

Most C6 Cells Are Cancer Stem Cells: Evidence from Clonal and Population Analyses

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Abstract

Cancer stem cells have been isolated from human gliomas and many other parenchymal tumors. It was previously assumed that many established malignant cell lines also contain a rare subpopulation of stem cells. This study was designed to investigate the fraction of cancer stem cells in the C6 glioma cell line using clonal and population analyses, rather than isolating methods, which are based on specific markers. Interestingly, in the serum-containing medium, each of the 67 single C6 cells plated per miniwell was able to generate a clone and subclones, which subsequently gave rise to a xenograft glioma in the BALB/C-nude mouse. The CD133⁻ C6 cells also possessed clonogenic, self-renewal, and tumorigenic capacities. Moreover, our findings indicated that brief exposure to Hoechst 33342 was harmful to the clonogenicity and proliferation of individual C6 cells. Therefore, the non-side-population cells may be deprived of their stem cell features in the process of Hoechst 33342 staining as a step in isolating a Hoechst-negative side population with flow cytometry. Thus, we concluded that the C6 line was mainly composed of cancer stem cells, although many of them were neither CD133⁺ nor side population. [Cancer Res 2007;67(8):3691–7]

Introduction

Gliomas consist of heterogeneous populations of cancer cells. The tumor is regarded as an aberrant organ containing a minor fraction of cancer stem cells and a major fraction of non-tumorigenic tumor cells (non-stem cells). Although both the stem cells and the non-stem cells contain the oncogenic mutations that result in tumorigenesis, the latter population lacks the capacity for perpetual self-renewal (1–3).

Recently, both the tumorigenic and nontumorigenic subsets of cancer cells have been isolated from human gliomas (4, 5) and other solid tumors (3, 6). Three methods have been introduced to isolate cancer stem cells from parenchymal tumors. First, several groups, using serum-free culture medium similar to that used to culture neural stem cells, reported cancer stem cells from gliomas or glioblastomas proliferating and forming “tumorspheres” (7–9); individual cells from the primary sphere were able to form a new sphere during serial passages, suggesting that these cells could self-renew. Second, cancer stem cells were isolated based on the expression of CD133 (10), a marker expressed by normal neural stem cells. Only the CD133⁺ cells collected using fluorescence-activated cell sorting (FACS) or immunomagnetic beads were

capable of forming spheres (11). These studies suggest that central nervous system tumors contain a subpopulation of stem cells. Third, the “side-population” (SP) in the tumor is composed of cancer stem cells (12). SP cells express ATP-binding cassette (ABC) transporters, which pump out the fluorescent nuclear dye Hoechst 33342. Therefore, they were not stained by Hoechst 33342 and could be isolated by FACS (13). Although different methods were used, studies of cancer stem cells from brain tumors still concluded that only a very small portion of the cancer cells are clonogenic, and they alone are capable of tumor propagation (1). These methods were also used to isolate stem cells from established cancer cell lines. It is unknown if this principle holds for the glioma cell line C6, which has been maintained *in vitro* over decades.

The C6 line contains only 0.4% SP cells (14). In serum-free medium, C6 SP cells, but not non-SP cells, can generate both SP and non-SP cells, and they are mainly responsible for the *in vivo* malignancy of this cell line. Therefore, only the C6 SP cells are considered cancer stem cells (14, 15). Here, however, we show that most C6 cells possess the main defining characteristics of cancer stem cells. We showed that most cells in this line generated cell clones when cultured in medium with, but not without, serum. Once the cell clones were transplanted into nude mice, they gave rise to xenograft tumors. The essential attribute of the cancer stem cell is the capacity to generate and perpetuate a tumor of its own origin. In summary, we showed that most C6 cells were cancer stem cells, whether or not they expressed CD133 or ABC transporter, and serum-free medium was dispensable in isolating and expanding cancer stem cells of glioma *in vitro*.

Materials and Methods

Cell culture. C6 glioma cells were provided by the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in serum-containing or serum-free medium at a density of 1×10^5 cells/mL. The serum-containing medium was composed of DMEM/F12 (Hyclone, Logan, UT) and 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY). The serum-free medium was composed of DMEM/F12, 20 ng/mL basic fibroblast growth factor (bFGF; Sigma, St. Louis, MO), 20 ng/mL epidermal growth factor (EGF; Sigma), and 20 μ L/mL B27 supplement (Life Technologies). Cells were incubated at 37°C with 95% air, 5% CO₂, and 100% humidity.

Preparation of single-cell suspension. For quantitative and clonogenic analysis, it is important to prepare single-cell suspensions without influencing viability significantly. The procedures were done as described previously (16, 17), with some modification. Briefly, cells were harvested, incubated in PBS buffer containing 0.05% trypsin and 0.012% EDTA for 20 min at room temperature, and triturated gently twice every 5 min using a fine polished Pasteur pipette. The suspension was centrifuged at 1,000 rpm for 5 min to collect the cells. The supernatant was removed carefully and completely. The cell pellet was resuspended in fresh medium (with or without serum). The resultant suspension was filtered through a fine mesh sieve. Cell viability was determined using the trypan blue dye exclusion assay, and the number of cells was counted using a hemocytometer.

