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ORIGINAL ARTICLE

Functional and molecular characterization of glioblastoma multiforme-derived cancer stem cells

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Summary

Purpose: *Brain tumors are the leading cause of cancer mortality in children and remain incurable despite advances in surgery and adjuvant therapies. The failure of malignant gliomas to respond to conventional treatment reflects the unique biology of these tumors, linked to a small population of stem-like precursors. This study describes the characteristics of stem cells isolated from glioblastoma multiforme (GM) and gives insight into the mechanism of brain tumorigenesis.*

Methods: *Tumor stem-like precursors were identified from primary human GM-derived cell culture using immunocytochemistry and reverse transcription polymerase chain reaction (RT-PCR). Cells were cultured in vitro in stem cell medium supplemented with growth factors and then the capacity of the surviving stem-like precursors to form tumor spheres and to continue to proliferate after chemoradiotherapy were tested.*

Results: *The tumor cells expressed the cellular markers CD133, CD105, CD90, Nanog, Oct ¾, CXCR4, nestin, glial fibrillary acidic protein (GFAP), neurofilament protein (NF) and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Cells also displayed a high proliferative potential despite chemotherapy and irradiation and also had the ability to form spheroids in suspension.*

Conclusion: *High grade gliomas contain stem-like precursors, which exhibit neural stem cell properties with tumorigenicity, establishing a novel developmental paradigm in the study of brain carcinogenesis and providing a powerful tool to develop patient-tailored therapy for this devastating disease.*

Key words: brain cancer stem cells, high grade gliomas, in vitro characterization

Introduction

Primary malignant central nervous system tumors are the most frequent form of solid malignancy in children [1]. Some of the most common histological types of this heterogeneous group of tumors are astrocytomas (52%), primitive neuroectodermal tumors, including medulloblastoma (21%), and high grade gliomas (19%) [2]. For high grade gliomas, the average life expectancy from the time of diagnosis is only 9 months to one year, even with the most up-to-date therapeutic modalities, combining aggressive surgery followed by concomitant radiation and temozolomide chemotherapy [3,4].

Most cases of malignant gliomas have an insidious onset, with a clinical presentation consisting of headaches, seizures, syncope, papilledema, nausea and vomiting, cognitive dysfunction, motor weakness, sensory loss or speech impairment. Imaging and pathology studies indicate that these tumors arise in the cerebral hemispheres, typically at the cortical/subcortical interface, and appear to be spreading through the white matter or occasionally to the opposite hemisphere through the corpus callosum, giving rise to the appearance of a butterfly tumor. Malignant glioma tumor cells are extremely infiltrative, often migrating along the basement membrane of blood vessels or along myelinated white matter, but rarely metastasize outside the central nervous system [5].

Biological properties such as resistance to chemotherapy and radiotherapy, their infiltrative nature, pro-

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liferative behavior and progressive character strongly support the suspicion that glioblastomas contain a population of stem-like tumor initiating cells. They share many properties with normal stem cells, including resistance to toxic drugs through the expression of several ABC transporters, active DNA repair capacity, resistance to apoptosis and lack of relative quiescent cell stages.

In eradicating invasive high grade gliomas, considered incurable using the treatment modalities presently available, the identification and proper characterization of stem cell-like precursors may shed light on brain oncogenesis and assist in the design of new therapeutic strategies.

Methods

Patient data

A 55-year-old woman presented with a 2-month history of headaches, nausea, vomiting and aphasic speech. Magnetic resonance imaging (MRI) revealed a supratentorial 3-cm heterogeneous frontal lobe lesion with enhancement by contrast medium. The patient underwent frontal lobe craniotomy for resection, followed by radiotherapy and temozolomide treatment. The pathological diagnosis was anaplastic astrocytoma. After one year the disease relapsed and the patient, after developing left hemiparesis, underwent neurosurgical tumor resection. Histological examination of the resected tumor showed atypical glial cells with highly pleomorphic astrocytic nuclei, occasional multinucleated cells and gemistocytes with necrosis. The pathological diagnosis was GM. After obtaining her written informed consent, a portion of the surgical specimen was submitted for cell culture under sterile conditions.

Tumor cell isolation and culture

One hour after tumor removal, the tissue was processed as described by Oka et al [6]. The biotic material was minced with fine scissors into fragments of 1×1×1 mm^3 and cultured in 1 ml of fetal calf serum (FCS) in Petri dishes. Four hours later, 2 ml of Dulbecco's Modified Eagles Medium (DMEM) were added, supplemented with FCS, glutamine, antibiotics and non-essential aminoacids (all from Sigma Aldrich, St Louis, MO, USA). The medium was replaced every day with identical fresh medium and the explants were kept in a 37° C incubator with 95% air and 5% CO_2. After one week, a monolayer of tumor cells was formed.

Neurosphere culture

After cells were detached using trypsin/EDTA, they were reseeded in serum-free DMEM growth medium supplemented with 20 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), 4 U/l insulin-like growth factor (IGF) and B27 supplement (1:50) (Sigma Aldrich), at a density of 100,000 cells/ml in a humidified atmosphere at 37° C and 7% CO_2.

