Induction of differentiation inhibits the tumorigenic potential of glioblastoma cancer stem cells

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Abstract

The outcome of the patients with newly diagnosed glioblastoma remains dismal, despite the use of surgery, radiotherapy and adjuvant temozolomide and while new agents like anti-angiogenic agents seem to offer some promise, a new approach is needed. Recent studies suggest that cancer stem cells (CSCs) may play an important role in malignant gliomas invasion and proliferation. Therefore, CSCs became new therapeutical targets, and one of the main experimental therapies which could be used against CSCs is the differentiation therapy.

The purpose of this study was to characterize the CSCs isolated from glioblastoma samples, to assess in vivo the tumorigenic potential of these cells and to induct the differentiation of the CSCs. The changes in invasive markers (matrixmetalloproteases-MMPs, cadherins and cathenins) expression were assessed. CSCs exposed to differentiation inductor factors have been inoculated in nude mice and their tumorigenic potential has been evaluated. The stemness biological feature was correlated with increased of MMPs, cadherins, catenin expression and with tumour contra-lateral invasion. The expression of MMPs, cadherins and cadherins decreased after exposure of the CSCs cultures to the differentiation inductor factors. In vivo experiments demonstrated the inhibition of tumorigenic potential of differentiated CSCs cultures.

In conclusion, differentiated CSCs showed a decreased expression of invasive markers in vitro and lost their tumorigenic potential in vivo.

Keywords: glioblastoma, cancer stem cells, xenografts, differentiation therapy.

Introduction

Malignant brain tumors remain severe diseases with a dismal prognosis despite the modern multimodal therapeutically management (5). For example in glioblastoma, the mortality is 100% and the medium period survival is approximately 12 months (27).

Embryonic stem cells could suffer several genetic mutations during specific proliferation phase of ontogenesis (12). These genetic mutations could lead to early development of various types of cancer early during childhood (8). Conversely, the adult stem cells could bear specific genetic mutation, and they can transform into cancer stem cells which secondary lead to oncogenesis (13).

Isolation and characterization of cancer stem cells (CSCs) from human

glioblastoma opened new opportunities in glioblastoma research and could offered alternative therapies for this severe disease (16; 14).

The cancer stem cells theory proposes that transformed neural stem cells could be the origin of gliomas (10). In adult brain, neural stem cells are located mainly in two important regions: hippocampus and periventricular region.

Adult human subventricular zone of brain is composed of several important layers: deep subcortical white matter, a periventricular ribbon of cells that can function as neural stem cells, a dense layer of astrocytic processes and the ependymal lining. Astrocytes from the subventricular zone exhibit a unique capacity for multipotency and self-renewal in vitro. The cancer stem cells theory proposed that tumoral transformation of the neural stem cells occurring at the periventricular zone, followed by migration of these transformed cells throughout brain parenchyma could be at the origins of gliomas (10).

Recent studies demonstrated that CSCs represent a very important tumor cell population, responsible for chemotherapeutic and radiotherapeutic resistance, because these cells secrete multi drug resistance proteins (like Breast Cancer Resistance Protein-1 (BCRP1) (6). Therefore CSCs become now one of the main attractive therapeutically targets within the multimodal management of glioblastoma (1).

According to Vescovi et al., induction of differentiation of CSCs, using BMP-4 molecule, is able to inhibit the tumorigenic potential of these cells (15). This strategy, called differentiation therapy, which has been initially used in malignant hemophaties (9; 11; 21) could represent in the future an important alternative therapy for glioblastoma.

The purpose of this study was to assess in vitro the expression of invasive molecules like MMPs and in vivo the tumorigenic potential of the CSCs after exposing the CSCs cultures to differentiation inductor factors.

Materials and methods
Cell cultures
Tumors from patients with confirmed glioblastoma multiforme (GBM) were mechanically and enzymatically dissociated and grown in neural stem cell expansion medium to generate neurospheres (DMEM supplemented with 10-20ng/ml FGF; 10-20 ng /ml EGF; 1x B27; 1xN2). U87 line was purchased from the European Collection of Animal Cell Cultures (ECACC No. 89081402).

