

Mini Review

Making of glioma-initiating cells

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There is an increasing body of evidence that suggests that malignant tumors, such as leukemia, breast cancers, and brain cancers, contain cells that maintain the characteristics of tissue-specific stem cells (TSSCs) and are malignant. Malignant gliomas, for example, contain proliferating cells expressing neural stem cell (NSC) markers, such as nestin and CD133, and differentiating cells expressing either neuronal markers or glial markers, raising the possibility that the tumors may contain NSC-like cancer cells. Additional evidence also exists, which indicate that malignant tumors might contain stem cell-like cancer cells, called “cancer stem cells” (CSCs) or “tumor initiating cells”. Although a number of anti-cancer drugs and irradiation therapy have been successful in eliminating cancers, occasionally some cancer cells survive and invade other tissues, leading to the recurrence of cancer; this indicates that the surviving cells are not only resistant to such anti-cancer drugs and irradiation but are also malignant. Previous studies have shown that various ATP binding cassette transporters, such as the multi-drug resistant protein encoded by the multi-drug resistant gene, and the breast cancer resistant protein 1 (BCRP1), contribute to drug resistance in cancers. Interestingly, some of these transporters are also expressed in many types of normal stem cells. BCRP1, for example, excludes the fluorescent dye Hoechst 33342, identifying a side population (SP) enriched with TSSCs. Taking advantage of the common characteristics that exist between TSSCs and cancer cells, many groups have demonstrated that CSCs in tumors or cancer cell lines can self-renew, express well-known TSSC markers, form tumors when transplanted *in vivo* and show resistance to anti-cancer treatments; this suggests that CSCs are important targets for curable therapy. In order to develop an effective therapy against tumors, characterizing and finding ways to kill CSCs is essential. In this review, I have summarized the recent progresses made in glioma CSC research and discuss the perspectives of this field.

Rec.4/21/2008, Acc.5/26/2008, pp537-542

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Key words cancer stem cells, cell of origin, oncogenes, tumor suppressor genes

Cell-of-origin for glioma

The concept of cancer stem cells (CSCs) was first proposed several decades ago. In the case of brain tumors, for instance, Globus and Kuhlenbeck suggested, in 1944, that malignant brain

tumors are generated from immature cells (NSCs or precursor cells) in the ventricular zone (VZ)¹⁾; this hypothesis was subsequently proven through a number of experiments. Hopewell and Wright discovered that brain tumors arose frequently from the

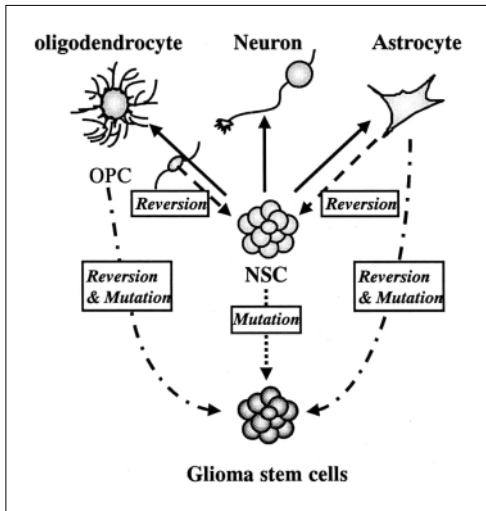


Fig.1 Cell of origin for glioma

Many lines of evidence suggest that astrocytes and oligodendrocyte precursor cells, both of which can acquire multipotentiality, and NSCs are cells of origin for malignant glioma.

VZ when carcinogenic pellets were randomly placed in the adult brain². Copeland and colleagues also showed that brain tumors were induced in the subventricular zone (SVZ) when they infected mouse brains with avian sarcoma viruses³. In addition, Doetch et al. have elegantly revealed that astrocytes in the SVZ can behave as multipotent NSCs in the adult brain⁴. Together with the evidence that NSCs and astrocytes in the VZ/SVZ survive and proliferate throughout life, whereas differentiated neural cells do not, these findings suggest NSCs and astrocytes in the VZ/SVZ have a higher probability of accumulating oncogenic mutations and transforming into CSCs that retain characteristics of NSCs and are malignant (Fig.1). In fact, it has been shown that malignant brain tumors, including glioblastoma multiforme (GBM) and medulloblastoma, are immunolabeled for both NSC markers, such as Nestin, CD133, Bmi1, Sox2, Musashi1/2 and Olig2, and differentiation markers, including the neuronal marker MAP2, the astrocyte marker GFAP, and the oligodendrocyte marker GC⁵⁻⁹.

