Journal of Cellular Biochemistry 88:11–19 (2003)

PROSPECT

Neural Stem Cells and Neuro-Oncology: Quo Vadis?

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Abstract Conventionally, gliomas are assumed to arise via transformation of an intraparenchymal glial cell that forms a mass that then expands centrifugally, eventually invading surrounding tissues. We propose an alternative model in which gliomas arise via initiation and promotion of cells within the brain's subependymal layer or subventricular zone, the source of a recently characterized pool of neural cells with the properties of self-renewal and multipotentiality (i.e., stem cells) that persists into adulthood. In this model, the particular histological subtype of glioma would represent the effects of temporal and spatial environmental influences rather than the particular cell of origin and the disease's centrifugal point would be the subependymal layer. The implications of such a model are discussed. J. Cell. Biochem. 88: 11–19, 2003. © 2002 Wiley-Liss, Inc.

Key words: neural stem cell; subependymal layer (SEL); subventricular zone (SVZ); glioma

THE CLINICAL PROBLEM

Brain tumors of glial histologies (gliomas) are the most common type of primary CNS neoplasms encountered clinically. Unfortunately, it has almost become a mantra for both clinicians and researchers to start any presentation on this topic with the fact that they remain difficult to treat despite intense research. The sad truth remains that although we have learned much about the basic biology of these tumors, little clinical progress has been made in increasing patient survival since the landmark BTSG studies over 20 years ago [Walker et al., 1978, 1980].

The discovery and characterization of a pool of cells that persists into adulthood and retains the capability to self-renew and differentiate, i.e., neural stem cells, potentially offers new ways for brain tumor researchers to approach their studies. To date, these new neurobiologic findings have been applied in two ways: (1) towards better classification of tumors, so as hopefully to improve prognostics and identify patients who may be better suited to certain types of therapy [Noble and Dietrich, 2002]; and (2) as a potential vehicle to improve delivery of therapeutic agents into tumors [Aboody et al., 2000].

We believe that a better application of the rapidly expanding field of neural stem cell biology is to investigate the early events that occur in situ before macroscopic tumors develop. We propose here a model in which gliomas arise from undifferentiated stem cells whose eventual fate is influenced by local factors in a manner similar to their normal counterparts. This model provides a framework from which investigators can begin to study heretofore neglected areas of glioma, including early diagnosis and prevention, as well as providing new potential therapeutic strategies.

How Do Gliomas Arise?

Reya et al. [2001] in a recent review made the cogent observation that although much is known about the genetic mutations that fuel the cancer process at the molecular level, we frequently do not know what the effects of these lesions are in the target cell in its own environment. Phrased differently, we know much about the *molecular* but not the *cellular* biology of solid tumors, glioma included.

Consider the following assumptions that guide brain tumor researchers. It is generally

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Received 29 March 2002; Accepted 2 April 2002
DOI 10.1002/jcb.10208

believed that glial tumors develop from a transformed glial, oligodendroglial or precursor cell. This cell escapes local control and begins proliferating aberrantly. In some cases, the growth is relatively slow, thus resulting in lower grades of glioma. In others, it occurs more rapidly, thus forming glioblastomas. This progression can occur stepwise, i.e., patients can first develop low-grade tumors that eventually become higher grade.

In this widely accepted model, tumors are therefore monoclonal, i.e., they develop from a single transformed cell and growth is *centrifugal*, i.e., cells spread outward as the mass enlarges and eventually invade the surrounding parenchyma. Consistent with this model is the observation that gliomas almost always arise in one intraparenchymal brain site. Furthermore, numerous instances exist of a patient having undergone a normal computed tomographic (CT) or magnetic resonance (MR) scan a few months before a large tumor is noted; this too, suggests that growth is centrifugal and arises from one site.

However, despite these examples, it is important to note that it has never been proven that gliomas arise from a resting parenchymal cell that undergoes transformation. This is because resolution of this issue requires that tumors be studied at very early time points in their development. Such study is obviously nearly impossible to accomplish using clinical brain-tumor specimens. To circumvent this, therefore, comparisons are made between established low- and high-grade gliomas on the one hand and normal brain on the other and inferences are made based on the differences. While the correlation of the differences between established tumors and normal surrounding tissue can provide some insights into early events, such information does not allow for study of what is happening at the critical moment that tumors develop.