Total RNA isolation and RT-PCR analysis

Total RNA was isolated from GM stem-like cells and from placenta mesenchymal stem cells which are used as a control, being already known to express the genes of interest. RNA isolation was performed from subconfluent monolayers of adherent cells plated on 6-well dishes, using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers instructions. 1 μg of total RNA was used for reverse transcription with the ImProm Reverse Transcription System (Promega, Madison, WI, USA). Only mRNA was transformed into cDNA by using oligo-dT primers in the reaction mixture, together with: AMV reverse-transcriptase 15 u/μg; buffer solution (10 mM Tris-HCl, pH=9.0; 50 mM KCl; 0,1% Triton X-100); dNTP solution, 1 mM each; $MgCl_2$ 5mM; recombinant ribonuclease inhibitor 1 u/μl; ultrapure nuclease-free water. The cDNA was amplified using GoTaq PCR Core System II (Promega). The primers used were designed according to the corresponding human genes: CXCR4 (sense, 5'-attcctttgcctcttttgcagatata-3'; antisense, 5'-atggccaggtagcggtccagactgatgaa-3'), Oct-3/4 (sense, 5'- aggagtcccaggacatcaaag-3'; antisense, 5'-tcgtttggctgaataccttc-3'), nestin (sense, 5'-aaacagggcctacagagccagatc-3'; antisense, 5'-aattcttggttcttaagaaag-3'), GFAP (sense, 5'- tatagacaggaagcagatgaag-3'; antisense, 5'-agactccaggtcgcaggtcaag-3'), NF (sense, 5'-tgaagatggctttggatattgagat-3'; antisense, 5'-tcttcttttgcttcttcagactct-3'), and GAPDH (sense, 5'- acaactttggtatcgtggaa-3', antisense, 5'- aaattcgttgtcataccagg-3') was used as internal control for PCR. Amplification reactions were performed on a Techne TC3000 thermal cycler (Bibby Scientific Ltd, Staffordshire, United Kingdom) at 95° C for 30 sec, t° C for 1 min (t=56° C for Oct-3/4, nestin, GFAP, NF, 50° C for GAPDH and 61° C for CXCR4), and 72° C for 2 min for 40 cycles. The PCR products were then separated by electrophoresis on 2% agarose gels and photographed with an UV transilluminator.

Immunocytochemistry

High grade glioma-derived adherent cells were

labeled with anti-human antibodies and fixed with 4% paraformaldehyde for 20 min. After blocking with Bovine Serum Albumin 10% (Sigma Aldrich), cells were incubated overnight with the following primary antibodies: CD133, Oct ¾, Nanog, CD90, CD105, GFAP and neurofilament, diluted 1: 100. For cell staining with secondary antibodies, the protocols used fluorescein isothiocyanate (FITC) Goat anti-mouse IgG and IgM phycoerythrin (PE), Goat anti-mouse IgG or Texas Red Goat anti-mouse IgG. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The fluorescent cells were visualized with an inverted phase Zeiss Axiovert microscope using filters of 488, 546 and 340/360 nm.

Tumor spheres cultured alternately in serum-based medium and serum-free stem cell based growth medium

Non-adherent tumor spheres were seeded in DMEM/F12 (1:1) medium, supplemented with FCS and the cells had attached to the bottom of the flask and grown into cell monolayers for one week. The flask was washed with PBS to remove the bovine serum and defined stem cell growth medium was later added. These procedures were repeated and morphological changes in the tumor cells were observed under an Olympus CKX 41 inverted light microscope, at 100× and 200× amplification.

Colony formation assay

Human tumor cells expanded in culture to 70-80% confluency were harvested using trypsin/EDTA. Cells were counted and then diluted in complete culture medium before being plated at about 100 cells per 100-mm tissue culture dish in complex DMEM medium. Cells were incubated for 10 days at 37° C in 5% humidified CO_2, washed afterwards with Dulbecco's Phosphate Buffered Saline (PBS) and visible colonies were counted [7].

Multidrug resistance assay

To assess the resistance of cells isolated from GM to chemotherapeutics under stem-cell conditions, cells were seeded at 3,000 cells/well in 96-well plates. The culture media was DMEM/F12 supplemented with growth factors. Cancer stem cells were compared regarding *in vitro* cell proliferation with HFL human lung fibroblasts (European Collection of Cell Cultures, Budapest, Hungary) and with MLS human ovarian tumor cells (a gift from Dr Yael Schiffenbauer, MedisEl Ltd, Yehud, Israel). After 6 hours, all cells were treated with 5 μg/ml temozolomide and 5 ng/ml bevacizumab. After 24 and 48 hours, the relative cell number was determined by standard MTT assay [8].

Rhodamine 123 efflux assay

Brain cancer stem cells, MLS ovarian tumor cells and HFL human lung fibroblasts were seeded in specific culture media supplemented with FCS, non-essential aminoacids, L-glutamine and antibiotics, at 20 x 10^4 cells/ml. Cells were stained with 10 μM Rhodamine 123 and then incubated for 3 hours at 37° C and 7% CO_2. After culture, all cell types were washed 3 times with PBS before intracellular fluorescence studies, according to Donnenberg et al. method [9].

