A ^{1}H MRS Study on Neurospheres of Cancer Stem Cells from Human Glioblastoma Multiforme shows the presence of markers of both glial and neuronal morphology

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INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and aggressive type of primary brain tumor both in children and adults. Available therapies usually reduce the tumor mass yet not avoiding tumor relapse. The presence of cancer stem cells (CSC) refractory to the usual therapies has been demonstrated for maintenance of a subset of self-renewing cells producing cancer recurrence. Numerous studies are currently performed to understand the CSCs characteristics in order to provide more effective therapies for these cancers. Among the possible *in vitro* models, the neurosphere systems have elicited much attention because they may provide a relative stable population of cells that share stemness properties. Neurospheres are floating structures obtained by the dissociation of cancer tissue into individual cells, growing as aggregates resembling spheroids in serum free medium supplemented with growth factors.

We here propose ^{1}H MRS as a feasible tool to add information on metabolism in two different CSC lines derived from primary GBM grade IV (WHO classification). Though this technique is much used in the study of the metabolic features of cancer cells, both *in vitro* and in *vivo*, MRS studies are scanty in the field of normal stem cells and nearly absent in CSCs. Two CSC lines from primary GBM grown as neurospheres are characterised and compared with T98G cells, a cultured cell line from GBM, in order to highlight similarities and differences.

MATERIALS AND METHODS

CSCs deriving from primary glioblastoma were isolated and kept in culture as exponentially growing neurospheres according to [1]. The criteria used to check the stem cell phenotype of CSCs, according to [2], were i) formation of primary spheres *in vitro*; ii) capacity of self-renewal on clonogenic and population analysis; iii) ability to differentiate under serum stimulation both into GFAP-positive astrocyte-like cells and into neurofilament expressing neuron-like cells; iv) generation of tumors upon orthotopic (intracerebral) transplantation in immunodeficient mice; v) maintenance of the chromosomal aberrations of the parental tumor. T98G cells were grown as adherent cells as described elsewhere [3]. ^{1}H MR spectra were run at 400.14 MHz on a digital Avance spectrometer (Bruker, Karlsruhe, Germany) equipped with a 1mm microprobe. Signals were acquired with a 90° RF pulse and a sweep width of 4006.4 Hz. Water suppression was obtained by irradiating water signal.

RESULTS AND DISCUSSION

The two CSC lines 1 and 2, CD133+ positive, and the glioblastoma derived T98G cells were compared. Line 1 CSCs display 80 % CD133+ cells in the neurosphere, while Line 2 reaches its maximum at about 2%. CSC Line 1 and T98G cells spectra are here reported. The spectral region between 0.5 and 4.5 ppm is shown in Fig 1a. Fig 1b shows the region of amide protons between 7.7 and 8.9 ppm. These spectra are resolution enhanced for better identification of the resonating amide groups. Finally, Fig 1c represents the region of N-acetyl and acetate resonances, also resolution enhanced. Many intense signals from brain metabolites are visible in the CSCs spectra, such as creatine, myoinositol, choline, and other metabolites with smaller signals. The intense alanine (ala) signal at 1.46 ppm is peculiar of Line 1 spectra, being present with lower intensity in Line 2. Signals from UDP-Hexosamines (Hex) recently assigned in tumor cells from both adenocarcinomas and gliomas are also clearly detected [4]. Most relevant observations can be done when comparing CSCs spectra with T98G cells. Prominent peaks from glutamine (gln) at 2.14, 2.45, 3.78 ppm are visible in glioma stem cells (Fig1a). Signals form gln are not usually abundant in cancer cells in culture and they are low or absent in T98G spectra where only signals from glutamic acid (glu) and glutathione (GSH) are visible, at high concentration in these cells [3]. In other glioma derived cells, namely A172 from GBM, glutamine signals are present at much lower extent (not shown). Such an intense glutamine concentration in GBM cells must be related to the glial origin of this cancer.