Flow cytometry
5 X 105 cells were collected, washed twice in PBS, 0.1% BSA, and then cells were incubated for 1 h with monoclonal antibody CD133/2-PE, CD 45 (Miltenyi Biotec, Germany), O4 and A2B5. The labeled cells were analyzed using a Beckman Coulter EPICS XL flow cytometer. Ten thousand events were acquired and data were analyzed with FlowJo software. Positive cells were determined as percentages of gated cells.

Microarray experiments
Microarray analysis was performed using Agilent technology according to One-Color Microarray- Based Gene Expression Analysis, version 5.5 / february 2007. We used microarray slides Whole Human Genome Oligo Microarray with SurePrint Technology 4 x 44 K. The changes in the expression level of MMPs, cadherins and cathenins have been assessed.

Induction of differentiation of CSCs
Glioblastoma derived CSCs cultures where exposed to DMEM supplemented

with fetal serum (10%) and several known differentiation inductor factors like BDNF, NT3 and all-trans retinoic acid (ATRA) which is known as a potent inductor of differentiation of cellular precursor in several malignant hemopathies (26), at different concentration levels. The effects of differentiation of CSCs were assessed in vitro, by evaluating the morphology of CSCs cultures and microarray expression of invasive markers. CSCs exposed to the differentiation factors have been inoculated in nude mice and their tumorigenic potential has been assessed.

In vivo tumor model

Three groups including 24 nude mice, 8–10-weeks-old (Crl: CD-1-Foxn1nu; Charles River Breeding Laboratories, Germany) were used in experiments. Animals were anesthetized by intraperitoneal injection of xylazine and ketamine. Mice were held in a stereotactic frame with ear bars (TAXIC-600, World Precision Instruments) and received stereotactically guided injections over 3 min into right forebrain. First group of eight animals received 5×10^5 cells of U87 culture in a volume of 3 μl PBS. The second group of eight animals received 2×10^5 cells of glioblastoma derived CSCs culture in a volume of 3 μl PBS. The growth pattern of glioblastoma CSCs xenografts were compared with U87 xenografts. In order to assess the effects of differentiation of CSCs, glioblastoma derived CSCs cultures exposed to fetal serum (10%) + differentiation inductor factors have been inoculated (2×10^5 cells) in the third group of 8 mice. All the surgical and experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee, in accordance with Romanian governmental guidelines for ethics in animal experiments.

Histology

Mice bearing intracranial human glioblastoma xenografts were sacrificed at 21 and 28 days (first group) and at two months (second and third group) after cells implantation. Brains were removed and were fixed in Bouin solution or 4% paraformaldehide in PBS, dehydrated in ethanol, cleared in toluene and embedded in paraffin. About 6 m-thick horizontal sections were used for hematoxylin and eosin (H&E) staining. The photomicrographs were taken by digital camera (AxioCam MRc 5, Carl Zeiss) driven by software Axio-Vision 4.6 (Carl Zeiss).

Results

In vitro results

We initiated several primary cultures from glioblastoma samples using special medium for neural stem cells cultures. The medium was changed every 3 days. Cells survived in cultures but the proliferation rate was low. The cells in the cultures organized in clusters similar to neurospheres, which are the morphological hallmarks of neural stem cells (Figure 1 A, B).

Cells cultures obtained from malignant gliomas samples expressed stem cells and oligodendrocyte markers at different levels (Table 1). However, all cultures expressed neural stem cells markers (especially CD133) at higher levels compared with U87 line (Table 1, Figure 2).

In vivo results

U87 developed intracranial xenografts with a specific growth pattern. The U87 xenografts were compact, round-shape, with no sign of infiltration of surrounding brain parenchyma (Figure 3 A, B). The size of the xenografts was in accordance with literature data.