Are there any other conceptual models that might fit the available information concerning the development of gliomas? One paradigm that has been very useful in better understanding epithelial cancers is the multistage model that was first proposed to describe the development of skin cancers after exposure to chemical carcinogens [Berenblum and Shubik, 1947, 1949] and since applied to other epithelial cancers, such as bladder, liver, and colon. This model posits that cancer develops in three stages: (1) an *initiation* stage, that presumably occurs through an irreversible or stable damage to cellular DNA (i.e., a genotoxic insult), (2) a *promotion* phase, which is an operational process that brings about the clonal expansion of initiated cells, and (3) a *progression* phase, which results when genetic instability leads to further mutagenic and epigenetic changes [Foulds, 1965; Trosko, 2001; Trosko and Chang, 2001].

In this model, the emergence of tumors from liver and skin after chemical carcinogenesis is explained by the initiation of pluripotent stem cells that are then suppressed by normal surrounding cells through inhibitory influences [Trosko and Chang, 2001]. Therefore, the early steps of promotion presumably result from a loss of intercellular communication that leads to the clonal multiplication and protection from apoptosis of single initiated cells.

Evaluating the Relationship of Neural Stem Cells and Glioma Using the Multistage Model

It is important to note, considering that the multistage model derived from studies of chemical carcinogenesis, that chemical exposure has been associated with brain-tumor development in epidemiologic studies [Selikoff and Hammond, 1982; Inskip et al., 1995; Carozza et al., 2000]. Furthermore, a model in which tumors emerge from initiation and promotion of a pluripotent stem cell seems an optimal framework with which someone with knowledge of the neural stem cell system could approach glioma.

To effectively link neural stem cells with glioma, however, there is a need to be able to study tumors at a very early stage. While not possible clinically, there is an experimental model of chemical neurocarcinogenesis that is ideally suited to this purpose. First described over three decades ago by Druckrey and colleagues [Druckrey et al., 1966, 1970; Ivankovic and Druckrey, 1968; Druckrey, 1971], it involves exposing rats during late gestation to a single dose of *N*-ethyl-*N*-nitrosourea (ENU). This results in the eventual development of neural tumors in virtually all offspring after a latency of greater than 4 months. Tumors are of variable types including mixed oligoastrocytomas, oligodendrogliomas, astrocytomas, and neurinomas [Zook et al., 2000]. Initiation occurs rapidly since ENU is cleared within minutes [Swann and Magee, 1971; Müller and Rajewsky, 1983] and transformation of brain cells isolated from exposed rats in the neonatal period has been reported [Laerum and Rajewsky, 1975;

Roscoe and Claisse, 1978]. The rapid clearance of ENU also allows the investigator to be fairly certain that any early changes observed are likely related to tumor development and not inflammation (which might be a problem with other models of chemical neurocarcinogenesis such as methylnitrosourea administration to adult rats or placing carcinogenic pellets in mouse brain [Zimmerman and Arnold, 1941; Seyfried, 2001]).

Several studies have assessed pathologic changes during the latency period between ENU administration and tumor development [Lantos and Cox, 1976; Schiffer et al., 1978, 1980; Lantos and Pilkington, 1979; Pilkington and Lantos, 1979; Mennel and Simon, 1985; Yoshino, 1985; Yoshino et al., 1985a,b; Ikeda et al., 1989]. As would be predicted in the multistage model where precursor lesions are observed before macroscopic tumors are noted [Farber, 1976; Solt and Farber, 1976], hyperplastic lesions with characteristics of early neoplasias could be detected several weeks before tumors are noted. These lesions were most frequently noted near the subependymal plate (at the time of these studies, this was still considered primarily a vestigial organ of unclear postnatal function and now is called the subependymal layer or subventricular zone), leading some to propose this area as a source of tumor cells [Lantos and Cox, 1976; Pilkington and Lantos, 1979].

Although detected on morphological grounds, these studies could not characterize these lesions using immunohistochemical markers. Thus, although macroscopic tumors contained cells that expressed GFAP, vimentin and Leu7 (a marker expressed on oligodendroglial cells), only one report noted the presence of the latter marker in what was deemed an early lesion [Galloway et al., 1990]. Therefore, the source of these early lesions remained uncertain.