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doi:10.1158/0008-5472.CAN-06-3912

Serial clonogenic analysis. To determine a cell's self-renewal ability, a single-cell suspension was prepared as described above (10 cells/mL), seeded into 96-well plates (100 μ L per well), and cultured in serum-containing or serum-free medium. Wells containing no cells or more than one cell were excluded, and those with only one cell were marked and checked daily under a microscope to count the number of cell clones. After 2 weeks, the clones were dissociated and cultured similarly in new 96-well plates to generate subclones.

Tumorigenicity. Subclones from the 96-well plates were transferred to six-well plates and expanded in serum-containing medium. The clonal cells (1×10^5) were then injected s.c. into the backs of 4-week-old BALB/C-nude mice (supplied by the Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai, China). Mice were treated according to the guidelines of the Zhejiang University Animal Committee. The mice were examined visually everyday. When the xenograft tumors grew to about 8 mm in diameter, the mice were sacrificed under deep anesthesia with pentobarbital. The tumors were then dissected and fixed with 4% paraformaldehyde in PBS solution. Paraffin sections were prepared following routine procedures. H&E staining and immunohistochemical staining for glial fibrillary acidic protein (GFAP) were done on the sections, which were then examined under a microscope to verify the tumorigenesis pathologically.

Immunohistochemistry. Paraffin sections of the xenograft tumors were hydrated, immersed in a solution of 3% H_2O_2 in PBS for 10 min, and blocked with rabbit serum for 1 h. The sections were then incubated with rabbit anti-GFAP polyclonal antibody (1:100; Immunon, Pittsburgh, PA), which is a marker of malignant tumors of glial origin, for 2 h at 37°C, followed by incubation with horseradish peroxidase-labeled secondary goat anti-rabbit immunoglobulin G (IgG) for 1 h at 37°C. The result was visualized using diaminobenzidine as the chromogen for 5 min.

BrdUrd incorporation assay. To determine whether the tumorsphere generated in the serum-free medium a cell clone derived from one mother stem cell (1, 11, 18) or just a mixture of many clones, we did a bromodeoxyuridine (BrdUrd) incorporation assay for three groups: BrdUrd labeled, BrdUrd nonlabeled, and mixed. Some C6 cells were cultured in BrdU-supplemented serum-containing medium for 3 consecutive days. The final concentration of BrdUrd was 3 μ g/mL. These cells were referred to as BrdU-labeled cells. The BrdUrd nonlabeled cells were cultured under the same conditions except for the supplement of BrdU. The BrdU-labeled and nonlabeled cells were harvested. A single-cell suspension was prepared for both types of cells in serum-free medium with a density of 4×10^4 cells/mL. For the mixed group, 2.5 mL BrdU-labeled cells and 2.5 mL nonlabeled cells were plated together on the 25-cm² culture plate. For the labeled and nonlabeled groups, 5 mL cell suspension was seeded separately on each plate. When the glioma spheres had expanded into hundreds of cells after 3 days, they were transferred to six-well plates containing poly-L-lysine-coated glass coverslips. After attaching to the coverslips, the spheres were fixed and immunostained for BrdUrd to determine the percentage of BrdUrd⁺ cells in the spheres.

Immunocytochemistry. Immunostaining was done as described previously (19–21), with some modification. Briefly, the cells attached to the coverslips were washed thrice with PBS and fixed using 4% paraformaldehyde in PBS solution for 30 min. The cells were then permeabilized with cold 0.5% Triton-X-100 in PBS for 10 min at room temperature and washed with cold PBS. PBS supplemented with 10% FBS and 5% nonfat milk was used as a blocking solution (30 min, 37°C). After removal of the blocking solution, the cells were incubated at 4°C overnight in primary mouse anti-BrdUrd monoclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-CD133 polyclonal antibody (1:200; Santa Cruz Biotechnology). After washing with PBS, the cells were incubated at 37°C for 30 min with horseradish peroxidase-labeled secondary goat anti-mouse or rabbit anti-goat IgG antibodies, respectively. The cells were then stained with diaminobenzidine, counterstained with hematoxylin to visualize the cell nuclei, and examined under a microscope. Triton X-100 treatment was canceled in immunostaining for CD133. Additionally, for immunostaining for BrdUrd incorporated into the genome, the cells were incubated in 1 N HCl for 30 min at 95°C, followed by washing with PBS before blocking.

Growth curve. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done to determine the growth curves of the C6 cells cultured in both serum-containing and serum-free medium. C6 cells were dissociated, adjusted to a density of 1,000 cells/mL, and seeded into seven 96-well microplates (180 μ L per well). Half of the cells (48 wells in each microplate) were cultured with serum-containing medium, and the other half were cultured with serum-free medium. A MTT assay was done daily (one microplate each time) from the second to the eighth day of incubation. The procedure was as follows. Briefly, 20 μ L MTT (5 mg/mL) was added to each well, and the cells were incubated for 4 h. The culture medium was then removed, and 150 μ L DMSO was added to each well. After shaking thoroughly for 10 min, the absorbance of each well was read in an enzyme immunoassay instrument at a wavelength of 570 nm. Because absorbance is in proportion to the number of living cells in a sample, the MTT assay reflects the extent of cell proliferation. Growth curves were drawn according to the absorbance.