Oligodendrocyte precursor cells (OPCs) may also be cells of origin for some glioma: Although OPCs are committed to differentiating into oligodendrocytes *in vivo*, they can also differentiate into GFAP-positive astrocytes and acquire NSC characteristics, including the expression of NSC markers and multi-potentiality, when cultured under specific culture conditions¹⁰⁻¹² (Fig.1). Moreover, transformed OPCs with oncogenic HRas and C-Myc

can form malignant glioma *in vivo*¹³.

Using a combination of transgenic mice and a retrovirus system, two groups have demonstrated that Nestin-positive NSCs and GFAP-positive astrocytes form malignant gliomas *in vivo*: Holland and his colleagues infected transgenic mice that expressed the avian leukosis virus (ALV) receptor under the regulation of either a nestin enhancer or a gfap promoter, with recombinant ALVs encoding oncogenic genes, such as platelet derived growth factor (PDGF) receptor beta, or activated Akt, or activated Ras, and found GBM had developed in the brain^{14,15}. De Pinho and colleagues overexpressed a constitutively active form of epidermal growth factor (EGF) receptor in either NSCs or astrocytes from Ink4a/Arf^{-/-} mice, transplanted them into the brain, and found that the cells formed high-grade gliomas¹⁶. Thus, these findings suggest that NSCs and astrocytes are cells of origin for brain tumors. However, since tumors would be, in theory, generated from one transformed cell, these tumor models, in which many transformed cells are generated or injected at the same time, do not provide an answer to whether NSCs and astrocytes are bona fide cells of origin for malignant glioma.

Characteristics of glioma stem cells

1) Anti-cancer drugs

A number of anti-cancer drugs have been successful in eliminating cancers; some cancer cells usually survive, however, causing the cancer to recur, indicating that the surviving cells are not only resistant to such anti-cancer drugs but are also malignant^{17,18}. It has been shown that various ATP-binding cassette (ABC) transporters, such as the multi-drug resistant protein (MRP) encoded by the multi-drug resistant gene (MDR) and the breast cancer resistant protein (BCRP1), contribute to such drug resistance in cancers (Fig.2). Among these transporters, some of which are expressed in many kinds of normal stem cells, BCRP1 excludes the fluorescent dye Hoechst 33342, identifying a side population (SP) that is enriched with various types of TSSCs¹⁹, although some research has shown that TSSCs exist in both SP and non-SP, and that SP cells do not express stem cell markers. A number of research groups have demonstrated that some established cancer cell lines and tumors, including glioma, AML, neuroblastoma, nasopharyngeal carcinoma, and ovarian cancer, contain a small SP and the SP cells, but not non-SP cells, self-renew in culture, are resistant to anti-cancer drugs including Mitoxantrone, and form tumors when transplanted *in vivo*²⁰⁻²⁶.

Since many brain cancer cells do not contain any SP fraction, it is of interest to investigate relationship between CSCs and other molecular mechanisms, such as glutathione, topoisomerase II,

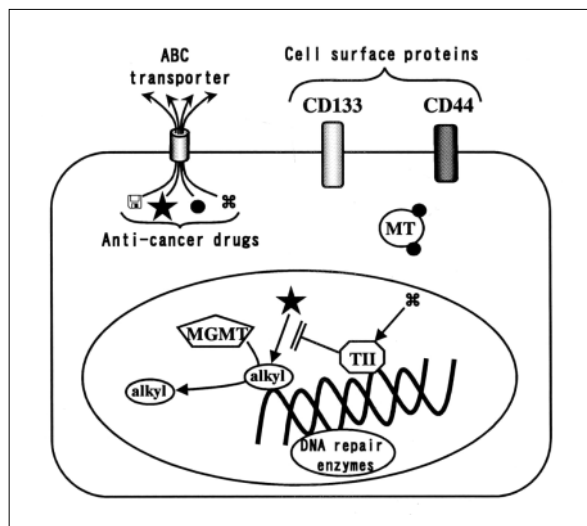


Fig.2 Characterization of glioma stem cells

It is likely that glioma stem cells express a number of factors: ABC membrane transporters that pump out anti-cancer drugs, Metallothionein and topoisomerase II which neutralize anti-cancer drugs, and DNA repair enzymes such as MGMT. Glioma stem cells can also express CD44, which regulates cell migration and invasion, and CD133, whose function is still unknown.

metallothioneins, and O6-methylguanine-DNA-methyl-transferase (MGMT), involved in the resistance to anti-cancer drugs²⁷⁾ (Fig.2).