At 28 days the U87 xenografts were voluminous, occupying more then a half of cerebral hemisphere (Figure 3B), which appeared bulge and expanded at macroscopic examination. The detailed growth pattern of U87 xenografts has been described elsewhere by the authors (7, 24).
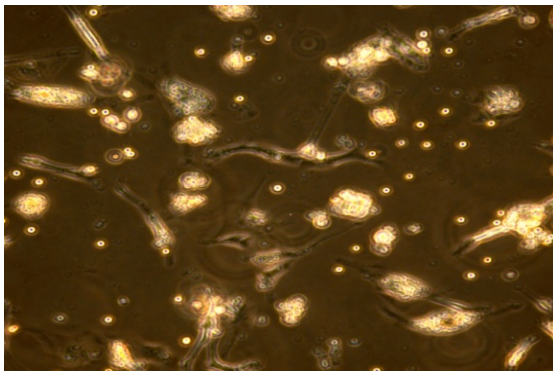
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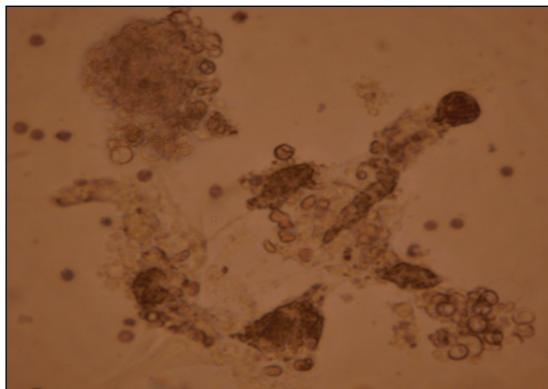
B

Figure 1 A, B Neurospheres in glioblastoma derived cells cultures grown in special stem media: DMEM: F12 supplemented with 10 ng/ml FGF, 20 ng/ml EGF, N2, B27, at 370C (Ob 20X)

TABLE 1

Expression of neural stem cells markers (CD133, O4) and oligodendrocyte markers (A2B5, O4) in cells cultures

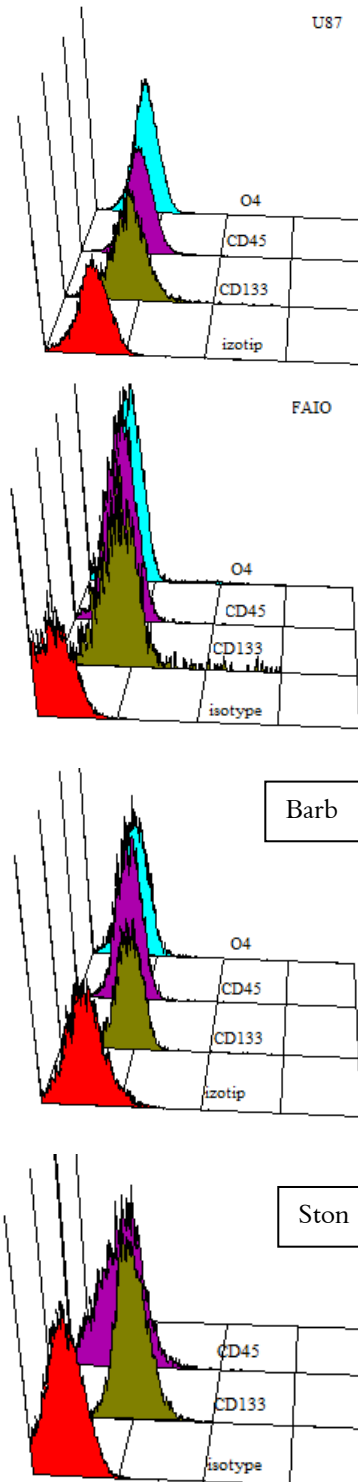| Culture	CD133	O4	A2B5
U87	2,56	5,51	20,24
STON	39,57	2,09	13,97
FAIO 10	29,82	3,84	70,38
BARB8	12,25	0,86	12,56

