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Letter to the Editor

Chemotherapy resistance of glioblastoma stem cells

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Dear Editor,

Glioblastoma Multiforme (GBM) is among the most devastating cancers, with a median survival of approximately 1 year.[1] GBM presents unique challenges to therapy due to its location, aggressive biological behavior and diffuse infiltrative growth. Despite the development of new surgical and radiation techniques and the use of multiple antineoplastic drugs, a cure for malignant gliomas remains elusive.[2] The scarce efficacy of current treatments reflects the resistance of glioblastoma cells to cytotoxic agents *in vitro*.[3,4] Moreover, the short interval for tumor recurrence in glioblastoma patients suggests that tumorigenic cells are able to overtake the treatments without major damage.

The cancer stem cell hypothesis asserts that solid tumors are maintained exclusively by a rare fraction of cancer cells with stem cell properties. The existence of cancer stem cells was first proven in the context of acute myeloid leukemia.[5] More recently, this principle has also been extended to other tumors, such as breast and brain cancer.[6–8] Cancer stem cells have been reported to be the only tumorigenic population in GBM, their unlimited proliferative potential being required for tumor development and maintenance.[8] Thus, these cells should represent the primary therapeutic target in order to achieve complete eradication of the tumor.

We isolated undifferentiated GBM cells from surgical specimens (Figure 1a,b) through mechanical dissociation of the tumor tissue and culture in a serum free medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) as previously described.[7] Isolated cells were expanded and characterized both *in vitro* and *in vivo*. GBM-derived cell clones were able to grow *in vitro* in aggregates called tumor spheres and maintain an undifferentiated state, as indicated by morphology and expression of stem cell markers such as CD133 and nestin (not shown). The *in vivo* tumorigenic potential of GBM tumor spheres was assayed by intracranial or subcutaneous cell injection in immunocompromised mice. GBM stem cells were able to generate a tumor identical to the human tumor in terms of antigen expression and histological tissue organization (Figure 1c,d and data not shown). Altogether, these features of GBM stem cells indicate that they may provide a reliable *in vitro* and *in vivo* model for studying glioblastoma response to treatments. Therefore, we ought to investigate the effect of different chemotherapeutic agents on GBM stem cell survival and expansion.

To evaluate the chemosensitivity of GBM stem cells, we treated stem cell clones derived from different GBM patients with commonly used antineoplastic drugs and assessed the rate of cell death in comparison with chemosensitive Jurkat leukemic cells and primary immature erythroblast. Hematopoietic cells were used as control because they represent the primary target of therapy-related adverse effects. Specifically, Jurkat cells may represent a good general control of chemotherapeutic drug activity, while primary immature erythroblasts are the specific target of chemotherapy-induced anemia.[9] After 48 h of treatment with chemotherapeutic agents, we observed a marked resistance of GBM stem cells to all the compounds used, whereas both Jurkat cells and erythroblasts displayed high rates of cell death (Figure 2a). We then analyzed the ability of GBM stem cells to recover and proliferate following treatment with chemotherapeutic agents. Cells were treated with antineoplastic drugs for 24 h, then the cytotoxic stimuli were removed and cell number was assessed after two additional weeks of culture. As a control, we used chemosensitive tumorigenic stem cells isolated from small cell lung cancer (SCLC). After treatment with chemotherapeutic agents, GBM stem cells were able to recover and proliferate, although at levels slightly lower than untreated cells. In contrast, SCLC stem cells treated with chemotherapeutic agents showed poor recovery and a limited increase in cell number (Figure 2b left panel). These results are consistent with the mode of disease progression in GBM patients, which show scarce or absent response to chemotherapeutic treatments, invariably followed by tumor recurrence.

New delivery modalities of antitumor treatments are currently under development to obtain higher drug concentration at the tumor site. Thus, although chemotherapeutic drug concentrations utilized in our experiments are in the higher range of serum peak levels transiently reached *in vivo* during high-dose chemotherapy, we tested whether longer treatment could increase cell death induction. We observed a significant induction of apoptosis in glioma progenitors only after prolonged exposure to anthracyclines (96 h), while the commonly used etoposide and temozolomide were scarcely effective (Figure 2b right panel). Of note, we did not observe a significant alteration of the cell cycle upon exposure to chemotherapeutic drugs, suggesting that cell cycle arrest is not a major event of drug-induced antitumor activity in glioma stem and progenitor cells (data not shown).

