Folia Neuropathologica

Imperfect oligodendrocytic and neuronal differentiation of glioblastoma cells

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Folia Neuropathol 2010; 48 (1): 27-34

Abstract

Previously, we have reported that glioblastoma (GBM) cells can be differentiated into cells showing neuronal, glial and non-neural (mesenchymal) phenotypes. Before the differentiation the GBM cells co-expressed GFAP, CD44, Beta III tubulin, MAP2, Vimentin, Nestin and SOX-2, whereas during the exposure to a neural differentiation medium the differentiation process was arrested at the early stages and the GBM cells presented features of four phenotypes: multi-lineage, non-neural (mesenchymal), intermediate of neuronal cells and glial cells.

Currently, we decided to check if changes in expression of: TH (tyrosine hydroxylase, marker of catecholaminergic cells) and GABA (neurotransmitter of GABAergic neurons) and markers of oligodendrocytic cells (O4, CNP) occur during the exposure of GBM cells to the differentiation medium.

After exposure to the PDGF alpha and thyroid hormones (oligodendrocytic differentiation medium 10-30 days) features of oligodendrocytic differentiation were presented by 0.2-2.4% of analyzed cells.

During the prolonged neural differentiation (GDNF, bFGF 20-30 days) only few cells showed expression of GABA. Moreover, in our cell cultures, there were not cells expressing markers of catecholaminergic neurons – TH.

Our work confirmed that the neuronal differentiation of GBM was inhibited at the stage of the neuronal intermediate phenotype. Moreover, we showed that the oligodendrocytic differentiation of GBM cells is very inefficient.

Key words: GBM, oligodendrocytic differentiation, neural differentiation, differentiation arrest, EGFR.

Introduction

Glioblastoma multiforme (GBM) is the most common and the most malignant of all glial tumors. It represents the leading cause of cancer death in adults with primary brain tumors, and the mean survival of GBM patients remains about 1 year. For better understanding of the invasive nature of the GBM and developing more effective therapies, it is important to evaluate differentiation ability of the GBM cells.

In cells isolated from high-grade gliomas, features of variable differentiation have been reported before [4,5]. More recent studies have demonstrated that the GBM cells differentiate not only into neuronal and glial cells but also into non-neural (mesenchymal) cells [8]. Furthermore, it has been shown that the

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neuronal differentiation of GBM was inhibited at stage of the neuronal intermediate phenotype, whereas mesenchymal differentiation was more advanced [10]. Up to now, differentiation features of GBM cells based on their ability to synthesis of known neurotransmitters were not described. Moreover, our previous work did not describe features of oligodendrocytic differentiation of GBM cells [10]. However, markers of oligodendrocytic cells were detected in GBM cells, and even GBM with oligodendrocytic differentiation is recognized by current classification of brain tumors [6].

To this end, we studied the neural differentiation characteristics of the GBM cells, especially capability of differentiation into TH-positive, GABA-positive and oligodendrocytic cells (O4+, CNP+).

The discovery of such differentiation properties might help in controlling this process and might provide considerable information to developing new therapeutic strategies.

Material and methods

Formation of GBM aggregates

Tissue samples were obtained from patients with GBMs treated in the Department of Neurosurgery of Medical University of Lodz, Poland. All samples were collected under protocols approved by Medical University of Lodz. The tumor cells were dispersed by means of collagenase type IV (20 U/mL, 37°C). Subsequently, the cells were for cultured 12 hours in expansion medium. Twelve hours later, the medium was changed to the serum-starvation medium, and aggregates were isolated after 1-4 days of incubation. For each tumor 20-40 aggregates were tested.

Propagation and characterization of GBM aggregates

The aggregates were isolated and transferred into cell culture dishes covered with Matrigel (BD Discovery Labware, Bedford, MA) and cultured in neural differentiation medium: DMEM/F12 supplemented with N2 (10×). After 12-24 hours of incubation, it was observed that cells were released from the aggregates. Then, the aggregates were gently removed by means of a 1-mL pipette and the cells which migrated out of the aggregates were left on the dish for further experiments. The aggregate-derived cells were immunocytochemically stained after 12-24 hours and at 5, 10, 15, and 20 days of growth. The experiments presented in this paper were performed after three to six transfers of the aggregates into new medium (no longer than five weeks of propagation).

