationship between tumor cell surface marker expression and tumorigenicity, the result has been used to call in question, the hierarchical interpretation of the CSC hypothesis. There are two important considerations concerning this study. First, the highly permissive *in vivo* condition used reflects an *in vitro* environment that lacks microenvironmental feedbacks that are believed to be critical for both normal and cancer stem cells (Gilbertson and Rich, 2007). As such, this highly artificial animal model may not represent the true *in vivo* niche. Second, the CSC hypothesis can function in concert with both hierarchical and stochastic models (Lagasse, 2008; Reya et al., 2001). Depending on the particular context, both hierarchical and stochastic processes are in play.

Experimental assays are helpful in improving understanding of concepts, but to what extent they may define glioma CSC remains unclear. Reliance on expression of intracellular and/or surface markers alone is insufficient in defining glioma CSC because unlike normal stem cells, the genetic dysregulation that occurs in cancer may lead to ectopic protein expression. Particular caution is warranted in using criteria that simply combine a "stem cell marker" and *in vitro* proliferative rate because enhanced division may instead identify a "transit amplifying fraction" capable of proliferation but not extensive self-renewal.

The *in vitro* environment assumes a reductionistic view that ignores the critical role of niche interactions with other cells (this may also apply to use of highly immunocompromised animals). The various putative markers of CSC may only identify subpopulations with enhanced *in vitro* propagation capability. Enhanced *in vitro* growth may also represent an artifact resulting from physical detachment of the cell from its native microenvironment. One potential means to overcome this difficulty is by performing live cell imaging to determine lineage mapping over generations of divisions to exclude transit amplifying cells, which may retain multilineage differentiation capacity, but only for brief periods (Park et al., 2008; Ravin et al., 2008). At this time we suggest heuristically that the CSC is characterized by persistent self-renewal capacity and tumor initiation in orthotopic animal models (Clarke et al., 2006).

It is difficult to know how best to study putative CSC. There is widespread agreement that long term *in vitro* exposure leads to irreversible changes in the identity of the cultured cells. For this reason, *in vivo* passage may be preferable. However, as discussed, even the *in vivo* conditions of rodent brains do not faithfully represent the native human brain microenvironment. This may be particularly true for artificially immunodeficient animals. Examination of human glioma specimens often demonstrates presence of CD45 positive (common leukocyte antigen) cells, implying interaction of cancer cells with the immune system. The *in vivo* animal model is incapable of providing this potentially critical input. In addition, we have observed that the molecular profile changes after treatment in unpredictable ways, suggesting evolution of over time (Horbinski et al., 2009). For example, some tumors acquire amplification of EGFR whereas others lose it. That the molecular profile of gliomas changes over time is hardly surprising considering that lower grade gliomas can transform to higher grades. Histopathologic grades represent an arbitrary classification applied to a process that is not punctuated, but rather existing on a spectrum, and the microenvironment likely plays a key role in the constant evolution of gliomas, an effect that cannot be reproduced in immunodeficient animals. The role of the microenvironment (cell nonautonomous influence) in tumorigenesis and maintenance is critical because cancer is more complex than a mere cell autonomous entity strictly dictated by intrinsic genetic and epigenetic programs. While recognizing the limits of both *in vitro* and *in vivo* systems individually, we submit that the combination of both methods is effective in advancing the field.

Significance of glioma cancer stem cells

The presence of glioma cells of variable differentiation status that correlates with self-renewal capacity has been demonstrated by numerous laboratories (in general, undifferentiated cells appear to have greater self-renewal capacity). Detailed studies with glioma cells enriched for the CSC subpopulation show increased resistance to irradiation, a major therapeutic modality for the treatment of malignant gliomas, because they activate the DNA damage response pathway, rapidly repairing the DNA damage induced by the radiation (Bao et al., 2006a). These cells also seem to play a critical role in recruitment of blood vessels, a necessary task to promote tumor growth (Bao et al., 2006b). Consonant with these observations, the presence of embryonic stem cell-like gene expression signatures in human cancers (GBM, breast, and bladder) is associated with aggressive histopathology, confirming clinical-prognostic significance for the stem-like phenotype of cancers (Ben-Porath et al., 2008). One conclusion is that the CSC subpopulation must be targeted to achieve complete and durable response, and considerable research efforts have been and continue to be devoted to this cause (Bao et al., 2008; Beier et al., 2008; Dietrich et al., 2008; Park et al., 2007; Rosen and Jordan, 2009; Schulenburg et al., 2006). According to a hierarchical model, only an identifiable CSC subpopulation is endowed with self-renewal capacity sufficient to repopulate the tumor. A competing stochastic idea suggests that self-renewal capacity is linked to the presence of key genomic alterations that may occur in a variety of cells. Therefore, non-CSC may form or develop into CSC. The argument is not simply a conceptual exercise because adoption of each model dictates obviously distinct therapeutic strategies. The available data, including clinical evidence for progression of well differentiated low grade gliomas to poorly differentiated gliomas suggest that both hierarchical and stochastic mechanisms may be involved (Lagasse, 2008; Odoux et al., 2008).