Classical multidrug resistance is attributed to an elevated expression of ATP-dependent drug efflux pumps belonging to the superfamily of ATP-binding cassette (ABC) transporters such as ABCB1 (also known as P-glycoprotein or MDR1) and ABCG2 (Breast Cancer Resistance Protein). Drug efflux mediated by ABC transporters leads to a decreased cellular accumulation of anticancer drugs and is considered a major

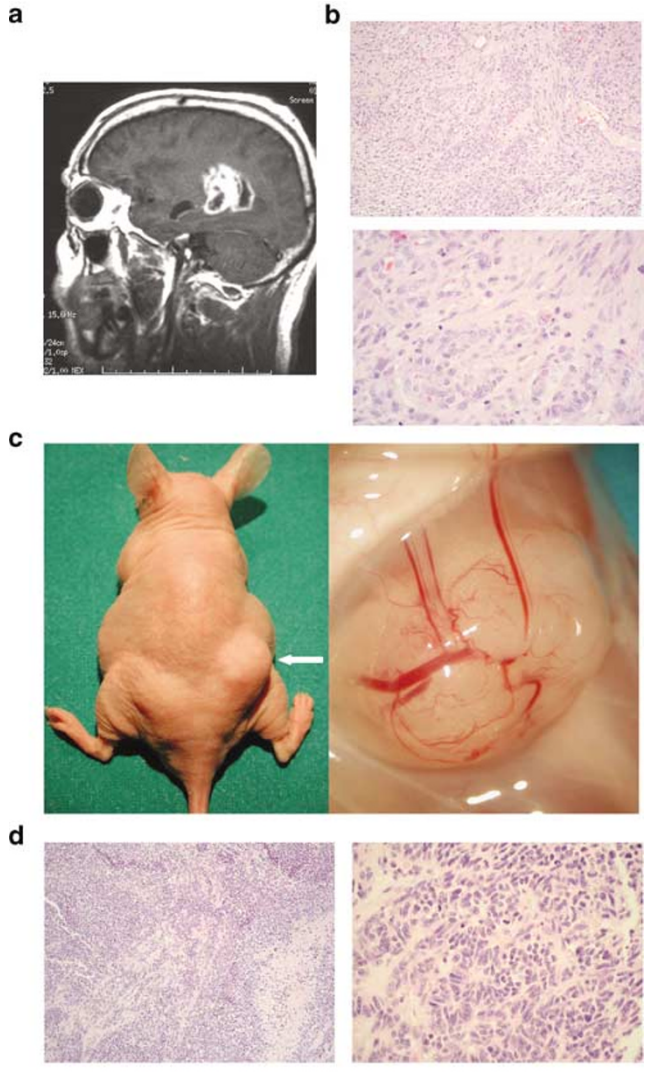

Figure 1 Stem cells derived from glioblastoma multiforme reproduce the original tumor in mice. (**a**) Brain nuclear magnetic resonance scan showing glioblastoma localization and size. (**b**) Hematoxylin/eosin staining of a histological section derived from one glioblastoma surgical specimen (upper and lower panel: 10 × and 40 × original magnification, respectively). (**c**) Subcutaneous human tumor xenograft generated by glioblastoma stem cells injection in nude mice. Surgical specimens were mechanically disrupted and recovered cells were cultured in serum-free medium containing 20 μg/ml EGF and 10 μg/ml bFGF, as previously described.[12] For generation of mouse xenografts, 10^5 cells were mixed to an equal volume of growth factor-reduced Matrigel (Beckton Dickinson, Los Angeles, CA, USA) and injected subcutaneously into one side of six week old athymic *nu/nu* mice (Charles River Laboratories, Wilmington, MA, USA). Mice were kept under pathogen-free conditions and observed daily for the visual appearance of tumors at injection sites. (**d**) Hematoxylin/eosin staining of a histological section derived from the mouse tumor xenograft (left and right panel: 10 × and 40 × original magnification, respectively)