Immunocytochemistry

Immunocytochemistry assays for double- or tripleimmunofluorescent labeling were performed. For immunofluorescence studies, cells were grown on tissue culture chamber slides or for single cell assay experiment, in 16-well chamber slides (Nunc). The cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature and blocked with 2% donkey serum in PBS for 1 hour at room temperature. For double or triple immunolabeling, fixed cells were subsequently incubated with appropriate primary antibodies (Table I) for 1 hour at room temperature. Double- or triple-labeling was achieved by simultaneous incubation with a combination of species-specific fluorochrome-conjugated secondary antibodies (1 hour, room temperature). For double immunolabeling, a mixture of donkey anti-rabbit AlexaFluor®488 (dilution 1 : 250) and donkey anti-mouse AlexaFluor®594 (dilution 1 : 250) antibodies (Molecular Probes) were applied. For triple labeling, the following combination of antibodies was used: donkey anti-rabbit AlexaFluor®488 (dilution 1 : 250), donkey anti-mouse AlexaFluor®594 (dilution 1 : 250), donkey anti-goat AlexaFluor®350 (dilution 1 : 250); (Molecular Probes). After a final rinse with PBS, the slides were mounted using Pro-Long®Gold Antifade Reagent (Molecular Probes). For nuclei staining, the Pro-Long®Gold Antifade Reagent with DAPI (Molecular Probes) was used. The slides were coverslipped and examined by use of an Olympus BX-41 fluorescence microscope. Semi-quantitative analysis based on measurement of fluorescence intensity was performed with the use of WCIF Image J software (Wright Cell Imaging Facility, Toronto Western Research Institute). MAP2+ high signal was defined based on current measurements and results published by Witusik *et al.* [12]. Cells showing intensity higher than 120 units/pixel were defined as MAP2+ high.

EGFR amplification analysis

In the present work FISH (fluorescent in situ hybridization) was performed to determine *EGFR* gene amplification in the cell culture. For analysis,

Table I. Primary antibodies used for immunocytochemical staining (IC)

I Ab	Host	Manufacturer	Dilution
anti-GABA	rabbit	Chemicon; AB141	(1 : 400)
anti-CNP	mouse	Chemicon; MAB 326R	(1 : 400)
anti-O4	mouse	Chemicon; MAB345	(1 : 400)
anti-GFAP	mouse	Chemicon; MAB360	(1 : 400)
anti-MAP-2	rabbit	Santa Cruz Biotechnology, Inc.; sc-20172	(1 : 100)
anti-βIII-tubulin	rabbit	Sigma; T 2200	(1 : 250)
anti-GFAP	goat	Santa Cruz Biotechnology, Inc.; sc-6171	(1 : 50)
anti-vimentin	goat	Chemicon; AB-1620	(1 : 40)
anti-βIII-tubulin	mouse	Chemicon MAB 1637	(1 : 200)
anti-TH	mouse	Santa Cruz Biotechnology, Inc.; sc-25269	(1 : 100)

1 ml of sample was centrifuged, resuspended in PBS and fixed with using of 4% paraformaldehyde. Slides were incubated in 2x standard saline citrate (SSC) at 72°C for 5 minutes. Then, the slides were placed in 70%, 85%, and 100% ethanol for 1 minute each, air-dried and placed on a 50°C slide warmer for 2 minutes. The FISH probe set mix (1 μl LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen, no 32-191053, Vysis, 7 μl LSI Hybridization Buffer and 2 μl water) was centrifuged and denatured at 73°C for 5 minutes. Denatured probe was placed in each specimen. The slides were then coverslipped and incubated at 37°C overnight in a humidified chamber. Afterwards slides were washed with 0.4x SSC/0.3% NP-40 at 73°C for 15 minutes and rinsed in 2x SSC/0.1% NP-40. 10 μl of DAPI II counterstain were placed on the slides and coverslipped. For the scoring, an Olympus BX-41 fluorescence microscope was used, equipped with a specially designed filter combination for spectrum green and spectrum orange. Ratios < 2 were considered as no amplification. Tumours with ratios between 2 and 5 were classified as low-level amplification, and if > 5 as high-level amplification.

