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Fourier transform infrared microspectroscopy reveals biochemical changes associated with glioma stem cell differentiation

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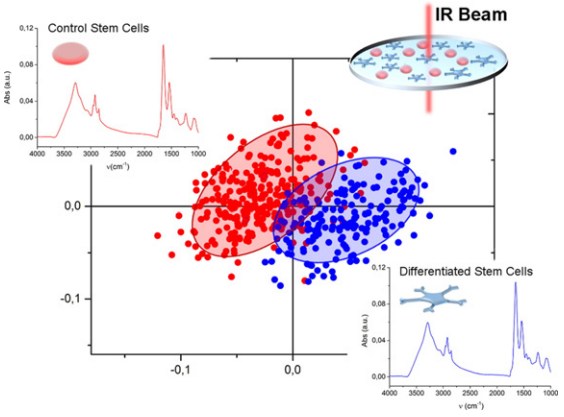
HIGHLIGHTS

- FTIR identifies spectral features that differ in glioma stem and non-stem cells.
- Glioma stem cell proteome and phosphorylation level differ from differentiated cells.
- Glioma stem cell plasma membranes are more rigid than those of differentiated cells.
- Glycogen level of glioma stem cells is affected by ATRA-differentiation.

GRAPHICAL ABSTRACT



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ABSTRACT

According to the cancer stem cell theory malignant glioma is incurable because of the presence of the cancer stem cells — a subpopulation of cells that are resistant to therapy and cause the recurrence of a tumor after surgical resection. Several protein markers of cancer stem cell were reported but none of those is fully reliable to grade the content of stem cells in a tumor. Hereby we propose Fourier transform infrared (FTIR) microspectroscopy as an alternative, labelfree, non-damaging and fast method to identify glioma stem cells based on their own spectral characteristics. The analysis of FTIR data revealed that in NCH421k cells, a model of glioma stem cells, the relative content of lipids is higher than in their all-trans retinoic acid-differentiated counterparts. Moreover, it has been assessed that stem cells have more rigid cellular membranes and more phosphorylated proteins, whereas after differentiation glycogen level increases. The ability of FTIR to estimate the content of stem cells in a heterogeneous sample, on the base of the identified spectral markers, and to classify stem and non-stem cells into two separate populations was probed. Although it was not possible to calculate the exact percentage of each subpopulation, we could clearly see that with the increasing amount of differentiated cells in a sample, more hits occupy the PC space previously identified as a space of differentiated cells. The present study is therefore an initial step towards the development of a FTIR based protocol in clinical practice to estimate the content of stem cells in a tumor sample.

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1. Introduction

Gliomas are a heterogeneous group of primary brain tumors, glioblastoma (GBM) being the most malignant one. Only 5% of glioblastoma patients survive 5 years after diagnosis and the majority dies within two years [1]. Standard therapy comprises maximal safe resection, followed by radiotherapy with concomitant systemic therapy using the alkylating agent temozolomide. However, patients respond differently to the standard therapy due to the high heterogeneity of the disease. The cancer stem cell (CSC) theory suggests that only a subpopulation of tumor cells in glioblastoma is able to initiate tumor growth and drive its development [2]. According to this theory all standard therapies will eventually fail if CSCs are not removed. The high content of CSCs has been correlated with worse prognosis or increased aggressiveness in many tumor types, such as breast [3], head and neck [4], oropharyngeal cancer [5], and glioma [6]. Given that CSCs are resistant to classical chemo and radiotherapy, several novel approaches to specifically target them have been suggested. Among these, inducing differentiation of CSCs is one of the most promising [7]. In particular, the bone morphogenetic protein 4 (BMP4) was found to induce CSC differentiation and to consequently block proliferation and tumor growth [8]. The bone morphogenetic protein 7 variant (BMP7) [9] and all-trans retinoic acid (ATRA) had a very similar effect [10].

Because of the central role of CSCs in tumor development and their prognostic value, it would be important to develop methods to monitor their presence/abundance in tumor samples [11], as well as the efficacy of stem cell-differentiating agents. Glioma stem cells have some known characteristics, such as ability of self-renewal, ability to give rise to different cell types, and the expression of molecular markers, such as nestin, CD133 and Sox2. These cells are also able to regenerate the heterogeneous cell populations similar to the original tumor, when transplanted to a nude mice. Most of these properties are however not an exclusive feature of CSC and do not provide a reliable strategy to study their abundance [12]. In the present study we suggest the use of Fourier transform infrared (FTIR) microspectroscopy as an alternative approach to find small differences between glioma stem cells and glioma cells without stem cell properties, and to estimate the content of CSCs in a heterogeneous sample.