Applying Concepts From Neural Stem Cell Biology to Neurocarcinogenesis

Farber [1976] proposed that morphology was the key to understanding early lesions and that the linkage depended on identifying markers that were present early in the course of carcinogenesis and persisted through progression. The lack of such a marker in the ENU model made linkage of these early hyperplastic lesions with macroscopic tumors somewhat tenuous. Furthermore, although the subependymal plate was a presumed source of tumor cells, its vestigial nature postnatally made it unclear what its role could be in driving the neoplastic process.

While the ENU model continues to be utilized by investigators [Blass-Kampmann et al., 1998; Adey et al., 2000; Zook et al., 2000; Kish et al., 2001], it is mostly to study the events that occur at initiation (i.e., genetic mutations) or progression (after large tumors are apparent). Little work is currently ongoing that examines the latency period, however, and to date, there are no data that suggests that a promotion stage, similar to that observed in epithelial cancers, occurs in the development of glioma [Koestner, 1990].

The recent characterization of an actively proliferating pool of cells in the SEL of adult mammals capable of responding to endogenous and exogenous influences and possessing robust migratory capacity raises several questions about what roles this area could be playing in the development of brain tumors. Our own interest in this question led us to investigate whether the SEL plays any role in glioma formation after ENU exposure. Our first experiments were based upon the assumption that if, as proposed, these early lesions represented tumors that had arisen in the subependymal layer, then they should share characteristics with this region [Jang et al., 2001a]. We therefore first assessed the expression of nestin during ENU neurocarcinogenesis. Nestin is a member of a unique class of intermediate filaments that is expressed by neural progenitors during development [Hockfield and McKay, 1985; Lendahl et al., 1990]. It is still widely expressed in brain at the time of birth, but its expression becomes restricted in normal adults to the SEL of mammals, including humans [Hockfield and McKay, 1985; Lendahl et al., 1990; Bernier et al., 2000]. Its expression can be reinduced, however, in reactive glial cells after various types of trauma [Clarke et al., 1994; Frisén et al., 1995; Duggal et al., 1997; Holmin et al., 1997; Brook et al., 1999; Kaya et al., 1999; Krum and Rosenstein, 1999; Li and Chopp, 1999], and nestin has been identified in clinical tumor specimens [Dahlstrand et al., 1992; Tohyama et al., 1992].

Since nestin expression occurs preferentially in undifferentiated, reactive and neoplastic neural cells, we reasoned that it may be expressed at all times during the development of brain tumors after ENU exposure. We noted first

(Jang et al., submitted) that nestin is detected in all tumors examined in rats that were sacrificed after 120 days of age. Nestin demarcated the tumors from surrounding parenchyma much better than GFAP and nestin-+ cells were much more frequently BrdU-+ than GFAP-+ ones. We then extended our analysis to assess nestin expression in ENU-exposed brains from birth and were able to detect nestin-+ cells in ectopic locations as early as 30 days of age. These cells were noted to occur singly or in multiple cell clusters and they tended to occur proximate to the SEL or around the corpus callosum, i.e., where early lesions are typically noted [Lantos and Cox, 1976; Schiffer et al., 1978, 1980]. We did not see such single or multiple clusters when sections were stained with vimentin or GFAP, nor did we ever see such clusters in control brains.

These nestin-+ cells were distinctive enough versus the background that they could be quantified. We therefore measured the number of single and multiple cell clusters over time and measured the greatest diameter of those multiple cell clusters observed. We noted that the size of the largest lesion increased with increasing age, although the median size of the lesions was not significantly different. A bit more surprising was the observation that the ratio of single to multiple cell clusters increased with increasing age. If initiated tumor cells had spread throughout the parenchyma soon after ENU exposure where they then underwent promotion; it would be predicted that the percentage of multiple cell clusters would have increased as the rat aged. Therefore, this finding is more consistent with the possibility that lesions were either involuting or that newly initiated cells continue to migrate into the parenchyma.