The CSC hypothesis represents an opportunity to emphasize developmental process in the study of tumorigenesis. Such effort involves examination of the stem cell niche and early developmental signaling pathways. Similar to normal neural stem cells, glioma-derived cancer stem cells seem to reside within a perivascular niche (Gilbertson and Rich, 2007; Palmer et al., 2000; Quinones-Hinojosa et al., 2006; Shen et al., 2004). This may suggest that therapeutic targeting of the tumor-associated vasculature may at least indirectly interfere with glioma CSC growth. The recent demonstration of clinical effectiveness of anti-angiogenic strategy with bevacizumab, a monoclonal antibody directed against vascular endothelial growth factor (VEGF), may in part be mediated by effect on the CSC (Kreisl et al., 2009; Vredenburgh et al., 2007). The significant therapeutic response in those patients receiving bevacizumab led to approval by the Food and Drug Administration of the United States of America for use of this agent in recurrent glioblastoma. There are ongoing studies of other anti-angiogenic agents in malignant gliomas that may provide pharmacodynamic insight into dependence of CSC on the perivascular niche for survival, growth, and proliferation. These studies may also provide an opportunity for comparative analysis and identity of cancer cells that adopt an enhanced migratory-invasive phenotype after exposure to anti-angiogenic agents. In addition to addressing the niche, the CSC may be directly targeted. Signaling cascades that are emphasized by normal stem cells, such as notch, hedgehog, Wnt, and the PI3K-Akt axis, have been a focus of increasing interest in cancer therapy because manipulation of these pathways may preferentially deplete the CSC (Androutsellis-Theotokis et al., 2006; Bar et al., 2007; Eyler et al., 2008; Miele et al., 2006; Rizzo et al., 2008; Taipale and Beachy, 2001). Because cells at different phases of development may rely on adoption of serial signaling pathways, it is possible that activation of particular pathways may aid in the identification and classification of CSC subtypes. For instance, glioma cancer cells that express similar markers indicative of the undifferentiated state may be further divided on the basis of activated signal transduction pathways. Cancer cells bearing identical surface markers may turn-on different pathways for self-renewal versus quiescence. In combination with surface markers, identification of activated signal transduction pathways may be used to provide highly discriminating targets and biomarkers of therapeutic response.

Glioma CSC cell of origin

An area of intense interest in CSC biology concerns the normal cells from which the CSC originate. Independent of providing insight into the controversy of hierarchical versus stochastic models, identifying these cells has the potential to fundamentally impact therapy, particularly those associated with familial

cancer syndromes. Tissue specific stem cells are reasonable candidates for respective cancer stem cells because the long existence may subject them to acquisition of multiple genetic abnormalities necessary for tumorigenesis (Dalerba et al., 2007; Hanahan and Weinberg, 2000). On the other hand because stem cells are in most circumstances quiescent, they may not experience an adequate number of divisions to acquire genomic errors. A possible stem cell origin of cancer is illustrated by a recent clinical report of neural precursor transplantation leading to formation of tumors of donor origin (Amariglio et al., 2009). The patient who had ataxia telangiectasia received intracerebellar and intrathecal transplantation of human fetal neural stem cells. Four years later he developed donor-derived multifocal glioneuronal brain tumors. There is also epidemiological evidence for role of precursors in brain tumor formation. Newborns with large heads appear to have an increased risk of developing brain tumors, suggesting a developmental dysregulation of cell number and/or size in contributing to subsequent brain tumor risk (Samuelsen et al., 2006). Reports of congenital brain tumors also link early organogenesis and tumorigenesis (Carstensen et al., 2006). None of these observations, however, exclude the possibility that the neoplastic transformation event occurred within differentiated cells. The paragraphs that follow will present some evidence for glioma cell of origin.

Experimental models with tumor suppressors indicate that undifferentiated cells may be more predisposed to neoplastic transformation. The hereditary tumor familial syndrome neurofibromatosis type 1 (von Recklinghausen disease) is caused by a germline mutation of the *NF1* gene that encodes for neurofibromin, a GTPase activating protein (GAP) that functions as a negative regulator of the Ras pathway (Marchuk et al., 1991). Optic glioma is one of many tumor types that these patients develop. Work by Luis Parada's group has elegantly identified the potential cell of optic glioma origin. Disruption of the *NF1* gene in mature neurons leads to abnormal cortical development but no tumors are produced (Zhu et al., 2001). Astrocytic-specific inactivation of *NF1* gene likewise fails to produce gliomas (Bajenaru et al., 2002). However loss of this gene function in the developing brain leads to proliferation of glial progenitors and optic glioma formation, thereby establishing a key neurofibromatosis 1 phenotype (Zhu et al., 2005b). In addition, sequential ablation of two tumor suppressor genes, *p53* and *NF1* in mice results in formation of astrocytomas only in the subventricular zone, region of the brain containing the multipotent neural stem cells (Zhu et al., 2005a). These data indicate that the tumor suppressor function of the *NF1* gene may be critical in preventing tumorigenesis primarily or even exclusively at the stem cell stage.