2) Resistance to irradiation

Irradiation is one of most effective therapies for glioma, however, a small population of cancerous cells tends to survive and cause tumor recurrence, suggesting that CSCs are radioresistant. Recently, Bao et al. have revealed that CD133-positive glioblastoma CSCs are much more resistant to irradiation than CD133-negative cells²⁸⁾.

3) Invasion activity

One characteristic of malignant glioma cells is their ability to invade and disseminate into normal brain tissue. Some of the infiltrating glioma cells cannot be removed by surgical operation and causes recurrence, suggesting that CSCs retain high invasion activity. In fact, Liu et al. have recently demonstrated that CD133-positive glioma cells highly express CD44, which mediates cell migration²⁹⁾.

Preparation of glioma stem cells

Several groups have succeeded in separating CSCs from cancers and cancer cell lines using the common features of TSSCs,

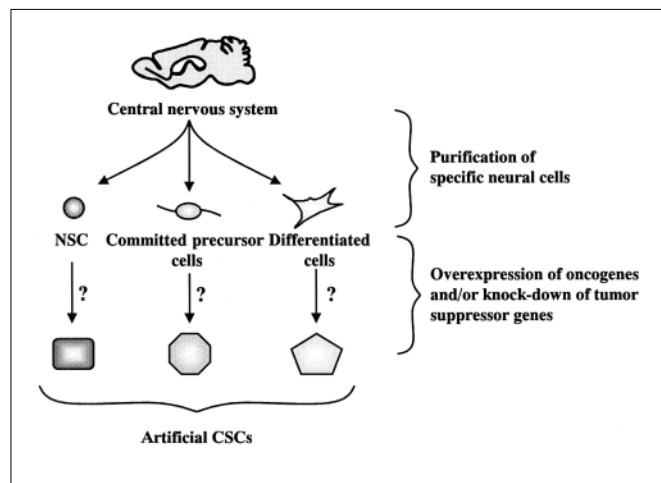


Fig.3 Making of glioma stem cells

Purified NSCs, committed precursor cells, and differentiated cells that are transfected with various types of glioma-related oncogenes and/or siRNA/shRNA for tumor suppressor genes, transform into CSCs that are capable of self-renewal, express NSC markers, and show malignancy.

such as cell surface markers, SP, and/or floating sphere formation assays.

1) Cell surface markers

Dirks and colleagues reported their success in separating brain CSCs from human medulloblastoma and GBM using an anti-CD133 antibody that recognizes a variety of different stem cells³⁰⁾. Here, as few as one hundred CD133⁺ GBM cells, although not CD133⁻ cells, formed tumors in NOD-SCID brains. Another study revealed that CD133 is expressed in a small subset of cancer cell lines and these CD133⁺ cells retain CSC characteristics: CD133⁺ cells predominantly formed tumors when transplanted *in vivo* and were resistant to irradiation^{31,32)}. However, since some reports suggested that CD133 negative cells from human tumor specimens could also form tumors in xenograft models^{33,34)}, it should be carefully checked whether CD133 negative cells give rise to CD133 positive cells *in vivo* and *in vitro*. Nonetheless, it seems likely that cell surface markers, CD133 in particular, are useful in separating CSCs from many types of tumors.

2) Side population

Several groups have already shown that many cancer cell lines contain CSCs. Using Hoechst 33342 staining and flow cytometry, it was demonstrated that a number of established cancer cell lines, including the rat C6 glioma cell line, human glioma cell lines, breast cancer cell lines, prostate cancer cell lines, neuroblastoma cell lines, myeloma cell lines, hepatocellular carcinoma cell lines,

head and neck squamous carcinoma cell lines, nasopharyngeal carcinoma cell lines, and pancreatic carcinoma cell lines, all of which have been maintained in culture for decades, contain a small SP²⁰⁻²⁶). These studies also demonstrated that the SP cells, but not the non-SP cells, self-renew in culture, are resistant to anti-cancer drugs such as Mitoxantrone, and form tumors when transplanted *in vivo*. In this way, the SP in cancer cell lines contain cells with characteristics of both stem cells and cancer cells.