Details of the Fig 1c highlight another relevant and constant difference between the CSCs and the glioma cell line. In fact, the N-acetyl-containing compounds (NAc) display differences, as two signals at 1.99 and 2.02 ppm are clearly detected in the CSCs spectrum, absent or less intense in T98G cells. Also the acetate-based region is different, where acetate is seen as a narrow intense peak at 1.92 ppm only in the glioma cell samples, a feature shared by A172 and adenocarcinoma derived cell lines (not shown). In the CSCs spectra a multiplet located in the same position accounts for the presence of γ-Aminobutyric acid (GABA). Assignment to this compound is confirmed by the concurrent presence of the resonances at 1.91, 2.28 and 3.03 ppm in 1D spectra, and of intense cross peaks in 2D COSY spectra (Fig 2) .GABA cross peak at 1.91-3.03 ppm is clearly visible and can be compared to the Lys cross peak of comparable intensity.GABA signals increase in confluent cells where neurospheres are larger, necrosis not being present at a large extent. The presence of an appreciable concentration of GABA is related to the multipotent nature of these CSC lines able to produce a progeny of neural cells with glial morphology. These spectral markers decrease going towards CSCs differentiation on Matrigel^{TM} support (not shown). Fig 1b shows the region of amide protons, where the amide signal of N-acetylgalactosamine (GalNAc), recently found in HeLa cells [5] is clearly detected at 8.04 ppm. This signal, present also in Line 2 spectra though at much lower intensity, is totally absent in T98G cells. Furthermore, as shown for Line 1 in Fig. 2, both cancer stem cell lines show the cross peak at 1.33-4.27 ppm attributed to fucose (Thr/Fuc) [5]. If confirmed, this would suggest that the CSCs neurosphere model allows to detect metabolic pathways of mutated protein O-glycosylation.

Finally, both CSC lines show the characteristic lipid signals at 0.89 and 1.28, ppm. In Line 2 cells the intensity of lipid signal increases approaching the plateau region, thus progressively obscuring metabolite information. Line 1 cells display very small lipid signals even when growing in larger neurospheres where spectra maintain narrow and intense metabolite peaks. This finding is unexpected as Line 1 cells grow as more stable and large neurospheres that may be largely hypoxic. Previous studies on MCF-7 tumor spheroids from breast cancer found that, at increasing spheroid size, lipid signals increased while metabolite peaks disappeared [6], with a behaviour more resembling that of Line 2 cells.

CONCLUSIONS

The overall picture point to a role for ^{1}H MRS in the study of CSCs metabolism, showing that the neurospheres, despite the hypoxic environment, host viable and metabolically active cells that show neuronal markers such as GABA and glial markers such as gln. Spectra of both CSC lines share differences with the glioma derived T98G cells. Intense lipid signals are not simply attributable to neurosphere related hypoxia. Finally, the occurrence of signals from sugars bound to O-glycosylation of proteins allows to consider MRS technique as an important tool for the study of these systems.

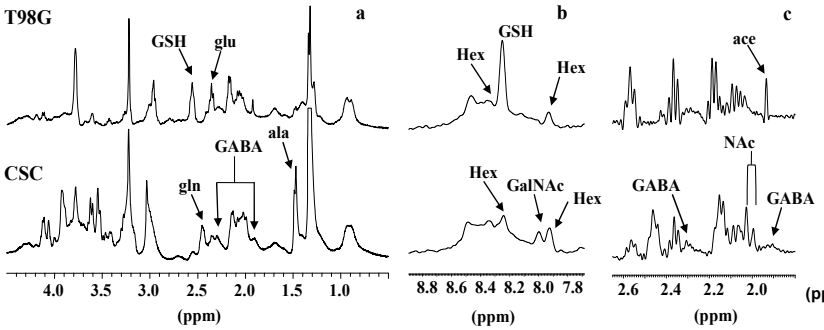

Figure 1. 1D spectra of T98G and CSC Line 1 cells. Regions: a) 0.5-4.5 ppm, b) 7.8- 8.9 ppm, and c) detail of a) with resolution enhancement.

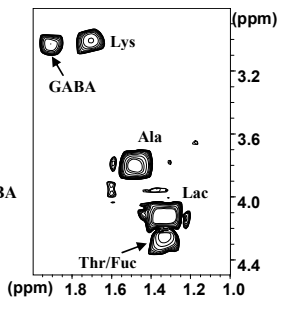

Figure 2. 2D COSY spectrum of CSC Line 1.

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