Purification of CD133⁺ cells. To determine whether CD133⁺ cells in the C6 line possessed the characteristics of cancer stem cells, we eliminated the CD133⁺ cells from the C6 cell line based on the cytotoxic effect of complement activated by the immune complex. Goat anti-CD133 polyclonal antibody (Santa Cruz Biotechnology) was added to C6 cells at a dilution of 1:200. After incubation at 37°C for 2 h, fresh guinea pig serum containing complement was added to the cells at 20% volume. After incubation at 37°C for another 4 h, the supernatant was replaced by fresh medium. Then, immunocytochemistry was done to verify the purity of the resultant CD133⁺ cells, which were subsequently tested for the capability for self-renewal and tumorigenicity, following the methods described above.

Effect of Hoechst 33342 on cancer stem cells. Previously, it was reported that only SP C6 cells are cancer stem cells (14). However, the possibility that Hoechst 33342 staining itself may lead to a false conclusion was not excluded. Because Hoechst 33342 is cytotoxic (22–25), it may interfere with the clonogenicity of stem cells. Thus, to determine whether Hoechst 33342 treatment could cause differences in results, C6 cells were incubated with Hoechst 33342 (Sigma) at a concentration of 5 μ g/mL for 2 h at 37°C as described in ref. (14), collected by centrifugation, resuspended in serum-containing Hoechst 33342-free medium, and finally seeded in 96-well plates. Clonogenic and tumorigenic analyses of Hoechst 33342-treated cells were done as described above. Control cells were prepared similarly without Hoechst 33342 staining.

Statistical analysis. Data were expressed as the mean \pm SD. Student's *t* tests or χ^2 tests were used where appropriate. *P* < 0.05 was accepted as statistically significant.

Results

Most C6 cells are clonogenic and tumorigenic. In the serial clonogenic analysis, 67 clones, each consisting of 300 to 500 cells, grew out of the 67 original single cells in the serum-containing medium after 2 weeks. The clone formation rate was 100%. The primary clones were dissociated and plated into 96-well microplates to generate subclones. The subclone formation rate was 99.6%, with 464 subclones from 466 single cells. Then, 67 representative subclones were expanded and injected into nude mice to test the subclone tumorigenicity. After 10 days, a s.c. tumor was observed at each of the 67 injection sites. Typical morphologic characteristics of glioma (26), such as marked cellular and nuclear pleomorphism, excessive cell division, coagulation necrosis, and microvascular hyperplasia, were evident in the tumor tissue sections (Fig. 1A). As expected, most of the glioma cells stained GFAP positive (Fig. 1B).

In the serum-free medium, only 46 clones formed from the 73 single cells after 2 weeks. The clone formation rate was 63.0%. Interestingly, when the clone reached about 1,000 cells after 4 weeks, it ceased expanding, even if sufficient, fresh, serum-free medium was supplied (Fig. 1C). However, once the serum-free medium