Radioresistance assay

Cells were trypsinized when near confluence and resuspended before being irradiated with 2 Gy at the Department of Radiotherapy, using a Theratron 1000 ^{60}Co source. According to current irradiation protocols, gliomas are irradiated with fractions of less than 2 Gy, usually 1.8 Gy. The only exceptions are stereotactic irradiation techniques used in neurooncology, such as Gamma Knife or Cyber Knife [10]. To ensure adequate build up and homogeneous irradiation, according to the debit of the source, we calculated the exposure times at 1 cm depth for a 10×10 field at source target distance of 100 cm. The dose rate value used was 0.65 Gy/min. Cells were irradiated in suspension instead of monolayer to avoid subjecting irradiated cells to further stress of manipulation, such as trypsinization, which may have interfered with cell recovery. During the irradiation time, control samples were kept outside the ^{60}Co source at the same temperature as the irradiated cultures. To prevent DNA repair immediately after irradiation, all cell samples were kept on ice and quickly transported to the Laboratory of Experimental Radiotherapy and Stem Cell Culture. Both control and irradiated cellular populations were further cultured in stem cell medium, in exactly the same conditions.

Statistical analysis

Statistical significance values were obtained using a one-way analysis of variance (ANOVA), with a 95% confidence level followed by Dunett multiple comparison test. Data were given as mean value ± standard error of the mean (SEM). Interpretation of the data was possible by using GraphPad Prism 5.0 statistics program (GraphPad, San Diego, CA, USA). All experiments were performed in triplicate.

Results

Pathology and MRI

Contrast-enhanced MRI showed a mass involving the right frontal hemisphere. The brightly enhancing tumor was infiltrative and had central necrosis, edema and irregular border. After surgery, the histopathological examination of the biopsy showed that the tumor was highly cellular with marked hyperchromatism and pleomorphism. Prominent vascularity with neoplastic cells palisading around an area of necrosis formed the image of a pseudopalisading necrosis, characteristic for GM (Figure 1).

Selection and expansion of stem-like cells from the human brain cancer

Tumor explants from a post-surgery specimen of GM were cultured using standard stem cell isolation protocols. Three days after plating, adherent cells were observed near the tumor explants. After another week, a monolayer of primary tumor cells was formed before being detached using trypsin/EDTA (Figure 2). Dissociated primary tumor cells were seeded for 24 hours in serum-free culture media. Most of the tumor cells became adherent, with a minority of floating cells forming tumor spheres composed of 3-5 cells (Figure 3).

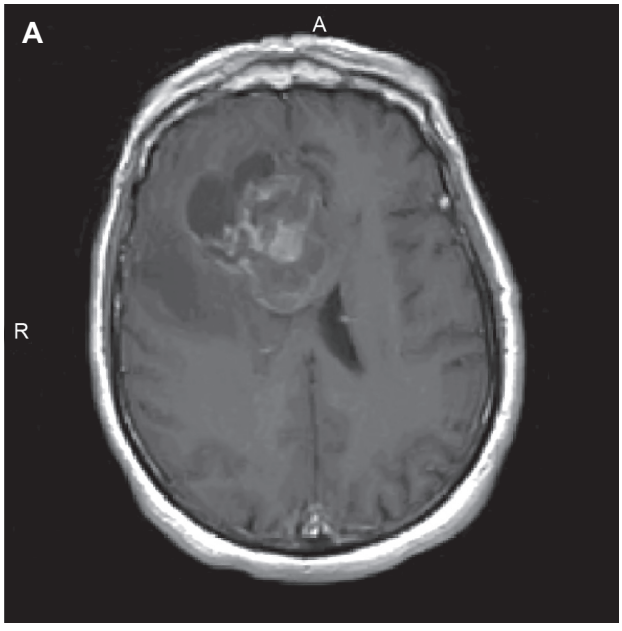
Stem cell specific marker expression

RNA from GM stem-like cells was analyzed by RT-PCR, demonstrating that cells were positive for CXCR4, Oct-3/4 and GAPDH in comparison with mesenchymal stem cells found in the placental chorion (Figure 4). We also analyzed the expression profile of some neural-related genes in the cultured tumor cells, proving that cells were also positive for CD90, CD105, CD133, GFAP, Nanog, and neurofilament (Figure 5).

Resistance to conventional chemotherapy and irradiation

Brain cancer stem cells were compared with both the human fibroblast cell line (HFL) and MLS ovarian tumor cell line. Using Bonferroni's multiple comparison test, we found statistically significant data ($p < 0.05$) between CSC vs. HFL (95% CI 0.1058 - 0.6675) and CSC vs. MLS (95% CI 0.05584 - 0.6175) (Figure 6). We also assessed the sensitivity of the cells to temozolomide and bevacizumab (Avastin) under stem cell conditions. Compared with HFL fibroblasts and MLS tumor ovarian cells, temozolomide IC_{50} values were greater ($p < 0.05$), as shown by the data analysis: CSC temozolomide vs. HFL temozolomide (95% CI, 0.6542- 0.9251), CSC temozolomide vs. MLS temozolomide (95% CI 0.6275 - 0.6584) (Figure 7).