Figure 2 Expression of stem cells markers CD 133, CD 45 and O4 in cells cultures

Figure 4 CSCs xenograft infiltrating the corpus callosum (arrow) in one specimen (a). Voluminous CSCs xenograft which infiltrates the opposite emisphere through the corpus callosum, in another specimen (b). H&E staining
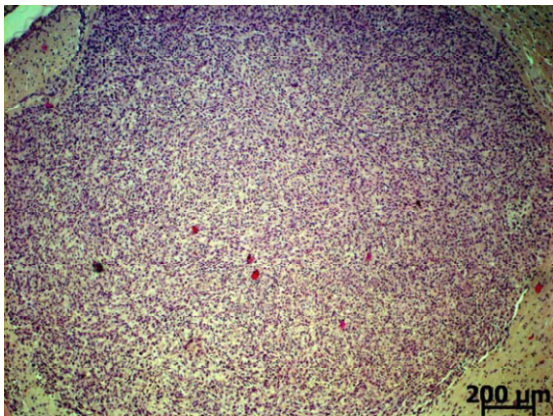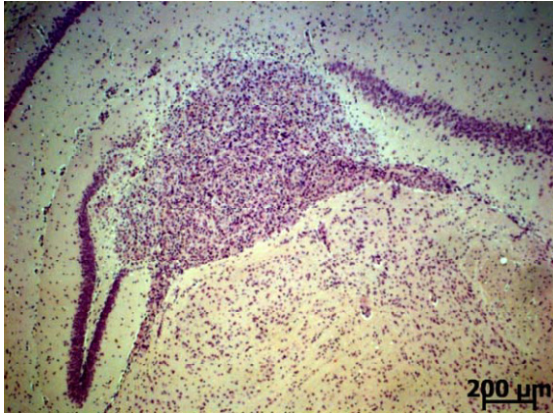
Figure 3 U87 xenograft at 21 days (a) and 28 days alter inoculation. H&E staining

The glioblastoma derived CSCs induced xenografts with a different growth pattern. Unlike U 87 xenograts, the CSCs xenografts exhibits a more infiltrating pattern (Figure 4).
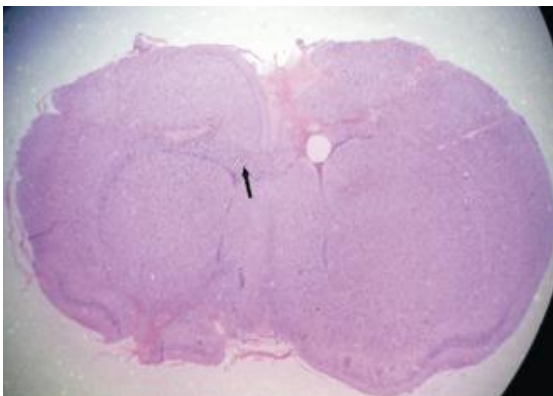

Interestingly, migratory tumor cells were found at distance from the CSCs xenografts, infiltrating surrounding brain parenchyma (Figure 5 A) or even the white commissural fibers (Figure 5 B).

Induction of differentiation of CSCs – results

Important changes in cultures phenotype has been noticed at approximate 24-48 hours after exposure of the cells cultures to fetal serum and differentiation inductor factors. Tumor cells lost their ability to form neurospheres and acquired a fibroblastic phenotype very similar to the morphology of standard glioblastoma lines (Figure 6).