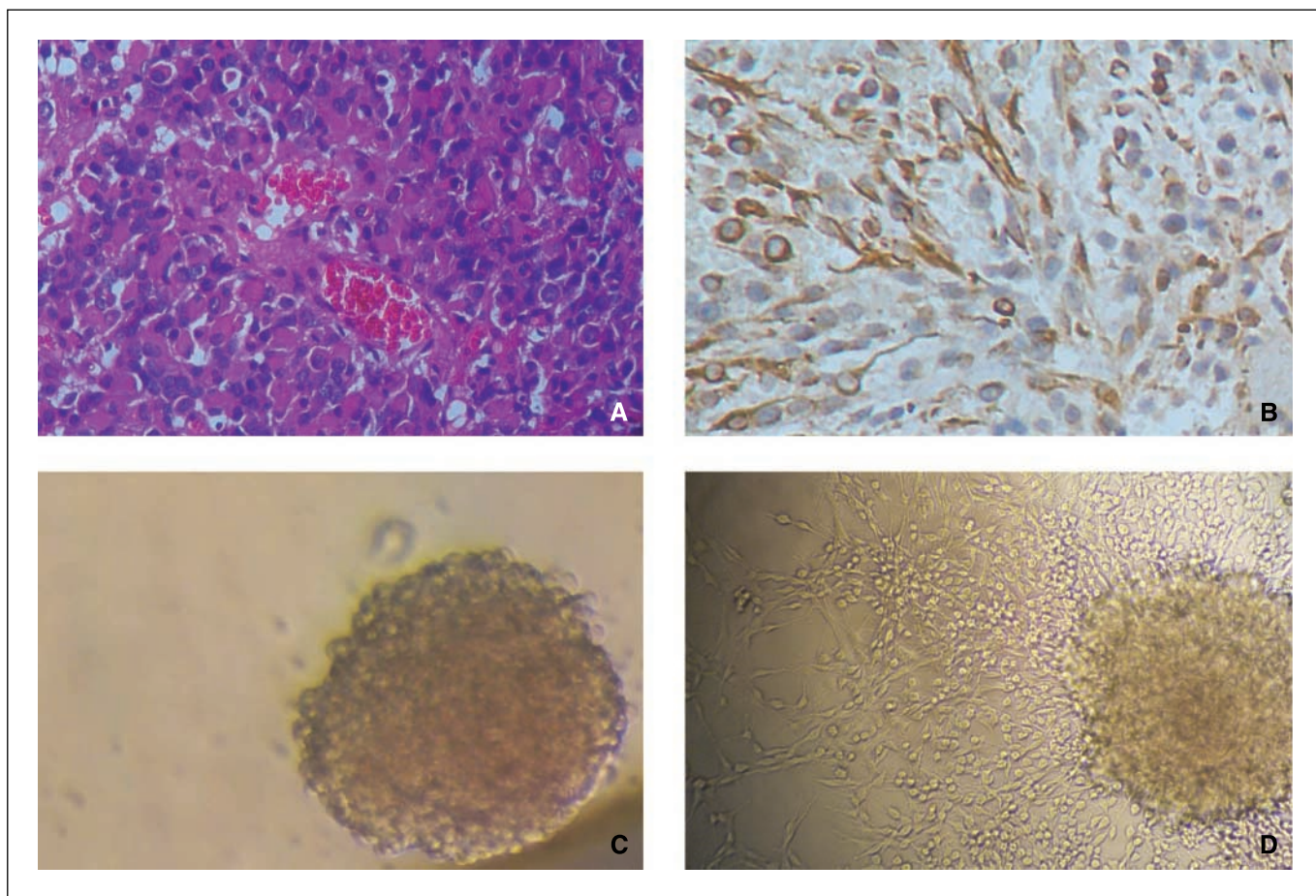


Figure 1. Clonogenic and tumorigenic capacity of C6 cells. *A*, the xenograft tumor initiating from a C6 subclone exhibited morphologic characteristics of glioma, including marked cellular and nuclear pleomorphism, excessive cell division, coagulation necrosis, and microvascular hyperplasia (magnification, $\times 200$). *B*, immunohistochemical staining indicated that most glioma cells in the xenograft tumor were GFAP positive (magnification, $\times 200$). *C*, C6 clones in serum-free medium did not expand further when they reached about 1,000 cells in size. *D*, after the serum-free medium was replaced by serum-containing medium, the quiescent clone continued to expand.

was replaced with serum-containing medium, the quiescent clones continued to expand (Fig. 1*D*). The 27 single cells that were incapable of generating clones remained quiescent but viable in the serum-free medium for at least 3 weeks; these began to divide once the medium was supplemented with 10% serum and eventually formed a typical tumorigenic clone. Thus, most C6 cells could generate clones that were capable of self-renewal and tumor generation, as long as they were cultured in serum-containing medium.

We prepared CD133⁻ cells by depleting the C6 line of CD133⁺ cells. The resultant population was verified immunocytochemically to be pure CD133⁻ cells (Fig. 2*A*), which were then examined for their ability to generate tumorigenic clones. Interestingly, 42 clones formed from the 43 CD133⁻ single cells. All of these clones also generated gliomas in nude mice. Moreover, the descendants of the CD133⁻ cells were a mixture of both CD133⁻ and CD133⁺ C6 cells (Fig. 2*A*).

Hoechst 33342 inhibited the clonogenicity of C6 cells. C6 cells were incubated in Hoechst 33342-containing medium for 2 h and cultured in 96-well plates in serum-containing medium. Only 7 of the 55 wells containing a single cell generated a clone after 2 weeks. These clones were confirmed to be tumorigenic *in vivo*. The clone formation rate was 12.7%, which was significantly

lower than that of the control (100%). Morphologically, many cells in the clones seemed fragmentary; even the viable cells exhibited a less vigorous status than the control clonal cells. Moreover, the average number of cells in clones of the Hoechst 33342-treated group was 26, whereas that of the control was 315 (Fig. 2*B*).

The glioma sphere generated in serum-free medium was not a clone. The BrdUrd incorporation analysis revealed that the BrdUrd labeling index (LI) of the mixed group was about half that of the BrdU-labeled group (mixed group: 47.6% versus labeled group: 97.2%, $P < 0.01$). If the glioma sphere generated in the serum-free medium were a cell clone, $\sim 50\%$ of the spheres in the mixed group would consist of pure BrdUrd⁻ cells. However, all glioma spheres in the mixed group consisted of both labeled and nonlabeled cells, indicating that the spheres were a mixture of more than one clone (Fig. 2*C*). This finding is consistent with a recent report (27) that showed that neurospheres were highly motile structures prone to fuse even under ostensibly "clonal" culture conditions.

If only a rare fraction of stem cells contributes to the formation of a glioma sphere, there must be three destinies for the original non-stem cells: die, persist without involvement in sphere formation, or proliferate in a monolayer form. However, we seldom found dead cells or debris in the serum-free cultures, which are

very common in primary cultures of neural stem cells. In fact, on the third day after passage, almost all cells were involved in the formation of floating spheres (Fig. 2D); neither a monolayer nor quiescent single cells were observed. On the fifth day after passage, the expanded sphere began to attach to the plate, and cells gradually emigrated (Fig. 2D). After all the spheres were detached and discarded, the leftover attached cells expanded and eventually generated new spheres (Fig. 2D), which in turn would detach by themselves and float again.

The gross population analysis provided another clue. Three days after seeding, we collected all of the spheres in a serum-free culture flask by centrifugation, dissociated them into a single-cell suspension, and counted the total number of cells as $\sim 5.2 \times 10^5$, which was somewhat greater than the original number of cells at $\sim 5.0 \times 10^5$.