Cancer stem cells showed little or no sensitivity to radiation, in comparison to both normal fibroblasts and ovarian tumor cells: CSC 2 Gy vs. HFL 2 Gy (95% CI 0.4176 - 0.5736) and CSC 2 Gy vs. MLS 2 Gy (95% CI 0.07842-0.6102) (Figure 8). These results support a role for these stem-like cells in brain cancer failure to eradicate progenitors resulting in tumor regrowth and death.



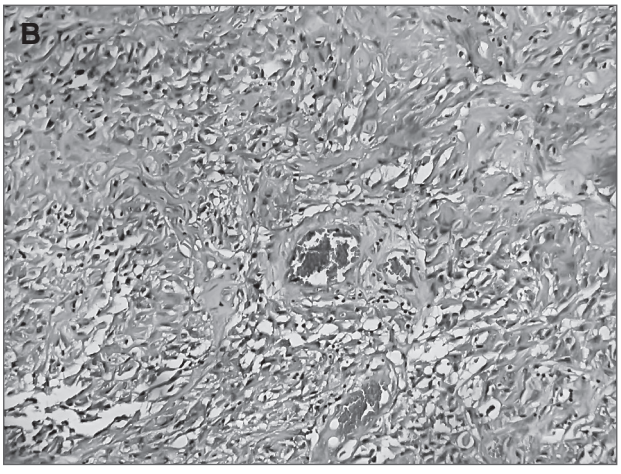


Figure 1. A: Magnetic resonance image showing a solid mass located in the right frontal lobe. **B:** Routine section shows a typical infiltrating glioblastoma multiforme.