We also borrowed another strategy from neural stem cell biology to culture SVZ cells obtained from neonatal rats exposed to ENU. To date, culture of brain cells involved either entire brains or cerebral hemispheres that were pooled [Roscoe and Claisse, 1976, 1978; Claisse et al., 1978; Yoshida et al., 1980; Laerum and Rajewsky, 1975; Blass-Kampmann et al., 1998]; occasional transformation was noted in these studies but the character of the cell involved in this event remained unclear. Since Reynolds & Weiss' report a decade ago [Reynolds and Weiss, 1992; Reynolds et al., 1992], it has been known that SVZ/striatal stem like cells can be isolated and maintained indefinitely in an EGF-supplemented chemically defined media. We therefore isolated such cells from ENU- and vehicle exposed pups at birth and serially cultured individual samples [Jang et al., 2001]. We found that normal cultures could not be maintained for more than 8 weeks in culture with serial passaging, after which growth ceased. By contrast, in 2 (22%) of the nine ENU-exposed cultures, a phenotypic change occurred after 50–60 days in culture, after which cells no longer grew as neurospheres but as a monolayer [Jang et al., 2001b]. Furthermore, they continue to grow well in the chemically defined media, and require weekly passaging. Histologically, these cells all express nestin and a smaller percentage express GFAP. Interestingly, although immortalized, these lines are not tumorigenic in either nude mice or syngeneic rat brains.

At this early point in our studies, we can therefore make the following observations/conclusions: (1) a nestin-+ otherwise undifferentiated cell is present at the earliest time points in tumor formation after ENU exposure and persists throughout tumor progression; and (2) SVZ cells removed at birth occasionally undergo spontaneous immortalization after in utero exposure to ENU.

While these results remain preliminary, they have several important implications that should serve as a guide to future experiments. For example, since the finding that the earliest lesions are nestin-+ suggests that the source of these cells is the SEL, an area proposed by others as the site of initiation, the question becomes when do these cells migrate from this area. Since it is know that SEL cells are capable of radial migration, providing a continual source of parenchymal cells even under normal conditions [Levison et al., 1993; Levison and Goldman, 1997; Kakita and Goldman, 1999], it is important to establish whether these initiated cells are immediately migrating or whether they migrate at a later time.

It will also be important to establish why the ratio of single cells to clusters increases with age. In regards to the possibility of regression, it is notable that studies examining the development of ENU-induced trigeminal neurinomas are consistent with the possibility that a percentage of early lesions regress [Swenberg et al., 1975]. Thus, a similar process could be occurring here. On the other hand, studies using MR imaging have noted that it is very

difficult to detect early lesions [Kish et al., 2001]. When serial scans are performed after ENU exposure, generally the scan before a new large tumor is found is normal. Thus, the possibility that these lesions become visible before regressing seems remote.

Considering our pathologic results, therefore, one potential explanation that would explain all these findings is that the observed tumor represents the descendants of a *recently* migrated cell, not the first one that migrated. This would in turn suggest that the SVZ is crucial in the effective promotion of initiated cells and that most of this process occurs there. It would also be consistent with our in vitro findings that SVZ cells can be immortalized when cultured at birth, but do not attain full malignancy, i.e., they have not yet been fully promoted. Recognizing that more work needs to be performed, these findings to date are consistent with the contention that much of the period of epigenetic promotion may be occurring in the SVZ.

CLINICAL IMPLICATIONS AND QUESTIONS ARISING

The proposed sequence of events described above represents a departure from the conventional view of brain-tumor development (Table I) and leads to some interesting possibilities. First, it leads to a different explanation for the histologic variability among gliomas. Thus, if the earliest tumor cell is an undifferentiated stem cell with, if not complete multipotentiality, at least the capacity of being susceptible to environmental and temporal influences, then histology would be more a reflection of the environment and time that initiation occurs than the cell of origin. In other words, the factors that determine whether a tumor ultimately becomes an astrocytoma or an oligoastrocytoma would reflect more the epigenetic effects on a nestin-+ undifferentiated cell rather than a different cell of origin.

This in turn suggests that instead of attempting to subclassify tumors based on their final appearance, we should be asking the following very important clinical question: *Does a glioma stem cell exist*? It has been known for decades that only a proportion of tumor cells are clonogenic or form tumors when xenografted [Park et al., 1971; Fidler and Kripke, 1977; Fidler and Hart, 1982; Nowell, 1986]. Two explanations can be offered for this finding, i.e., either all tumor cells have a general low probability of dividing or that most cancer cells have only a limited proliferative potential, i.e., there must be a stem cell.