The CSCs cultures express MMPs, cadherins and cathenins at higher level (up to ten times) compared with the fibroblastic aspect of the same cultures after exposure to the differentiation inductor factors (microarray studies) (Figure 7).
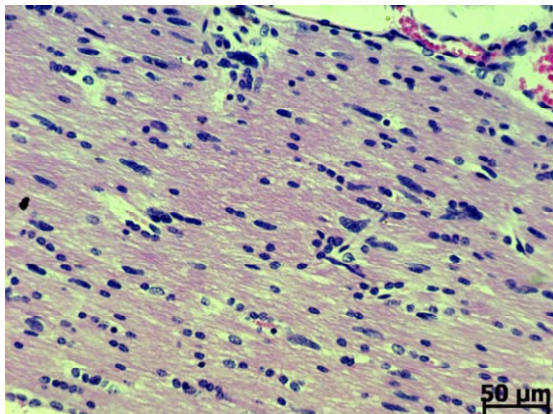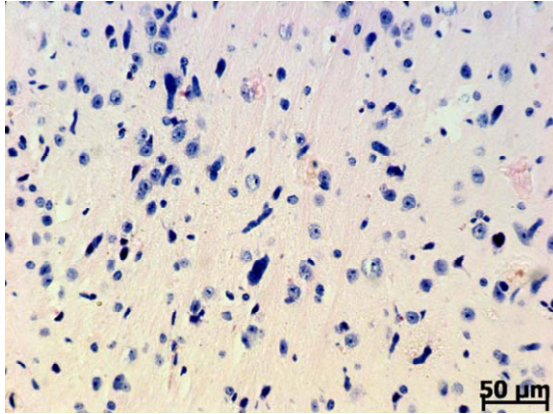
Figure 5 Tumor cells located near the CSCs xenograft (arrows) infiltrating the hippocampus (a). Migratory tumor cells (arrows) distant located from the CSCs xenografts, in corpus callosum, migrating to the opposite hemisphere (b). H&E staining
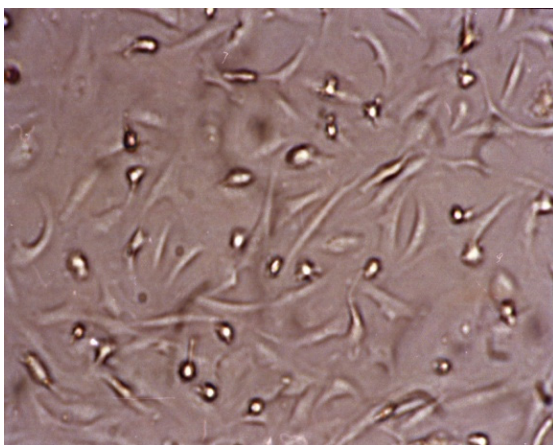

Figure 6 The fibroblastic phenotype of the CSCs after exposure of the cells cultures to the differentiation inductor factors
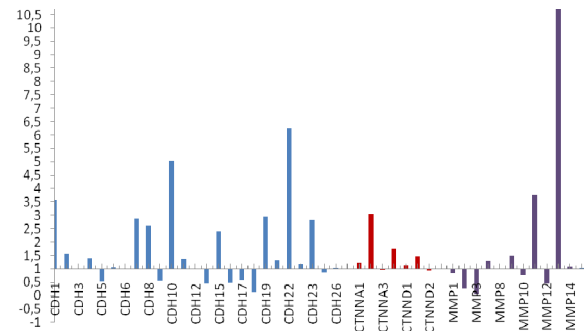

Figure 7 MMPs (purple), cadherin (blue) and cathenins (red) expression in CSCs cultures before and after exposure of the cell cultures to the differentiation inductor factors

Discussions

Initiation of CSCs cultures

We observed formation of cells clusters similar to neurospheres at approximately 14-21 days after initiation (Figure 1 A, B). Neurospheres could be passaged multiple times by mechanical dissociation of large spheres and reseeding in fresh proliferative medium every 2–3 weeks. Some of the initiated cultures formed the free-floating structures generated by these cells in vitro, the "neurospheres", considered to be a characteristic feature of tumor neural stem cell, which were morphologically and functionally heterogeneous.

The expression of neural stem cells markers (CD133, O4) were assessed by flow-cytometry analysis. All the newly initiated cultures expressed high level of neural stem cells markers compared with U87 cultures (Table1, Figure 2). Among several markers considered to be specific for neural stem cell and CSCs isolated from glioblastoma, CD 133 was the first and the most used (17, 23). Despite of several published evidence that identified glioblastoma derived CSCs population negative for CD 133 (3, 20), this marker remain one of the most used for identifying

glioblastoma derived CSCs. Our glioblastoma derived cultures cells expressed high level of CD133 compared with U87, indicating the presence of a large population of CSCs cells. The using of serum-free culture (neurosphere assay) allowed the selection of CSCs containing subpopulation that were able to reproduce original tumor aspect in orthotropic xenografts.