FITR is a well-established label-free analytical methodology for the analysis of biological samples [13]. It provides spatially resolved information on the composition and structure of the most relevant biomacromolecules [14], probing their vibrational modes without inducing sample damaging [15]. In the last decades, advances in detector technology [16] and source brightness [17] have made it possible to perform single cell analysis, allowing the fine characterization of heterogeneous cell populations.

The topic of cell differentiation has already been studied using FTIR in different systems [18,19,20,21,22]. Nevertheless, each of the investigated samples (cell type, tissue) is biochemically different: in adipose cells there is an accumulation of triglycerides, in hepatocytes and muscle cells there is a high level of glycogen, in brain cells typical lipid constituents are different to other cell types and the panel of expressed proteins is different for each. Therefore, the marker regions of infrared spectra that could distinguish glioma stem cells from differentiated ones would likely be different from those previously identified in other cell types.

In this work, we report the changes in biochemical composition that occur during the differentiation of NCH421k as a glioma stem cell model. Using Principal Component Analysis (PCA), we identified clear signatures of differentiation affecting proteome and lipidome cellular profiles, but also the extent of protein phosphorylation and intracellular glycogen level. Moreover, we tested the ability of the technique to estimate the content of stem-like cells in heterogeneous samples demonstrating the predictive capability of the technique and its potential for diagnostic purposes.

2. Materials and methods

2.1. Cells and differentiation

NCH421k cells as a glioma stem cell model were purchased from CLS cell lines service and grown as floating neurospheres in DMEM/F12 medium supplemented with 0.25% BSA, 1% ITS, 20 ng/mL epidermal growth factor and 20 ng/mL basic fibroblast growth factor at 37 °C in 5% CO_2 atmosphere (control cells from here on). Cells were routinely passaged every 4 days. Differentiation of neurospheres was induced as described by Campos [10] by growing them in the same medium containing 10% FBS and 10 nM ATRA for 72 h (ATRA-differentiated from here on). To follow cell cycle phase distribution, PI staining was performed as described elsewhere [23].

2.2. Sample preparation

From control cells (neurospheres) single cell suspension was prepared. ATRA-differentiated cells were instead collected by trypsinization. Each sample was washed in physiological solution and divided into two parts, one for FTIR microspectroscopy analysis and the other for parallel IF, WB or PCR.

2.3. PCR

RNA was isolated using Isol-RNA lysis reagent following the manufacturer's instructions (5Prime) and 2 μg of RNA was transcribed to cDNA using a cDNA Archive kit (Applied Biosystems). Expression levels of GFAP, nestin, CD133 and β-actin as endogenous control were measured by PCR (BioRad) using the SYBR Green master mix (BioRad) and the following primer pairs (sequences selected from primerdepot NIH): GFAP F: acagacttggtgtccaggct, R: gagatcgccacctacaggaa; nestin F: gggagttctcagcctccag R: ggagaaacagggcctacaga; CD133 F: gcattggcatcttctatggtt, R: cgccttgtccttggtagtgt; and β-actin F: ccttgcacatgccggag R: gcacagagcctcgcctt. PCR conditions were 50 °C for 2 min, 95 °C for 10 min and 45 cycles of 95 °C for 15 s and 60 °C for 1 min; the data were analyzed by the ΔΔCt algorithm. Statistical significance between expressions was determined by two tailed Student's *t*-test and $p < 0.05$ was considered significant.

2.4. GFAP and CD133 immunofluorescent staining

To determine the percentage of differentiated cells, staining of astrocyte-marker GFAP and stem cell marker CD133 was performed. Staining was performed on cells in single cell suspension. Cells were then fixed in 3.7% paraformaldehyde, blocked in 4% BSA and incubated in anti-GFAP primary antibody (Novus Biologicals, 1:50 in 1% BSA in PBS). For CD133 primary antibody incubation (Miltenyi, 1:11) was done before fixation. Afterwards, cells were incubated in Alexa488 anti-rabbit (Invitrogen, Molecular Probes, 1:300) and nuclei were counterstained with Toto3 (Invitrogen Molecular Probes, 1:5000). Images of at least 200 cells were acquired using a Zeiss LSM 510 Meta confocal microscope and the percentage of differentiated cells was calculated as the number of GFAP positive cells versus all cells stained by Toto3.

2.5. Apoptosis assessment

Annexin V staining was used to detect early stages of apoptosis. For positive control, cells were treated with 10 μM camptothecin for 4 h. Control, ATRA and camptothecin treated cells were washed in PBS and resuspended in Annexin V binding buffer: 10 mM Hepes, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$. The staining was performed according to the instructions of the producer (Roche) and 10,000 cells were analyzed on FACSCalibur (Becton Dickinson) using CellQuestPro software.