LOH and MSI analysis

DNA was isolated from the cells obtained from the aggregates and from immunostained cells, original tumor cultures and blood isolated from the patients, by means of Macherey-Nagel DNA/RNA/Protein purification kit. LOH and MSI analyses were performed using tumor specimen and corresponding peripheral blood sample. The following LOH markers were used: D1S2734, D1S197, D1S162 D1S156, D9S319, D9S162, D10S587, D10S1267, D17S1828. MSI markers have already been described [11]. Forward primers were 5'-end fluorescencelabeled. PCR was performed in thermocycling conditions individually established for each pair of primers. PCR products were denatured and gel electrophoresis in LiCor automatic sequencer system was applied to the separation and analysis of PCR-generated alleles.

Nucleotide sequence analysis of *P53*

Four genomic regions of *P53* gene (exons 5-8) were amplified by PCR using sets of primers encompassing each exon [7]. Sequencing was performed as described before [7] using the dideoxy termination method, SequiTherm Excel DNA Sequencing Kit (Epicentre Technologies) and LiCor automated sequencer.

Exposure of GBM cells to the oligodendrocytic differentiation medium

The aggregate-derived GBM cells were plated in 3-well tissue culture dishes, and cultured in alpha-MEM medium containing 10% FBS (fetal bovine serum) until 80% confluence. Medium was changed to oligodendrocytic induction medium and cultures

were incubated for 10-30 days. The medium was repleaced every 3 to 4 days. The oligodendrocytic medium contains 20 ng PDGF alpha and 50 nM T3 and T4 (thyroid hormones) [2]. After differentiation cells were stained with proper antibodies.

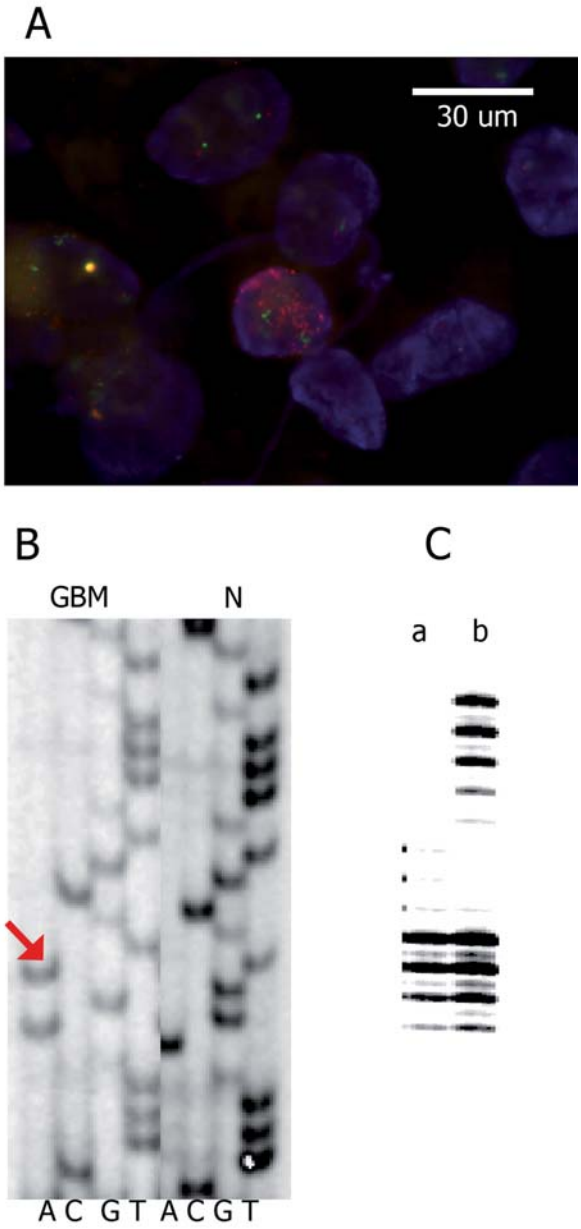

Fig. 1. Molecular characterization of glioblastomas. A) FISH to show *EGFR* amplification. B) Sequencing of *P53* normal sample and sample presenting mutation of *P53* (GBM1). C) LOH analysis. Lane a – blood sample, lane b – lack of cells with retention of heterozygosity (normal cells), DNA isolated from aggregate.