Most C6 cells took part in the population expansion. The MTT proliferation assay revealed that the growth curves of the serum-containing and serum-free groups were similar in shape (Fig. 3), although the absolute value of the serum-containing group

was much higher. Both groups exhibited an exponential growth phase.

Cells in the exponential growth phase (5 days) were exposed to 3 $\mu\text{g}/\text{mL}$ BrdUrd for 1 h, cultured for another 6 h, and then fixed and stained immunocytochemically for BrdUrd. The BrdUrd LI of the serum-containing group was $19.4 \pm 2.2\%$, and that of the serum-free group was $18.9 \pm 3.5\%$ ($P > 0.05$). When the BrdUrd exposure time was increased to 3 days, the LI of the serum-containing group was $97.2 \pm 1.5\%$, and that of the serum-free group was $98.4 \pm 1.1\%$ ($P > 0.05$).

Discussion

The C6 glioma cell line has been used widely for decades. Although it has been cultured *in vitro* for thousands of passages, its tumorigenicity remains quite stable. Thus, it is logical to assume that most cells in the line are tumorigenic. Without any classification based on a specific marker, we simply did single-cell cloning culture of unfractionated C6 cells using routine

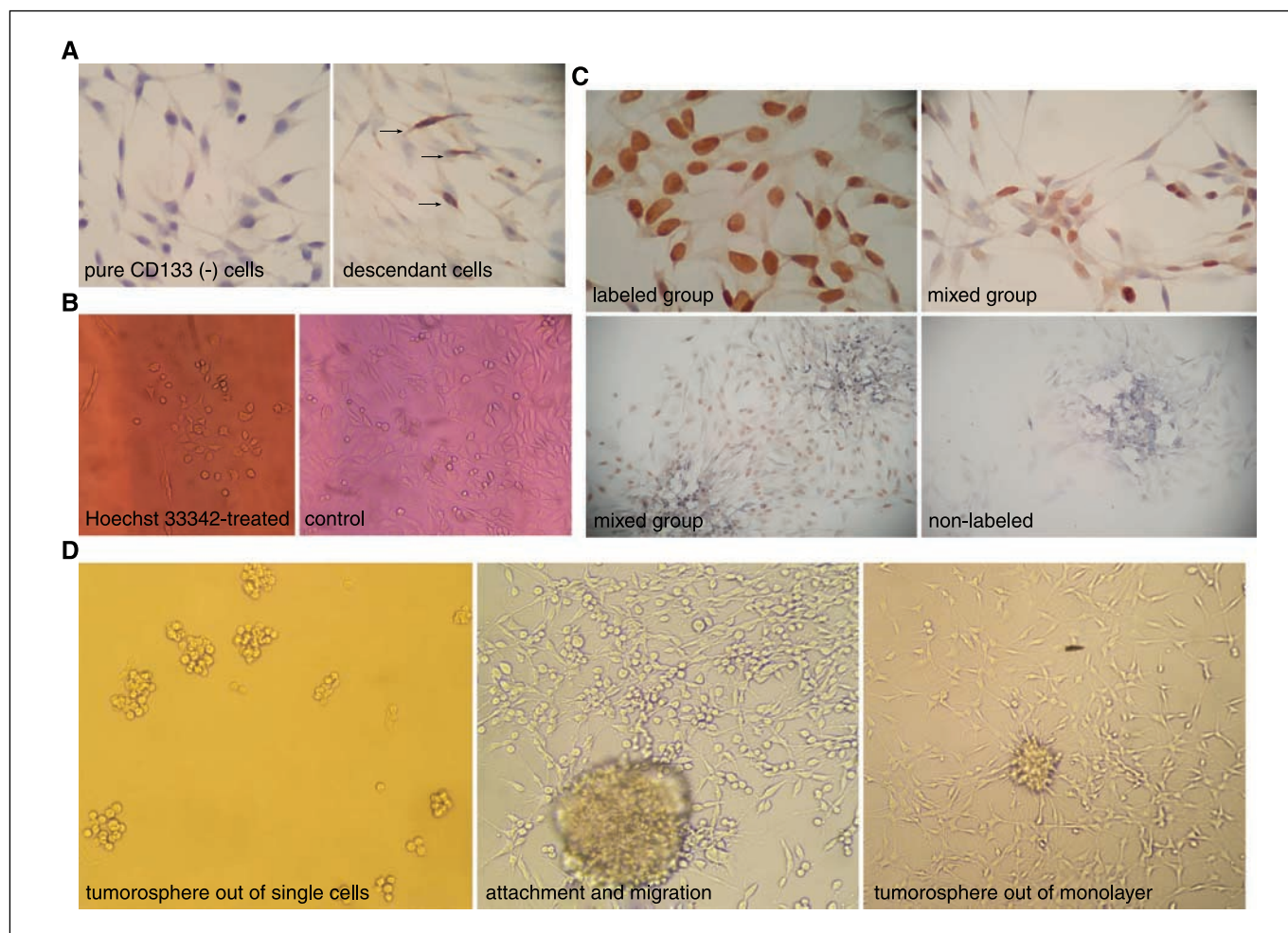


Figure 2. Results of clonal and population analyses. *A*, the C6 line was depleted of CD133⁺ cells by complement-dependent microcytotoxicity. The resultant population was verified immunocytochemically as pure CD133⁻ cells. However, their descendants comprised both CD133⁺ (arrows) and CD133⁻ cells (magnification, $\times 250$). *B*, clone originating from Hoechst 33342-treated cell contained much less cells than the control (26 versus 315 on average) after 2 wks. Many cells in the Hoechst 33342-treated clones seemed fragmentary; even the viable cells seemed to have a less vigorous status than in the control. *C*, results of BrdUrd incorporation analysis. Immunocytochemistry indicated that 97.2% of cells in the labeled group were BrdUrd positive. About 47.6% of cells in the mixed group were BrdUrd positive. All cells in the nonlabeled group were BrdUrd negative. *D*, most single C6 cells cultured in serum-free medium took part in the formation of tumorospheres. The tumorospheres expanded and attached to the plate; many cells emigrated from the attached spheres. The emigrated cells proliferated and formed a monolayer, which in turn generated new spheres.

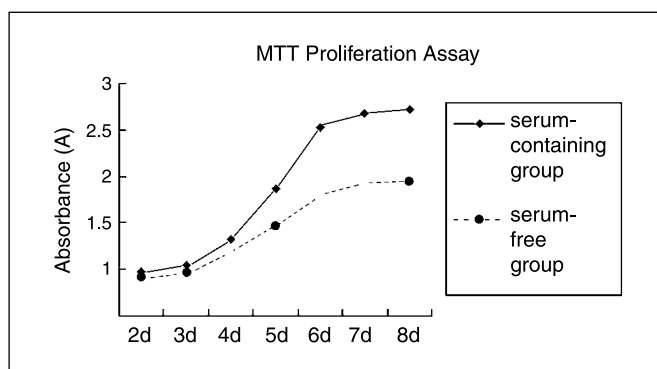


Figure 3. Results of the MTT proliferation assay. The growth curves of the serum-containing and serum-free groups were similar in shape, although the absolute value of the serum-containing group was much higher.

procedures of end-point dilution and showed that in serum-containing medium, every viable C6 cell could generate a self-renewable clone, which could subsequently give rise to glioma *in vivo*. Several definitions need to be clarified with reference to the concept of cancer stem cells, including the meaning of the word “stem.” In the context of normal development, stem refers to multipotentiality or a cell’s ability to give rise to multiple cell types of a particular lineage. In the context of cancer, it defines the tumor-forming ability of an undifferentiated cancer cell that might express normal stem cell markers without necessarily exhibiting other behaviors attributable to