Activation of oncogenes shows similar tumorigenic predisposition for precursor cells whereas combined manipulation of an oncogene and a tumor suppressor yields more surprising results. Stimulation of platelet-derived growth factor receptor signaling by infusion of the ligand induces formation of tumor-like proliferation of neural stem cells confined to the subventricular zone (Jackson et al., 2006). Eric Holland's group reported that the combined activation of two oncogenes, *Ras* and *Akt*, can drive tumor formation from progenitor cells but is insufficient for neoplastic transformation of differentiated astrocytes (Holland et al., 2000). The same group found that the combination of oncogene activation (*Ras*) and disruption of a tumor suppressor (*Ink4a-Arf*) provides an adequate oncogenic stimulation for tumorigenesis of both nestin-positive neural progenitor cells and differentiated astrocytes (Uhrbom et al., 2002). Similar findings have been reported by others (Bachoo et al., 2002). Inappropriate signaling of the epidermal growth factor receptor and inactivation of the *INK4a-Arf* tumor suppressor pathway are considered to be a defining molecular feature of GBM. In experimental models, combination of this signaling dysregulation was found to be gliomagenic for both neural stem cells and differentiated astrocytes (Bachoo et al., 2002). Experiments conducted by several other laboratories seem to indicate that although the undifferentiated neural progenitors may be more tumorigenic, gliomas can arise from both undifferentiated and differentiated cells, and maintain stem-like features. These data also suggest that the lower threshold for tumorigenicity of neural stem cells may be an intrinsic property (e.g. activation of particular signaling pathways) rather than merely a consequence of their longevity as previously suggested.

Experimental models, then, indicate that gliomas can arise from both undifferentiated multipotent cells and well differentiated astrocytes. If this is true, one must ask whether the cell of origin defines the disease. It may be that the differences between the primary/de novo and secondary/progressive GBM may reflect a different cell of origin. For example, primary GBM may have a stem-progenitor origin while the secondary pathway might reflect transformation of a differentiated cell into a low grade glioma. Given the possibility of differentiated cell of origin, some investigators take issue with the term "cancer stem cell," and suggest alternative names such as "cancer recapitulating cell" or "cancer maintaining cell." However, the argument is largely semantic because the cancer stem cell hypothesis does not necessarily imply that all cancers originate from normal stem cells. Nor does it require that some cancers are derived from the direct malignant transformation of stem cells. The conceptual importance is that certain cancers are maintained by a population of malignant cells that exhibit stem-like properties of self-renewal and multipotency, irrespective of the cell of origin which may be a stem cell, a progenitor cell beyond the stem cell stage, or a differentiated somatic cell that has reacquired the stem-like properties.

Conclusions and perspectives

Over the past several decades, clinical oncology has benefited greatly from laboratory research. Leukemia, previously universally fatal, is now often curable. Breast cancer, in many instances, becomes after treatment, a chronic illness with prolonged survival. However, in spite of improved understanding of underlying molecular genetics, improvements of surgical and imaging techniques, development of highly advanced irradiation methods and application of multiple chemotherapeutic agents, the prognosis of GBM has changed little over the past 30 years. For this reason, primary brain tumors have become increasingly responsible for disproportionately high mortality, a figure expected to rise as treatment for other cancer types continue to improve. Recently, the field of brain tumor research and clinical neuro-oncology has been consumed by the excitement over cancer stem cells in malignant gliomas. The attention has been generated by both supporters and skeptics. Whether or not the cancer stem cell hypothesis will have a meaningful therapeutic impact, the field is likely to benefit from the increase in attention and possibly research funding.

The cancer stem cell hypothesis is compelling because the stem-like behavior of cancer is difficult to ignore. Cancer cells possess striking regenerative capacity, although the outcome adversely affects the host. Stem-like characteristics of cancer were appreciated many years ago, but only descriptive analyses were performed. We believe the renewed interest in the self-renewal properties of cancer is timely because there are scientific tools now available to investigate questions concern-

ing lineage analysis, differentiation, quiescence, and cell of origin. The cancer stem cell hypothesis places fundamental investigations into development at the center by reinforcing the notion of viewing cancer in the developmental context of each particular tissue or organ. The opportunity to tie together developmental biology and cancer biology may be the greatest contribution of the cancer stem cell hypothesis because cancer may very well be a disease of failed differentiation (Harris, 2004; 2005).

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