setback of currently applied chemotherapeutic regimens. As drug efflux capacity has been associated with stem cells derived from both normal and neoplastic tissues,[10] we investigated whether chemoresistance of GBM stem cells could result from reduced drug uptake or rapid drug extrusion from the cytosol. Different clones of GBM stem cells were exposed to spontaneously fluorescent doxorubicin. Fluorescence emission was evaluated immediately after drug exposure and after drug removal followed by overnight culture. GBM stem cell clones and control SCLC stem cells displayed similar levels of drug uptake following incubation with doxorubicin. However, GBM stem cells did not show a decrease in fluorescence intensity after drug removal, indicating that doxorubicin was not actively extruded from the cell, whereas doxorubicin content of SCLC cells decreased over time (Figure 2c). In addition, confocal analysis of GBM stem cells exposed to doxorubicin showed that the drug was retained inside the nucleus, even after washing and overnight cell culture, thus excluding the possibility that drug inactivity could result from its nuclear extrusion and cytoplasmic compartimentalization (Figure 2c). This observation suggests that drug resistance of GBM stem cells is not due to reduced cellular drug concentration caused by defective drug uptake or by active drug extrusion.

Activation of the apoptotic program has been shown to be responsible for chemotherapy-induced cytotoxicity in tumor cells, while alterations in the apoptotic machinery have been related to chemoresistance in several tumor types.[11] Drug resistance observed in GBM stem cells may therefore depend on abnormalities of the cell death pathway such as overexpression of antiapoptotic factors or silencing of key death effectors. The altered expression of apoptosis-related proteins renders normal neural stem cells strongly resistant to death receptor ligands and inflammatory cytokines.[12] It is, therefore, possible that stem cells derived from brain tumors exploit similar mechanisms to escape the effect of cytotoxic drugs. Further studies aimed at understanding the mechanisms of chemoresistance in GBM stem cells are required to address this issue and might contribute to the development of new effective pharmaceutical approaches for the treatment of brain cancer.

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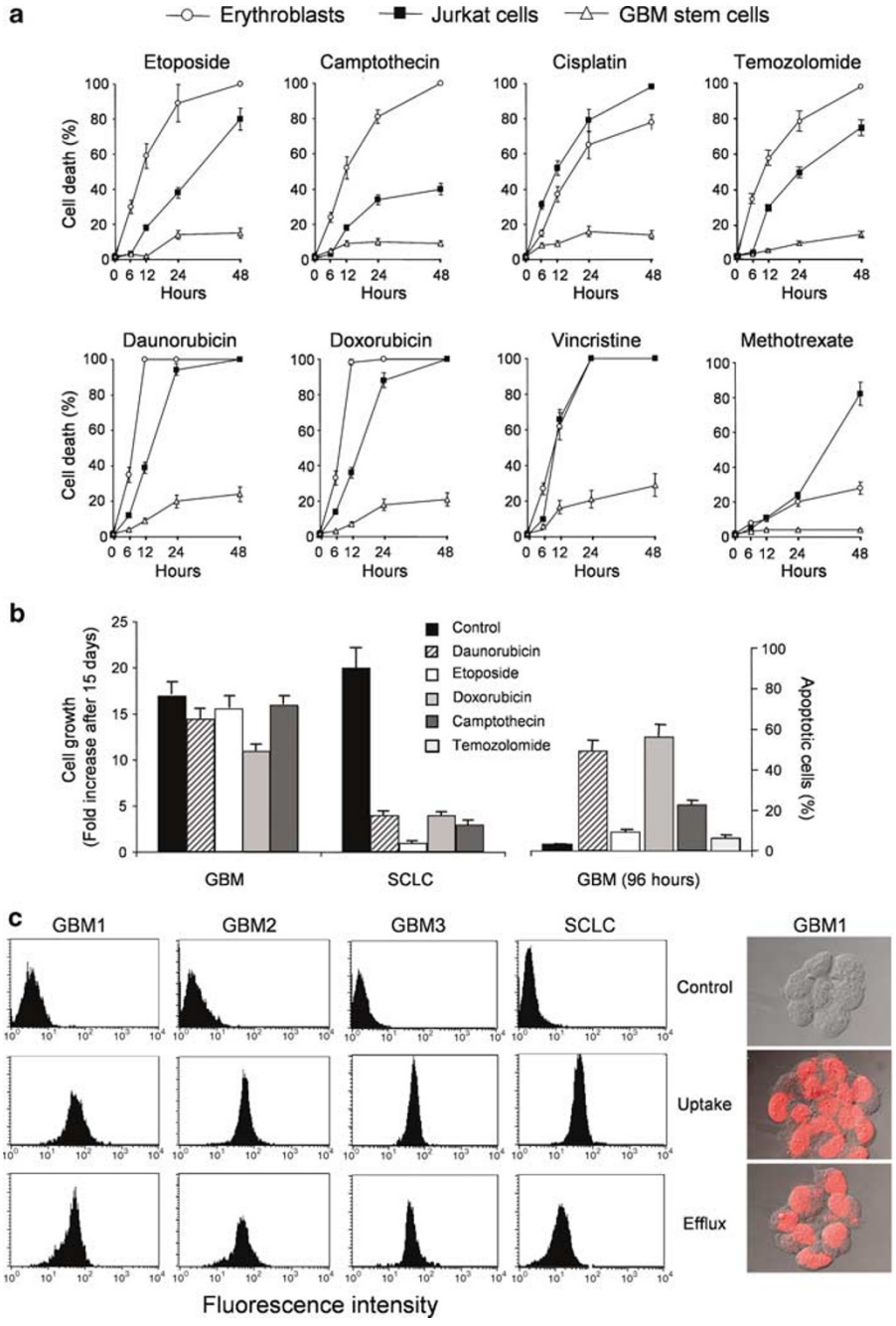