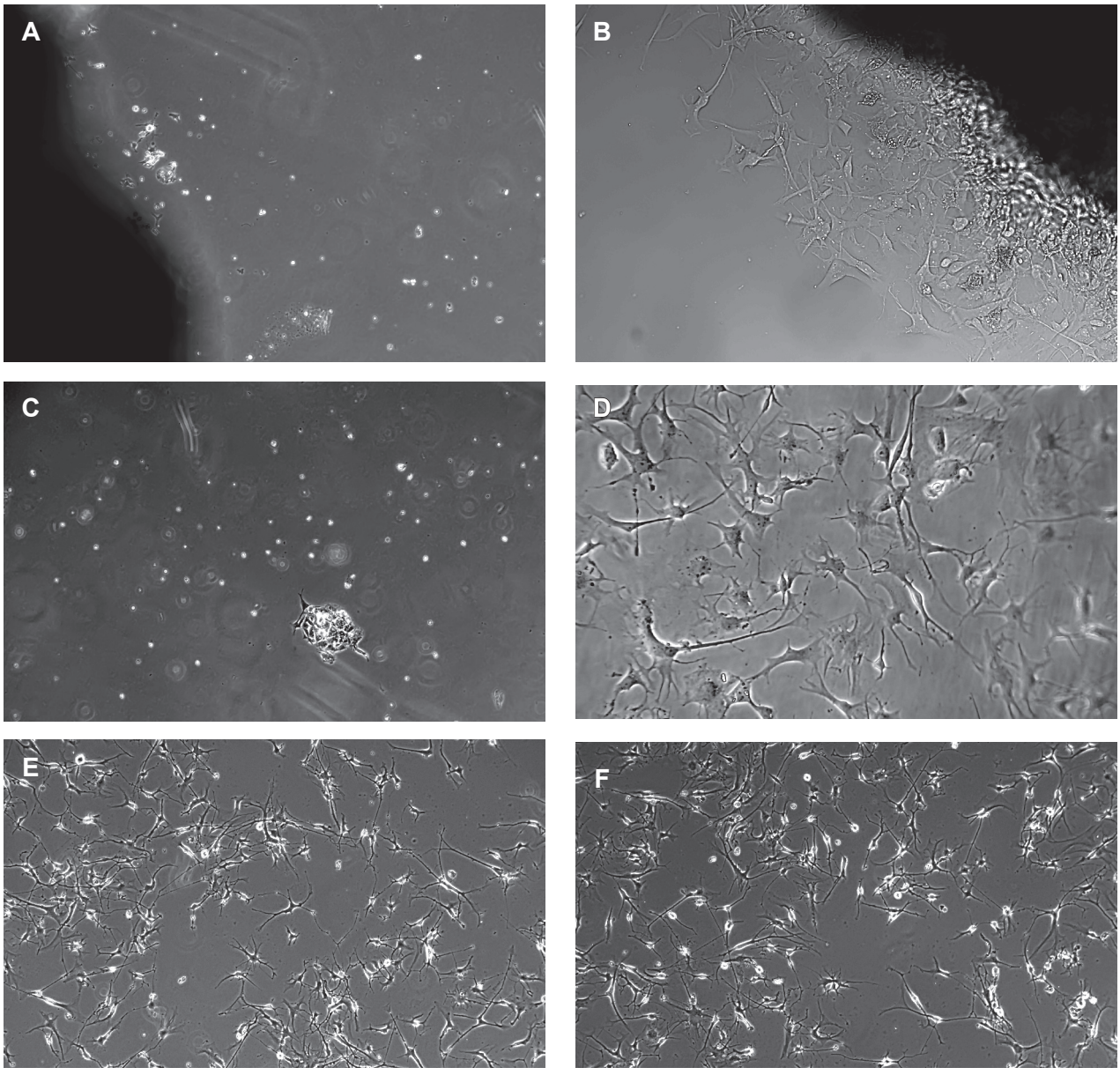


Figure 2. After 3 days tumor cells were visible near the explants (**A, B**). Cells had the characteristics of clonal expansion, also demonstrated by the colony forming assay (**C**), and showed the phenotypical characteristics of neuronal progenitors and high mitotic activity (**D, E, F**) (×100).

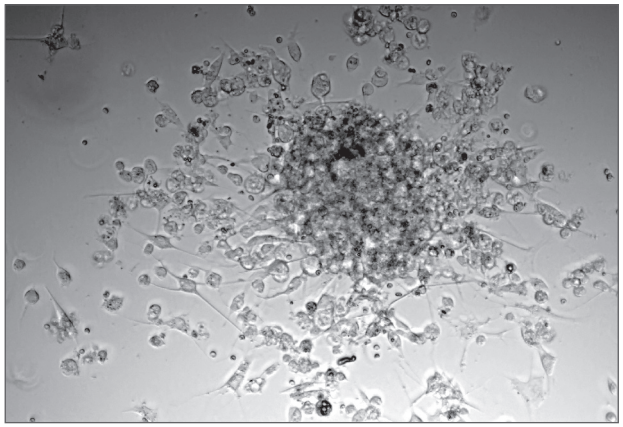


Figure 3. Neurospheres formed in serum-free cultured medium.

Discussion

Tumors of the central nervous system represent the leading cause of cancer-related death in children and the 4th leading cause in adults, with typically less than one year life expectancy after diagnosis. Glioblastomas, the most common malignant tumors, lack the ability to penetrate the basement membrane of the blood vessels and disseminate systemically, but have a high propensity to infiltrate into the surrounding healthy tissue and give rise to multifocal tumors. GM cells preferentially invade along myelinated fibres in the white matter tracts, although subpial, perivascular, perineuronal or

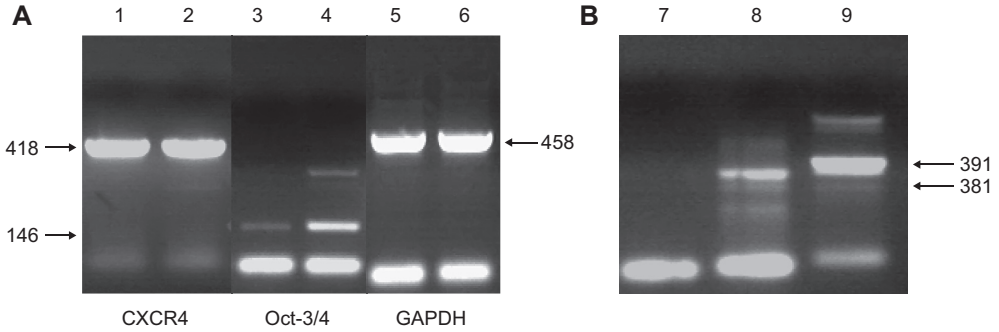


Figure 4. A: Expression levels of CXCR4, Oct-3/4 and GAPDH as determined by RT-PCR in stem-like cells from glioblastoma (lanes 1, 3 and 5) compared to placenta mesenchymal stem cells (lanes 2, 4 and 6); **B:** Expression of nestin (lane 7), GFAP (lane 8) and neurofilament protein (lane 9) in glioblastoma. The numbers near the arrows represent the size in bp of the PCR amplification products corresponding to the studied genes. CXCR4: CXC chemokine receptor 4, Oct-3/4: Octamer ¾, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, GFAP: glial fibrillary acidic protein.