Prolonged exposure of GBM cells to the neural differentiation medium

The presence of tyrosine hydroxylase (TH) and neurotransmitter of GABAergic neurons – GABA was determined by prolonged culturing of GBM aggregates in neural differentiation medium. GBM cells were plated in 3-well tissue culture dishes. After cells reached about 80% confluence, they were incubated for 20-30 days in neural medium, containing 20 ng GDNF, 20 ng bFGF. The medium was replaced every 3 to 4 days. After differentiation cells were stained with proper antibodies.

Results

Molecular background of tested GBMs

To examine whether GBMs were genotypically divergent, isolated cells underwent basic molecular tests. Considering two predominant genetic pathways typical for primary (*EGFR* amplification) and secondary GBMs (mutations of *P53*) [1,3], we performed sequencing of *P53* gene and *EGFR* amplification analysis by FISH. We observed *EGFR* amplification in 2 GBMs and *P53* gene mutations in 1 GBM (Figs. 1A, 1B). To rule out contamination of GBM cultures by non-tumor cells, we preformed LOH and MSI analysis of the aggregates. GBM cells, from all examined aggregates, consisted of only tumor cells (Fig. 1C).

Multilineage phenotype of cells released from the GBM aggregates

Our previous studies demonstrated that GBM cells cultured under serum-starvation conditions formed stable aggregates and the multilineage phenotype of these cells had been observed for several months. Subsequently by, changing culture conditions to those of neural differentiation conditions, GBM cells migrated from aggregates forming a monolayer. After 5-days exposure to neural differentiation medium, phenotypic characterization of the monolayer cells released from GBM aggregates showed that these cells co-expressed CNP (low level), GFAP (high level), and MAP2 (low level) (multilineage phenotype), cells showing high expression of CNP and O4 were not detected (Fig. 2). In current studies, we characterized those cells further and examined the putative oligodendrocytic and neural differentiation.