The optimal way to distinguish tumor stem cells from other tumor cells is using separation techniques with selective markers to demonstrate that tumorigenicity, often equivalent to tumor production after xenografting, occurs with only a certain cell subset. These conditions have been best worked out for leukemia, where tumorigenicity is confined to particular pools of cells that can be detected using specific markers [Bonnet and Dick, 1997; Reya et al., 2001]. Its presence in solid tumors has been much more difficult to establish and scant attention has been paid to this issue in glioma.

From our work with the ENU model to date, we would predict that the glioma stem cell would be nestin-+. This is of limited help, however, since such an intracellular marker would not be helpful in planning separation strategies. Therefore, an important area for further study in this model (and for glioma in general) will be to define markers that can be used to selectively separate these cells at early stages of tumor formation. The question of whether a glioma stem cell exists is an important issue to resolve, since its presence would

TABLE I. Explanations of Common Glioma Characteristics According to Conventional and Proposed Models of Glioma Formation

	Conventional model	Proposed model
Tumor histology	Dependent on cell of origin	Dependent on epigenetic influences on common undifferentiated precursor/stem cell
Glioma stem cell	Perhaps	Yes
Centrifugal point	Intraparenchymal	SEL
Reason for incurability	Invasiveness; tumor cell resistance to treatment	SEL continued source of promoted tumor cells

imply that treatments aimed at just reducing tumor cell number without affecting the stem cell pool would ultimately fail.

Another issue that is raised from our studies on the ENU model is the sensitivity of our detection methods. Despite the sensitivity of MR scanning, early lesions that can easily be detected pathologically are not seen using conventional MR sequences such as T2 and diffusion weighted imaging. The reason for this is unclear but could be a reflection of the fact that the pulse sequences generally used are intended to pick up changes in water content and cellular density; such an approach is clearly not optimal for detecting early lesions from their pathological appearance, which suggests that their presence is associated with little cellular distortion and edema. From a clinical perspective, it would also suggest that the availability of MR imaging has given us a false sense of security in thinking we can identify all possible tumor lesions and would serve to weaken the contention that the multistage model cannot be applicable to the clinical situation in glioma because most often only one lesion is noted. The findings to date using the ENU model would suggest that further work needs to be done in this area to improve our detection capability.

Finally, the issue of why treatment of gliomas almost always ultimately fails needs to be addressed in the context of this model. As stated above, in our current brain tumor model in which tumors develop and spread centrifugally from some intraparenchymal point, this is usually explained by some inherent property of the tumor cell such as invasiveness or heterogeneity in treatment susceptibility. The model proposed here, that tumor cells are not only initiated but also promoted in the SVZ before migrating to the intraparenchymal destination from which they proliferate, forces a reconsideration of just where the true source of tumor cells lies, i.e., are they continually being produced in the SVZ and are local therapies failing because this area is not being addressed. In this regard, it is notable that many studies have demonstrated how responsive the normal SVZ cell is to growth factors placed in the CSF [Craig et al., 1996; Kuhn et al., 1996; Zigova et al., 1998; Fallon et al., 2000; Benraiss et al., 2001; Pencea et al., 2001]. That tumor formation and patient outcome might be influenced by intraventricular therapies would not be seriously considered using our conventional model, but with the present one, it would certainly merit attention.

FINAL THOUGHTS

Several years ago, Cairncross [1987] wrote a short piece in which he used several similarities between astrocytoma and chronic myelogenous leukemia to suggest that the former was also a stem cell disease. He noted, however, that the "... [hematopoietic system] ... contains recognizable self-renewing stem cells whereas gliogenesis is severely limited in the adult primate brain and true stem cells have not been found." With the identification and characterization of a CNS stem cell that persists into adulthood, however, the similarities become more compelling.

We believe it is no longer purely a speculative exercise to view glioma as a disease of "brain marrow." Furthermore, hypothesizing that glioma represents a transformation of a neural stem cell within the SVZ challenges some basic assumptions about glioma, including its "centrifugality." If verified, this model provides a framework with which to understand not only the early events of glioma but would also suggest strategies that could lead to earlier diagnosis, preventative therapies, and perhaps cures for this terrible disease.

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