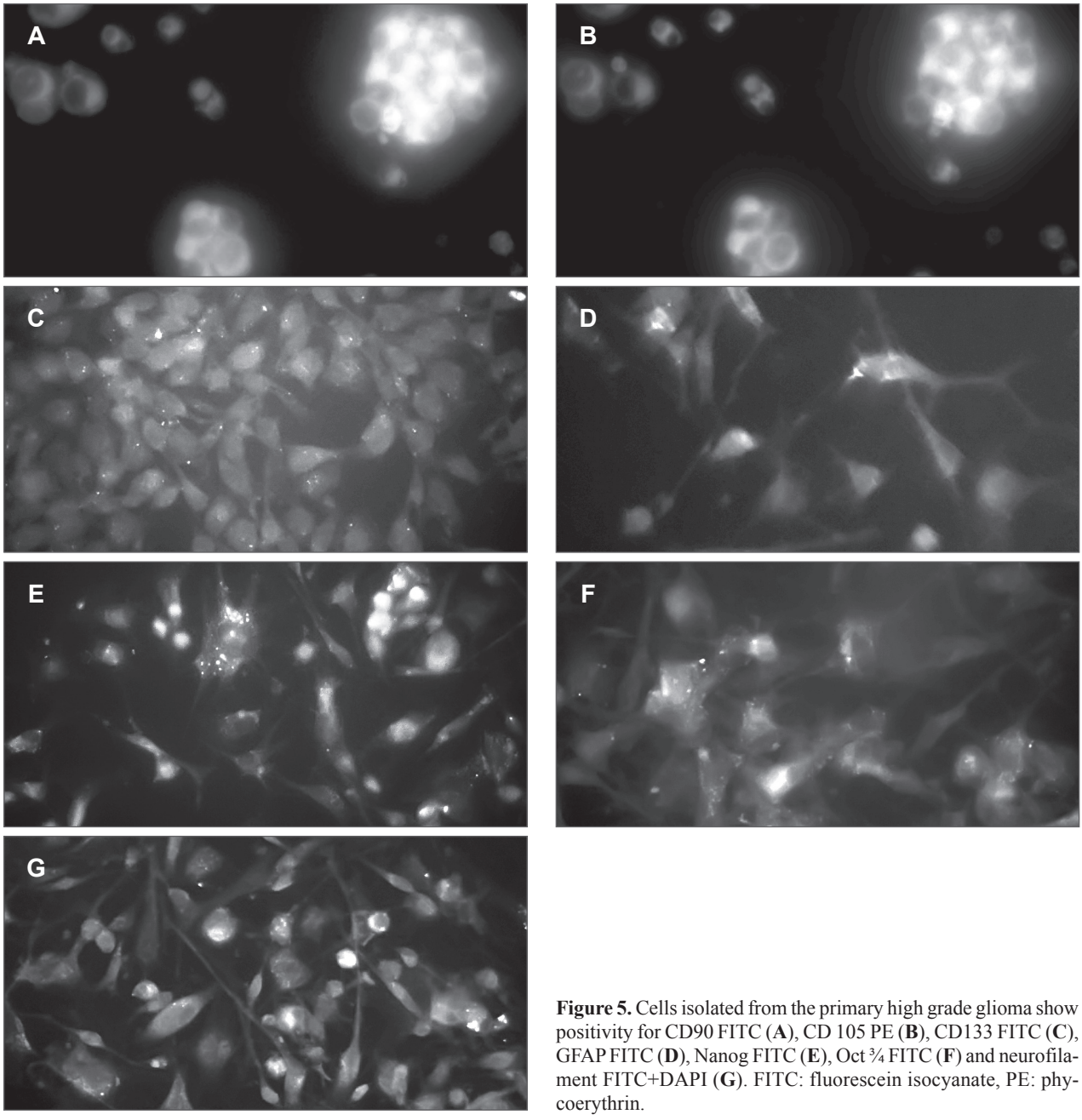


Figure 5. Cells isolated from the primary high grade glioma show positivity for CD90 FITC (**A**), CD 105 PE (**B**), CD133 FITC (**C**), GFAP FITC (**D**), Nanog FITC (**E**), Oct ¾ FITC (**F**) and neurofilament FITC+DAPI (**G**). FITC: fluorescein isocyanate, PE: phycoerythrin.

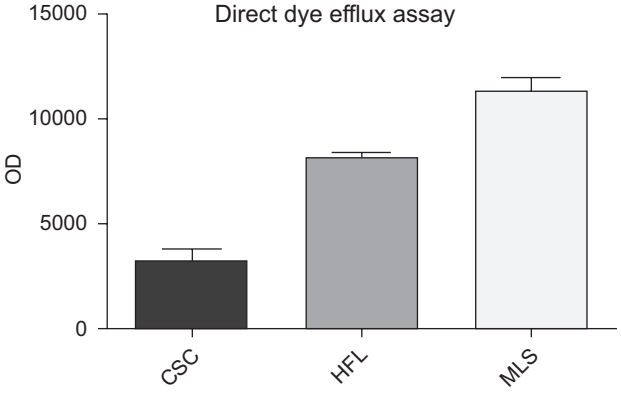


Figure 6. After culture for 3 h in a humidified atmosphere, brain cancer cells had the capability for accelerated efflux from the cytoplasm of fluorescent Rhodamine 123 using the ABC family proteins. The fibroblasts also expressed, to some extent, this characteristic but the ovarian tumor cells were not resistant, failing to eliminate the dye. CSC: cancer stem cells, HFL: human lung fibroblasts, MLS: ovarian tumor cells, OD: optical density.