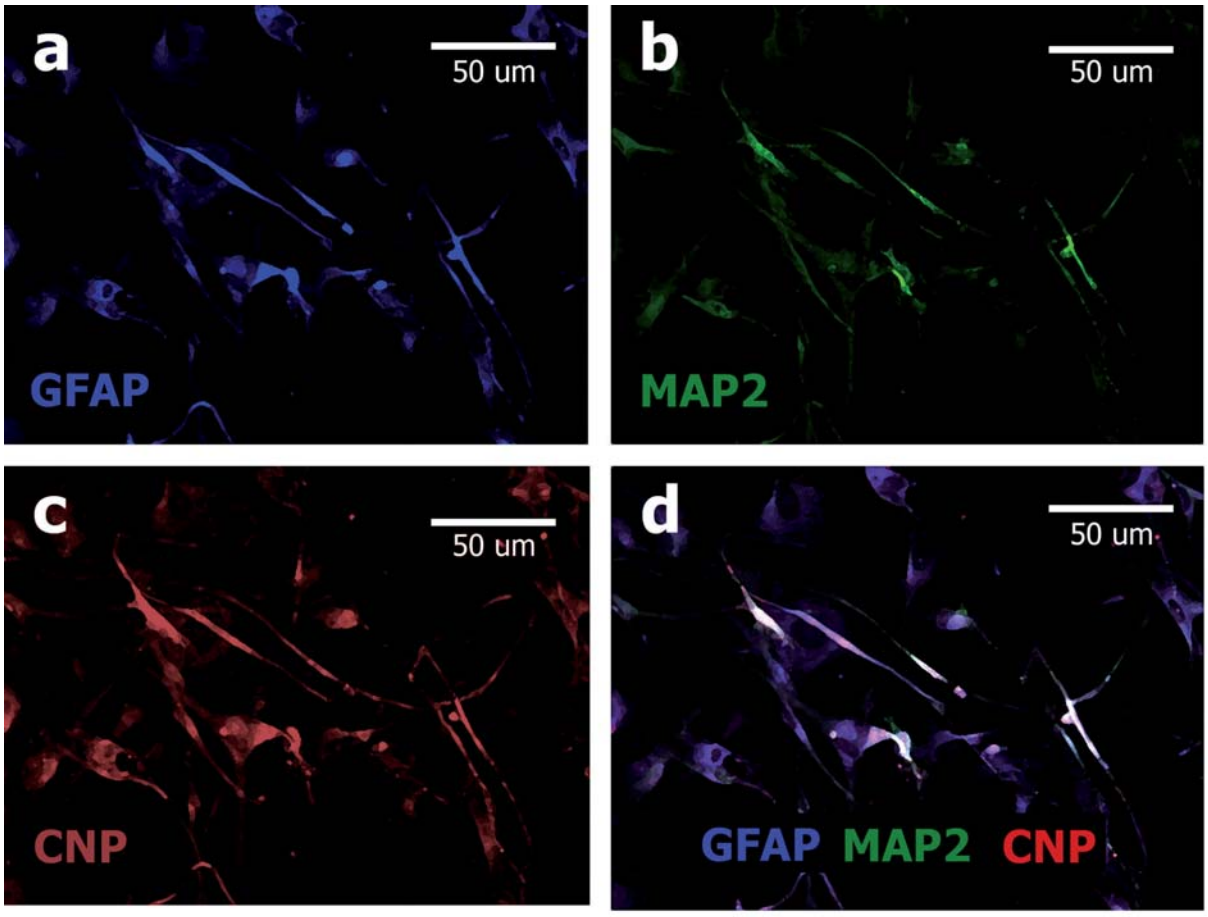

Fig. 2. CNP and MAP2 expression presented by GBM. (a-d) CNP, GFAP and MAP2 coexpression observed in the cells released form aggregate. Intensity of AlexaFluor®350 antibodies (blue signal) is weaker than intensity of AlexaFluor®488 (green signal) or AlexaFluor®594 (red signal). Compare Fig. 2a (blue GFAP) with Fig. 3c (green GFAP).

Inefficient oligodendrocytic differentiation of GBM aggregate-derived cells

To examine wheather the GBM cells exhibit the potential to generate oligodendrocytic cells, these cells were sequentially cultured in oligodendrocytic differentiation medium supplemented with PDGF alpha and thyroid hormones for 10-30 days. The aggregates of three GBMs were characterized by immunostaining: O4, GFAP and MAP2, GFAP, CNP. During 30-day culture, the appearance of markers underwent changes. Immunocytochemical analysis after 20-25 days showed that a few cells expressed O4, which is typically present in oligodentrocytic cells (Fig. 3).

Next, in three independent experiments, we examined the presence of CNP, that is known to characterize the oligodendrocytic cells. In all of cases (GBM1, GBM2 and GBM3), immunocytochemical staining revealed very low percentage of cells with oligodendrocytic phenotype after oligodendrocytic differentiation, approximately 0.2-2.4% of cell populations (Table II). Oligodendrocytic cells were not detected before differentiation.

Arrested neural differentiation of GBM cells – an update

In parallel with oligodendrocytic differentiation of GBM cells we cultured GBM aggregate-derived cells under conditions supporting the growth of neural cells, in serum free medium supplemented with 20 ng GDNF, 20 ng bFGF. The neural differentiation potential of GBM cells was assessed by expression of cate-

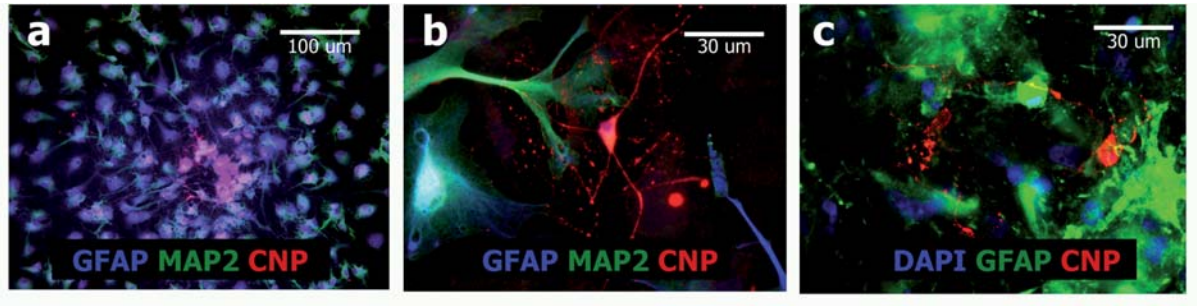

Fig. 3. GBM oligodendrocytic differentiation. (a) GBM cells released form aggregate, 25 days of oligodendrocytic differentiation. Cells positive for GFAP, MAP2 and O4. Only small "island" of cells is strongly positive for O4. (b) GBM cells released form aggregate, 20 days of oligodendrocytic differentiation. One cell strongly positive for CNP. (c) GBM cells released form aggregate, 20 days of oligodendrocytic differentiation. Two cells CNP-positive.

cholaminergic and GABAergic neurons specific genes, respectively TH and GABA.