CSCs xenografts

CSCs xenografts exhibit specific growth pattern, very different from the compact growth pattern of the U87 xenografts. At two months after inoculation, CSCs induce usually smaller xenografts (Figure 4 A) compared with 28 days U87 xenografts (Figure 3 B), but with more extensive infiltrating pattern. However there were also CSCs xenografts with a volume comparable with U87 xenografts (Figure 4 B). U87 xenografts have a compact growth pattern with no obvious signs of brain infiltration. Even if the xenografts reached considerable volume, tumor developed only in the ipsilateral cerebral hemisphere, at the inoculation area. In contrast with the U87 xenografts, CSCs xenografts were highly infiltrating. The final behaviour was to infiltrate the opposite cerebral hemisphere, through the corpus callosum or dorsal hippocampal comissure (Figure 4 A, B). This infiltrating growth pattern seems to be specific not only for glioblastoma but also for anaplastic astrocytoma derived CSCs xenografts (4). When higher magnification was used, we could notice tumor cells with migratory features infiltrating not only the surrounding brain parenchyma, but also the white commissural tracts, explaining the infiltration of the tumor into the opposite hemisphere. These findings, which suggest that CSCs could drive the invasive

phenotype of malignant gliomas, are supported also by the in vitro results. CSCs cultures expressed at higher levels (up to ten times) MMPs and cadherins, compared with the differentiated cells cultures (Figure 7). MMPs and cadherins are important markers associated with cells invasion; therefore the high expression of these molecules could explain the infiltrating growth pattern of the CSCs xenografts. According to Beadler et al., the infiltrating glioblastoma cells and migratory precursor neural cells used the same molecular mechanisms (2). One of the main molecular mechanisms is the LIS-1/dynein complex (18). This molecular mechanism could be used also by glioblastoma derived CSCs. On the other hand, according to our previously reports, there are also other molecular markers potentially involved in CSCs migration, like VCAM-1, which could explain the high migratory feature of malignant gliomas CSCs xenografts (25).

Induction of differentiation

In order to see how the differentiation of CSCs will influence the expression of invasive markers and their tumorigenic potential, CSCs cultures were exposed to a combination of fetal serum (10%) and differentiation factors like BDNF, NT 3 and ATRA. The first change to be noticed was in the morphology of the cells. Thus, the neurospheres become smaller and eventually disappeared in 24-48 hours, and the cells exhibited a fibroblastic phenotype and adhered to the bottom of the culture dish (Figure 6). The expression of MMPs, cadherins and cathenins, evaluated by microarray experiments, decreased after exposure of the CSCs cultures to the differentiation inductor factors (Figure 7). Important to mention that in vitro changes was noticed only when the differentiation

factors (BDNF, NT3 and ATRA) were used together with fetal serum.

Differentiated CSCs with fibroblastic phenotype were not been able to induce the formation of tumor xenografts in mice brain. Therefore in vivo experiments demonstrated the inhibition of tumorigenic potential of CSCs cultures exposed to the differentiation factors.

MMPs and cadherins are key molecules involved in tumor invasion (19; 22). The marked decreased in the MMPs and cadherins expression after exposure to the differentiation factors could explain the lost of tumorigenic potential of cells cultures.

These findings suggest that differentiation therapy, which target CSCs, could be successfully applied in glioblastoma. However, these are only preliminary results and further studies are needed in order to find the optimal combination of molecules able to induct a strong and irreversible differentiation of CSCs and to block the proliferation and invasion of malignant gliomas cells.

Conclusions

Serum-free culture allowed the selection of a subpopulation containing CSCs with increased tumorigenic potential. When exposed to a specific combination of differentiation inductor factors, CSCs cultures showed a marked decrease of expression of invasive markers (MMPs and cadherins) and lost their tumorigenic potential. Our findings confirm the potential of differentiation therapy as a novel experimental therapy for infiltrative glioblastoma and further studies should be performed in order to assess the clinical potential of differentiation inductor molecules as a novel therapeutically agents in glioblastoma.

Abreviations

ATRA: all - trans retinoic acid
CSCs: cancer stem cells
H&E: hematoxylin and eosin
MMPs: matrixmetalloproteases
VCAM-1: vascular cell adhesion molecule 1

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