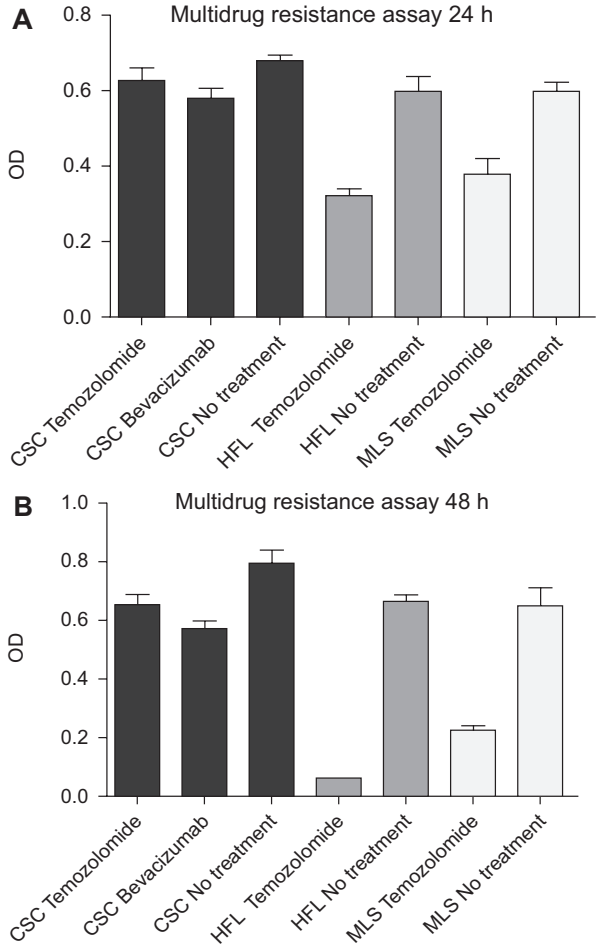


Figure 7. Glioma CSC under stem cell conditions are highly resistant to conventional chemotherapies. Cells were treated with 5 μg/ml temozolomide and bevacizumab for 24 h (**A**) and for 48 h (**B**). Stem-like tumor cells (both treated and the control group) were compared with fibroblasts and ovarian tumor cells and cell survival was determined by standard MTT assay. CSC: cancer stem cells, HFL: human lung fibroblasts, MLS: ovarian tumor cells, OD: optical density.

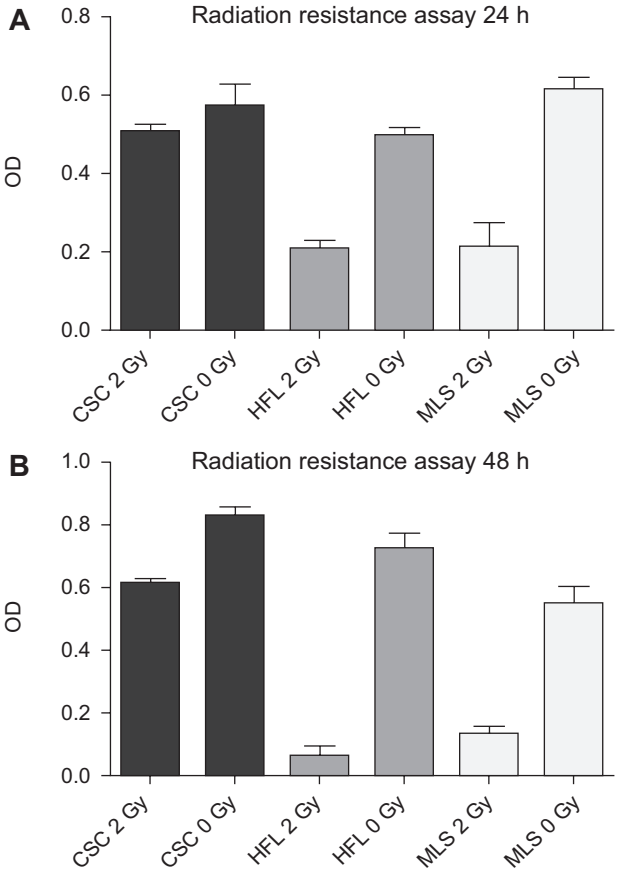


Figure 8. Glioblastoma-derived stem cells were also resistant to ionizing radiation at a dose used clinically for curative purposes in the case of low grade gliomas. Standard MTT assay was used to determine the growth rate at 24 h (**A**) and 48 h (**B**). All experiments were performed in triplicate. CSC: cancer stem cells, HFL: human lung fibroblasts, MLS: ovarian tumor cells, OD: optical density.

even the extreme example of diffuse infiltrative gliomatosis cerebri are also encountered [11,12].

Inevitably, GM recur after standard treatment with surgery, radiation and chemotherapy and are generally incurable. The overall survival for responsive patients has improved only slightly in the last 20 years because of the highly invasive behavior of these tumors, which allows the active invasion of cells from the main tumor mass into the surrounding normal brain tissue and evades local therapies, such as neurosurgery and radiotherapy. GM growth is a very complex process that involves cell detachment from its original site, modification of the receptor-mediated adhesion to extracellular matrix proteins, proteolytic degradation of the matrix and active cell movement [13,14]. The invasive growth of neoplastic cells bears a remarkable resemblance to the migration of glial and neuronal cells and their precursors during brain embryogenesis, with increasing evidence suggesting that GM arises from neural stem cells rather than from dedifferentiated mature astrocytes.