normal stem cells (2, 10, 28). It has been proposed that a single cell capable of giving rise to a tumor of its own origin *in vivo* should be termed a cancer stem cell (28, 29). Therefore, most viable C6 cells are cancer stem cells, according to this criterion. Consistent with this conclusion, purified CD133⁻ C6 cells also generated tumorigenic clones, and the descendants of CD133⁻ cells contained both CD133⁻ and CD133⁺ cells. Similarly, the opinion that non-SP C6 cells are not stem cells is questionable. Exposing C6 cells to Hoechst 33342 for 2 h severely affected their clonogenicity and proliferation. The flow-cytometric procedure may further harm the stained cells. It is reasonable to deduce that SP cells free of Hoechst 33342 would be less affected than non-SP cells. Therefore, the FACS process alone is sufficient to deprive most non-SP cells of their stem cell nature and results in differences in clonogenicity between SP and non-SP cells. However, we have not shown definitely that non-SP C6 cells are clonogenic and tumorigenic. Because the phenotype of the side population involved a complex mechanism (12, 30), we were unable to prepare pure and viable non-SP cells other than by using FACS.

However, the current prevailing opinion is that most C6 cells do not possess tumorigenic capacity, and only about 1%, i.e., the so-called “stem cells,” retain unlimited self-renewal and tumorigenic capacity (14, 15). There are several paradoxes to this idea, as listed below.

The stem cells can perform both symmetrical and asymmetrical cell division and give rise to both stem and non-stem cells (1). The model in Fig. 4 illustrated that the cell line cannot maintain a rare but stable subpopulation of stem cells unless the stem cells always divide symmetrically. Otherwise, the proportion of stem cells would decrease quickly and disappear. Actually, the model is based on two premises. First, most C6 cells proliferate and take part in the population expansion *in vitro*, as shown in our results. Second, stem cells cycle at lower or equal speed compared with non-stem

cells in the same culture because the stem cells are generally quiescent. If the stem cells always divide symmetrically, from where did the presumed non-stem cells originate?

In contrast to the stem cells, the non-stem cells are thought to possess very limited self-renewal capacity (1). Thus, all descendants of a non-stem cell will die within a given period. It follows logically that the immortality of the C6 line would then lie in the rare stem cell subpopulation. However, it is not clear how many times the non-stem cell and its daughter cells could divide before dying. If four times, all of the parent “non-stem cells” and their descendants would have died within a passage of 7 days. If eight times, then at least half of the population would have died within a passage of 7 days. However, we never found great cell losses in the C6 cultures unless they had been contaminated.

Another paradox is that the proportion of stem cells in the C6 line (0.4%; ref. 14) is lower than that in glioma specimens (3–5%, ref. 18; 1–25%, ref. 9). The establishment of the glioma cell line involved successive subcloning to achieve a highly homogeneous tumorigenic cell population. It is difficult to imagine that this purification process did not increase the proportion of stem cells.