After 20-30 days of culture, we immuno-stained the GBM cells in three independent experiments. Immunocytochemical results showed that expression of GABAergic neurons marker was rarely presented while expression of catecholaminergic neuron genes was undetectable (Table II). We observed that all examined tumors showed a high resistance to neural differentiation conditions. In all of these GBMs the neural differentiation was arrested at early stages. Advanced stage characterized by expression of GABA and TH was almost not observed (Fig. 4). These results confirm our previous findings that induction of neural phenotype in the GBM aggregate-derived cell was significantly inhibited.

Discussion

The differentiation of GBM cells was frequently described within the last few years [4,5]. It is obvious that features of astrocytic, neuronal and oligodendrocytic differentiation of those cells can be detected. However pathways of GBM cells differentiation seem to be very enigmatic, since these cells frequently coexpress markers belonging to very different cell lineages. For example, problems with defining oligodendrocytic differentiation of GBM cells were very strongly articulated because of observed coexpression of markers such as CD44 and O4. The first one was considered as a marker of astrocytes restricted cells, and the second as a marker of oligodendrocytic progenitors. We have proposed a new model of differentiation, which may help to resolve those kinds of issues. We have shown several times, that coexpression of different lineages markers at low level can be temporarily presented even by normal neural progenitors [9, 10]. Those observations encourage us to compare a normal neural progenitors showing multilineage phenotype with GBM cells coexpressing markers of different lineages. Our model allows to investigate certain subpopulation of tumor cells – aggregated tumor cells presenting multilineage phenotype. Based on our data, we suggest that multilineage phenotype can be strongly expressed and stably presented by GBM cells as a consequence of differentiation arrest [10].

Current work supports already suggested hypothesis suggesting that multilineage phenotype is expressed by GBM cells. We have found a low expression of CNP oligodendrocytic marker in cells released from aggregates and expressing MAP2 and GFAP. It shows that GBM cells can simultaneously coexpress markers of oligodendrocytic, astrocytic and neuronal cells. As we already described, neural (astrocytic and

Table II. Percent cells of glioblastoma expresing CNP or GABA

Features	GBM1	GBM2	GBM3
% of cells expressing CNP	I* – 0.2	I* – 0.6	I* – 1.6
	II* – 0.8	II* – 0.4	II* – 2.4
	III* – 0.6	III* – 0.2	III* – 1
% of cells expressing GABA	I* – 1	I* – 0.4	I* – 0.6
	II* – 1.6	II* – 0.2	II* – 1
	III* – 1	III* – 0	III* – 1.2

** I – the first experiment; II – the second experiment; III – the third experiment*

neuronal) differentiation of GBM cells can be triggered even in 50% of GBM cells, although this differentiation is arrested at early stage. Previously, we did not focus on oligodendrocytic lineage and now we have decided to fulfill this gap. Oligodendrocytic differentiation of GBM cells released from aggregates presenting multilineage phenotype appeared to be very inefficient. Only 0.2-2.4% of GBM cultures presented clear features of oligodendrocytic differentiation. We confirm that the oligodendrocytic differentiation of GBM cells cannot be induced easily.

Our previous data suggested than neuronal differentiation of GBM cells is arrested at early stage. We decided to reconsider this suggestion analyzing the expression of neurotransmitter GABA and the expression of an enzyme required for a catecholamines synthesis (TH). We detected less than 1% of cells released from aggregates expressing GABA, but not TH. Our data, once again showed that despite of MAP2 and beta III tubulin expression GBM cells are extremely rarely able to reach advanced stage of neuronal differentiation.

This analysis broadens our previous work on differentiation of GBM cells. The major finding of this study is that the GBM cells cannot be efficiently differentiated to catecholaminergic and GABAergic neuronal cells. The neuronal differentiation of GBM was arrested at stage of the neuronal intermediate phenotype. In addition, oligodendrocytic differentiation of GBM cells was very unsuccessful.