3) Sphere formation assay

An increasing amount of evidence points to the fact that CSCs as well as NSCs can form floating aggregates (tumor spheres) and be enriched in the spheres when cultured in serum-free medium with proper mitogens, such as bFGF and EGF. Although many CSC researchers use sphere formation methods to concentrate their CSCs in culture, little is known why CSCs — as well as NSCs — are enriched in the spheres. Among a number of potential mechanisms, it is of interest to investigate a role of cell adhesion molecules, particularly N-cadherin, in the spheres, as it has been shown that N-cadherin is strongly expressed in neurospheres and ventricular zone^{35,36}.

It is therefore crucial to characterize bona fide CSCs in any attempt to reach a curable therapy. As it seems likely that the cells separated by any of the three methods remain a mixture of CSCs and other cells, then a combination of these methods might help in purifying CSCs; failing this we will have to establish new methods to obtain pure CSCs.

Niche and CSCs

The number of TSSCs is precisely regulated by both intrinsic mechanism and extracellular signals derived from specialized microenvironment “niche”. For example, it was demonstrated that niche provides a limited number of physical anchoring sites, including beta1-integrin and N-cadherin, for TSSCs and secretes both growth factors and anti-growth factors, including Wnt, FGF, hedgehog, bone morphogenic proteins and Notch^{37,38}). Moreover, it was shown that the ablation of niche results in loss of TSSCs. It seems likely that CSCs also need niche for tumorigenesis. Kaplan and his colleagues have elegantly demonstrated that bone marrow derived progenitors form the pre-metastatic niche in the tumor-specific pre-metastatic sites before cancer cells arrive and that the ablation of the niche prevents tumor metastasis³⁹). However, since transplanted cancer cells form tumors in any area *in vivo*, CSCs might be independent of the niche regulation or have a capability to make a new niche by recruiting bone marrow stem cells and other component cells.

Making of glioma stem cells

It still remains controversial whether CSCs arise from TSSCs, committed precursor cells, or differentiated cells. In addition, the relationship between cell of origin for brain CSCs and genetic alterations have not yet been elucidated, although a number of oncogenes and tumor suppressor genes have been well characterized in tumorigenesis. Using neural lineage markers and new methods including FACS, it is now possible to separate pure neural lineage cells. We can then overexpress oncogenes or knock down tumor-suppressor genes in the cells and examine the relationship between cell of origin for malignant glioma and genetic alterations (Fig.3). Indeed, it has been demonstrated that overexpression of exogenous oncogenes can induce hematopoietic stem/progenitor cells to transform into leukemic stem cells⁴⁰⁻⁴²). We also succeeded in generating glioma stem cells in culture. In brief, we transfected glioma-related oncogenes to neurosphere cells, oligodendrocyte precursor cells (OPCs), oligodendrocytes and astrocytes, and examined their proliferation, colony formation ability in soft agar, expression of NSC markers, and malignancy *in vivo*. We found that neurosphere cells and OPCs, but not either oligodendrocytes or astrocytes, transformed to CSCs that express NSC markers, including CD133, and are highly malignant *in vivo*, suggesting that neurosphere cells- and OPC-derived CSCs are enriched with glioma stem cells (unpublished observation). Using similar methods, we might generate brain CSCs from NSCs, OPCs and/or mature neural lineage cells, characterize them, and identify targets for curable therapy.

Concluding remarks

Over the last few decades, numerous progresses have been made toward characterizing gliomas and identifying many genes that are mutated in gliomas; there has been, however, no significant progress made in finding a cure for malignant glioma. In the case of GBM, for example, the mean survival time is about 12 months. As CSCs have been found in malignant gliomas, there is now a move toward characterizing these cells and finding targets to kill them. Using oncogenic gene mutations, purified neural lineage cells, and a number of characterized markers, we are now able to make CSC lines in culture, characterize them, and develop ways to kill them for potential uses in curable therapy.

Acknowledgements

I would like to apologize to the authors whose works were not referenced due to limitations of space. I thank Hazuki Hiraga for critical reading of the manuscript. TK was supported in part by a Grant-In-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of Interest Statement

None declared

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