Previous studies reported that conditions such as attachment, serum-containing medium, and withdrawal of mitogens (EGF/bFGF) were essential to induce cancer stem cells to differentiate into committed non-stem cells (18). Considering that the C6 cell line has been cultured in the form of an attached monolayer in serum-containing medium for several decades, how did the small fraction of stem cells maintain their undifferentiated state under this selection pressure? This is yet another paradox. These controversies may arise from drawbacks in the definition, identification, and isolation of cancer stem cells. In our opinion, most C6 cells are cancer stem cells, which seldom give rise to committed non-stem cells in both serum-containing and serum-free medium *in vitro*. These paradoxes were resolved in our opinion.

Although many exceptions have been reported, CD133 is generally considered a marker for stem cells, as is the ABC transporter, which is mainly responsible for drug resistance in C6 SP cells (31). FACS based on Hoechst 33342–negative staining is also a standard method for isolating stem cells from many sources. CD133 and the ABC transporter are important for stem cells to survive or maintain their primitive state *in vivo* (5, 14, 31–33), but this is not the case for the C6 cell line. In the *in vitro* culture system, there is no strong factor inducing the primitive cancer stem cells to die or differentiate; otherwise, no stem cells would remain in the line after decades. Thus, we deduce that in the absence of selection pressure, the expression of CD133 and ABCG2 is likely altered in the glioma cells, without the loss of their capability for self-renewal, clonogenicity, and tumorigenicity. Although this hypothesis remains to be examined, we think it is likely the case, considering the marked phenotypic and genotypic differences between primary human tumor-derived tumor stem cells and the established glioma cell lines (4, 34, 35).

Serum-free medium containing mitogens (EGF and/or bFGF) is reported to selectively favor the proliferation of cancer stem cells rather than nontumorigenic cancer cells (7, 8, 18). We showed that most C6 cells proliferated in both serum-free and serum-containing medium, although at different velocities. If serum-free medium has a selective effect on cancer stem cells, most C6 cells have been selected. In the serum-free medium, the status of living cells varied between a floating sphere and an attached monolayer. Therefore, it is illogical to assume that cancer stem cells merely take the

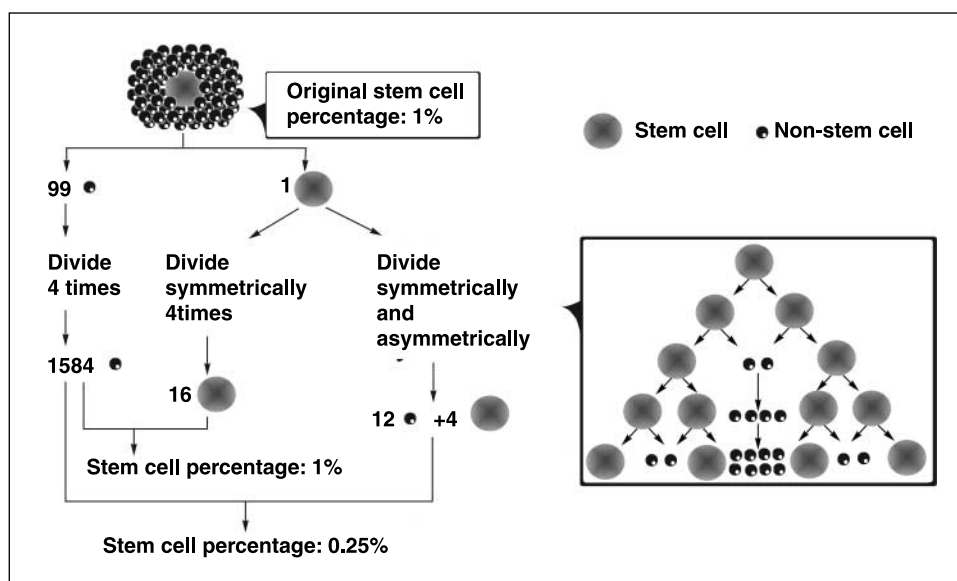


Figure 4. Model of cancer stem cell division. The model indicates that the cell line cannot maintain a rare but constant fraction of stem cells unless the stem cells always divide symmetrically.

appearance of a floating sphere because differentiation is generally irreversible. In fact, attachment is very common in serum-free culture, especially when the spheres become too large to remain in suspension. The suspension or attachment status did not seem to distinguish different populations with different capabilities for self-renewal and tumorigenicity. Furthermore, only in the serum-containing medium could every viable single C6 cell give rise to a clone. The serum-free medium was sometimes insufficient to stimulate clone formation and individual cell expansion.

Conclusions

In contrast to several previous studies, we found that the C6 glioma cell line is composed mainly of cancer stem cells. In serum-containing medium, every viable C6 cell generated a clone that was

capable of giving rise to a glioma *in vivo*. Serum-free medium was not necessary for the generation of a cancer stem cell clone. In this line, the CD133⁻ cells also possessed a cancer stem cell nature. Moreover, we showed that Hoechst 33342 is harmful to the clonogenicity and proliferation of single C6 cells. Therefore, the non-SP cells may be deprived of their stem cell characteristics in the process of Hoechst 33342 staining and FACS.

Acknowledgments

Received 10/23/2006; revised 12/12/2006; accepted 2/20/2007.