Induction of efficient differentiation of GBM cells can be considered as putative therapeutic approach, since maturation of cells decreases malignancy of tumor cells, inhibits their proliferation and even induces apoptosis. The data presented here showed that induction of advanced oligodendrocytic and neuronal differentiation of GBM cells would be rather difficult. Surprisingly, from all considered by us pathways of GBM cells differentiation so far: mesenchymal, neuronal, astrocytic and oligodendrocytic the mesenchymal one seemed to be most promising therapeutically, because of its high efficiency [10]. To this end, we cannot recommend induction of oligodendrocytic and neuronal differentiation by means of exposure to growth factors as a proper approach, to design GBM therapy. However, we do not exclude that more efficient method to induce oligodendrocytic or neuronal differentiation of GBM cells exists.

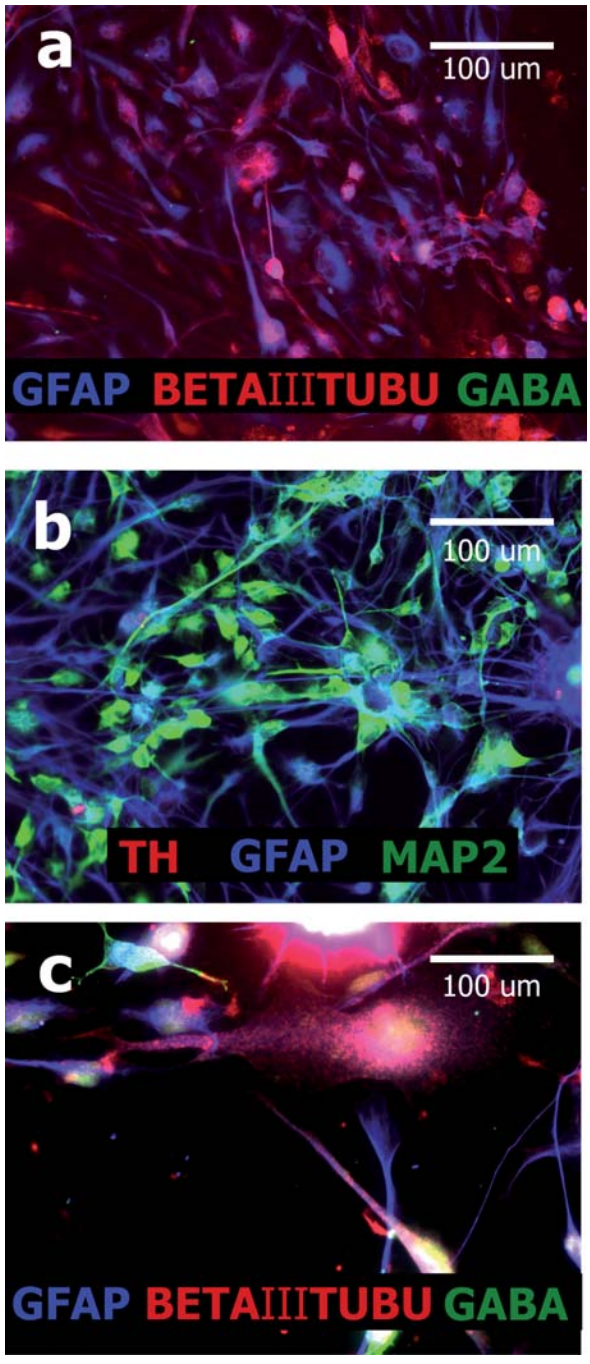

Fig. 4. GBM neural differentiation. (a) GBM cells positive for GFAP and BETA-III-TUBULIN and negative for GABA (25 days of neuronal differentiation). (b) Single cells positive for GABA (20 days of neuronal differentiation). (c) Lack of TH positive GBM cells in spite of GFAP and MAP2 expression in two differentiated populations of cells (25 days of neuronal differentiation).

Acknowledgements

All experiments were performed with the understanding and consent of each subject, with the approval of the Bioethics Medical University Committee (RNN/97/09/KE April 21 2009). This study was supported by Polish Ministry of Scientific Research and Information Technology N40104631/1195.

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