Figure 2 Glioblastoma stem cells have a high resistance to chemotherapeutic drugs that is not caused by altered drug trafficking. (**a**) Death percentage of glioblastoma stem cells (triangles), primary human erythroblasts (circles) and leukemic Jurkat cells (squares) treated for 48 h with etoposide (10 μM), camptothecin (100 ng/ml), cisplatin (5 μg/ml), temozolomide (250 μM), daunorubucin (1 μM), doxorubicin (1 μM), vincristine (0.1 μM) or methotrexate (10 μM). All the chemotherapeutic agents were purchased from Sigma (St Louis, USA), but Temozolomide (Schering-Plough, Kenilworth, NJ, USA). Cell viability was evaluated by both MTS assay (Promega, Madison, WI, USA) and cell count by trypan blue exclusion. (**b**) Left panel: Glioblastoma stem cells are able to recover after treatment with chemotherapeutic drugs. Glioblastoma stem cells (GBM) and chemosensitive small cell lung cancer stem cells (SCLC) were exposed to chemotherapeutic drugs for 24 h at the concentrations indicated above. After 24 h drugs were removed and cells were cultured for two additional weeks. Drug effect on cell growth is indicated as fold increase in cell number of treated cells versus untreated cells at the end of the culture. The experiments in **a**–**b** have been repeated three times with stem cell clones derived from six different glioblastoma patients. Right panel: Prolonged drug treatment results in increased apoptosis induction with some drugs but not with others. Glioblastoma stem cells were incubated with the indicated chemoterapeutic agents, at the same doses as in Figure 2a, for 96 h and apoptotic cells were dectected by propidium iodide staining and flow cytometry analysis. (**c**) Glioblastoma stem cell chemoresistance is not due to altered drug trafficking. Left panel: cytofluorimetric profile of glioblastoma stem cells clones from three different patients (GBM1-3) and small cell lung cancer stem cells (SCLC), untreated (Control), after 2 h of exposure to 5 μM doxorubicin (Uptake), or after 2 h of drug treatment followed by washing and overnight incubation in fresh culture medium (Efflux). Right panel: Phase contrast confocal analysis of glioblastoma stem cells treated as described above. Cells were fixed, cytospinned on glass slides for immunofluorescence microscopy and visualized with 40 $\times$ objective lens

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