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We thank Dr. Chunlei Zhao and Dr. Zhaohui Gong for their help in linguistic editing. We are grateful to Dr. Jiaping Peng for instruction on the experiments.

References

- Singh SK, Clarke ID, Hide T, Dirks PB. Cancer stem cells in nervous system tumors. *Oncogene* 2004;23:7267-73.
- Fomchenko EI, Holland EC. Stem cells and brain cancer. *Exp Cell Res* 2005;306:323-9.
- Jordan CT. Cancer stem cell biology: from leukemia to solid tumors. *Curr Opin Cell Biol* 2004;16:708-12.
- Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA. Human cortical glial tumors contain neural-stem-like cells expressing astroglial and neuronal markers *in vitro*. *Glia* 2002;39:193-206.
- Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003;63:5821-8.
- Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* 2003;100:3983-8.
- Galli R, Binda E, Orfanelli U, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res* 2004;64:7011-21.
- Hemmati HD, Nakano I, Lazareff JA, et al. Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci U S A* 2003;100:15178-83.
- Al-Hajj M, Clarke MF. Self-renewal and solid tumor stem cells. *Oncogene* 2004;23:7274-82.
- Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature* 2004;432:396-401.
- Dirks PB. Brain tumor stem cells. *Biol Blood Marrow Transplant* 2005;11:12-3.
- Zhang M, Rosen JM. Stem cells in the etiology and treatment of cancer. *Curr Opin Genet Dev* 2006;16:60-4.
- Zhou S, Schuetz JD, Bunting KD, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 2001;7:1028-34.
- Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. *Proc Natl Acad Sci U S A* 2004;101:781-6.
- Setoguchi T, Taga T, Kondo T. Cancer stem cells persist in many cancer cell lines. *Cell Cycle* 2004;3:414-5.
- Kukekov VG, Laywell ED, Thomas LB, Steindler DA. A nestin-negative precursor cell from the adult mouse brain gives rise to neurons and glia. *Glia* 1997;21:399-407.
- Kukekov VG, Laywell ED, Suslov O, et al. Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. *Exp Neurol* 1999;156:333-44.
- Yuan X, Curtin J, Xiong Y, et al. Isolation of cancer stem cells from adult glioblastoma multiforme. *Oncogene* 2004;23:9392-400.
- Itoh T, Satou T, Hashimoto S, Ito H. Isolation of neural stem cells from damaged rat cerebral cortex after traumatic brain injury. *Neuroreport* 2005;16:1687-91.
- Ekdahl CT, Mohapel P, Elmer E, Lindvall O. Caspase inhibitors increase short-term survival of progenitor-cell progeny in the adult rat dentate gyrus following status epilepticus. *Eur J Neurosci* 2001;14:937-45.
- Ricci-Vitiani L, Casalbore P, Petrucci G, et al. Influence of local environment on the differentiation of neural stem cells engrafted onto the injured spinal cord. *Neural Res* 2006;28:488-92.
- Chen AY, Yu C, Bodley A, Peng LF, Liu LF. A new mammalian DNA topoisomerase I poison Hoechst 33342: cytotoxicity and drug resistance in human cell cultures. *Cancer Res* 1993;53:1332-7.
- Singh S, Dwarakanath BS, Mathew TL. DNA ligand Hoechst-33342 enhances UV induced cytotoxicity in human glioma cell lines. *J Photochem Photobiol B* 2004;77:45-54.
- Singh S, Dwarakanath BS, Lazar Mathew T. Role of topoisomerases in cytotoxicity induced by DNA ligand Hoechst-33342 and UV-C in a glioma cell line. *Indian J Exp Biol* 2005;43:313-23.

25. Siemann DW, Keng PC. Cell cycle specific toxicity of the Hoechst 33342 stain in untreated or irradiated murine tumor cells. *Cancer Res* 1986;46:3556-9.
26. Sanai N, Alvarez-Buylla A, Berger MS. Neural stem cells and the origin of gliomas. *N Engl J Med* 2005;353:811-22.
27. Singec I, Knoth R, Meyer RP, et al. Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. *Nat Methods* 2006;3:801-6.
28. Oliver TG, Wechsler-Reya RJ. Getting at the root and stem of brain tumors. *Neuron* 2004;42:885-8.
29. Al-Hajj M, Becker MW, Wicha M, Weissman I, Clarke MF. Therapeutic implications of cancer stem cells. *Curr Opin Genet Dev* 2004;14:43-7.
30. Patrawala L, Calhoun T, Schneider-Broussard R, Zhou J, Claypool K, Tang DG. Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2⁺ and ABCG2⁻ cancer cells are similarly tumorigenic. *Cancer Res* 2005;65:6207-19.
31. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer* 2005;5:275-84.
32. Hirschmann-Jax C, Foster AE, Wulf GG, et al. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. *Proc Natl Acad Sci U S A* 2004;101:14228-33.
33. Zhou S, Morris JJ, Barnes Y, Lan L, Schuetz JD, Sorrentino BP. Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells *in vivo*. *Proc Natl Acad Sci U S A* 2002;99:12339-44.
34. Lee J, Kotliarova S, Kotliarov Y, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer Cell* 2006;9:391-403.
35. Sibenaller ZA, Etame AB, Ali MM, et al. Genetic characterization of commonly used glioma cell lines in the rat animal model system. *Neurosurg Focus* 2005;19:E1-E1.