During normal embryonic and fetal development of the central nervous system the proliferation and migration of stem cells is crucial. By contrast, these phenomena are only present in a few locations in the adult brain, such as the subventricular zone, dentate gyrus, hippocampus and subcortical white matter [15,16]. The molecular biology underlying neural stem cell migration in an active area of investigation and a link between chronic inflammation, stem cells and gliomagenesis is given by the strong inducing effects of molecules such as chemokine monocyte chemoattractant protein-1, stem cell factor, stromal cell-derived factor (SDF)-1, bFGF, EGF or IGF [17-19].

Increasing evidence strongly supports the key role of stem cells in brain carcinogenesis [20]. GM cells can be grown under conditions originally designed for the isolation of normal neural stem cells, proliferate indefinitely, self-renew and be induced to differentiate along neural, astrocytic or oligodendroglial lineages, similar to normal neural stem cells. This genetically stable population of tumor cells also highly express proteins associated with stem and progenitor cells, such as CD133, CD 90, CD105, Oct 3/4, Nanog, NF or GFAP. Cancer stem cells, just like their non-neoplastic counterparts, can be maintained as neurospheres and can also generate new tumor spheres from a single cell (self-renewal).

Oct-3/4 is essential for developmental processes, such as embryogenesis or stem cell expansion, but is also expressed in various types of cancer (e.g. in testicular germ cell tumors). CXCR4 is a chemokine receptor found on numerous mesenchymal cells (including those with stem-like properties) with important roles in homing and metastasis, whereas GAPDH is one of the most commonly used housekeeping genes. Nestin is an intermediate filament protein that is expressed predominantly in stem cells of the central nervous system in the neural tube. Upon terminal neural differentiation, nestin is downregulated and replaced by neurofilaments. This situation is reflected very well in our genetic data. The GFAP gene encodes one of the major intermediate filament proteins of mature astrocytes, which is well known to be positive in immunohistochemistry studies in GM. The monoclonal antibody staining protocols described demonstrate that cells, either forming a monocellular adherent layer or in suspension as spheroids, are positive for markers specific for both the stem cells (CD133, CD90, CD105, Nanog and Oct ¾), and for neuronal progenitors (GFAP and neurofilament).

Apart from all these functional and proteomic properties, also proven by our results, what truly defines a cancer stem cell is multidrug resistance. Cancer stem cells are naturally resistant to chemotherapy through their quiescence, their increased capability for DNA repair and ABC-transporter expression. Malignant tumors have a population of drug-resistant pluripotent cells that can survive chemotherapy and regrow. The rapid relapse observed in some cancers, such as GM, at times within on cycle of chemotherapy, has a normal tissue parallel in the repopulation of the bone marrow by normal hematopoietic stem cells and the recovery of the mucosa from the gastrointestinal tract, both of which usually occur within one 3-week cycle.

In clinical neurooncology, radiation is the most successful non-surgical treatment of brain tumors. High grade gliomas usually respond to radiation treatment but subsequently recur due to radioresistant cells with extensive proliferation, self-renewal and pluripotency. Bao et al. have proven that tumor-initiating cells derived from GM surgical specimens and xenografts display resistance to radiation because of increased DNA repair capacity by activation of the DNA damage checkpoints, including phosphorylation of the checkpoint proteins Chk1 and Chk2. Both normal and malignant stem cells are able to repair double-strand breaks caused by γ-irradiation through increased activity of the ataxia-telangiectasia mutated (ATM) DNA repair pathway. Conversely, inhibition of c-src tyrosine kinase (CSK) homologous kinases (CHK), an enzyme involved in the activation of ATM, makes both normal and neoplastic stem cells more sensitive to radiation in laboratory experiments [21].

As in the case of the brain cancer cells isolated by our research team, resistance to chemoradiotherapy and the very aggressive growth and infiltration have led to little or no improvement in the management of malignant brain cancers. Thus, there is general agreement that therapies must be targeted to glioma stem cells, therapies that include gene therapy, radiosensitization by anti-epidermal growth factor receptor monoclonal antibodies, targeted oncolytic virus therapy or blockade of embryonic signaling pathways reactivated in cancers [22-25].

Conclusion

The inadequacy of standard therapies of high grade gliomas is currently being considered as a failure of the existing chemotherapeutics to target brain cancer stem cells, resulting in inevitable clinical relapse and death. As cancer stem cells are responsible for perpetuating recurrent and chemoradiorefractory disease, a great need exists for therapies that target the small percentage of tumorigenic progenitors. The much needed breakthrough in the treatment of malignant brain tumors is only possible by continuing the identification and study of cancer stem cells. Only then will early diagnostic and therapeutic strategies be able to cure brain cancer.

Acknowledgements

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