

Brain tumor stem cell dancing

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Abstract

Background. Issues regarding cancer stem cell (CSC) movement are important in neurosphere biology as cell-cell or cell-environment interactions may have significant impacts on CSC differentiation and contribute to the heterogeneity of the neurosphere.

Aims. Despite the growing body of literature data on the biology of brain tumor stem cells, floating CSC-derived neurospheres have been scarcely characterized from a morphological and ultrastructural point of view.

Results. Here we report a morphological and ultrastructural characterization performed by live imaging and scanning electron microscopy. Glioblastoma multiforme (GBM) CSC-derived neurospheres are heterogeneous and are constituted by cells, morphologically different, capable of forming highly dynamic structures. These dynamic structures are regulated by not serendipitous cell-cell interactions, and they synchronously pulsate following a cyclic course made of “fast” and “slow” alternate phases. Autocrine/paracrine non canonical Wnt signalling appears to be correlated with the association status of neurospheres.

Conclusions. The results obtained suggest that GBM CSCs can behave both as independent cells and as “social” cells, highly interactive with other members of its species, giving rise to a sort of “multicellular organism”.

Key words

- brain tumor stem cells
- pulsations
- live imaging
- scanning electron microscopy
- Wnt5a

INTRODUCTION

Cancer arises from a series of mutations that occurs in few or even single cells. These cells eventually acquire unlimited and uncontrolled proliferation potential. Two hypothetical models can explain this phenomenon. The stochastic model proposes that tumor cells are heterogeneous, but that virtually all of them can function as a tumor founding cells, although this may happen only rarely. Conversely, the hierarchical model implies that only a small subpopulation of tumor stem cells can proliferate extensively and sustain the growth and progression of a neoplastic clone. The latter hypothesis fits with the cancer-stem-cell (CSC) theory and is now supported by a plethora of literature data [1].

Besides the properties shared with normal stem cells (self-renewal and the ability to differentiate into other cells), candidate cells must present the following properties to be considered as CSCs: (i) the unique ability to engraft giving rise to experimental tumors, (ii) the ability of these xenografts to recapitulate the tumor of origin morphologically and immunophenotypically in xenografts, and (iii) the ability to be serially transplanted [2]. These criteria are the standard to identify other CSCs not only in hematopoietic tumors but also in solid tumors. In fact, cells with “stem cell” properties have been isolated from different malignancies [3-5] in-

cluding glioblastoma multiforme (GBM).

Due to the highly dismal prognosis of brain tumors, particular attention has been focused on CSCs isolated from GBM. The first evidence of the existence of cells with characteristics of stem cells in brain tumors was reported by Ignatova *et al.* [6]. These authors isolated precursor cells capable of forming neurosphere *in vitro* from human GBM. These neurosphere-forming cells expressed both neuronal and astroglial differentiation markers, thus showing multilineage potency. After Ignatova *et al.*, other researchers isolated cells with stem cell properties from GBM [7-9]. In particular, long-term expanding GBM CSC lines were isolated from surgical specimens of GBM by exposure of tumor cells to the culture conditions typical of the NeuroSphere Assay (NSA) [10], *i.e.* in the presence of specific mitogens (epidermal growth factor and fibroblast growth factor-2) in serum-free medium [7, 8]. GBM CSC lines are endowed with the essential features of CSC, as they can be long-term cultured *in vitro*, as neurospheres [7-10]. Upon differentiation, these cells are multipotent: they may undergo mesenchymal differentiation [11] and can generate new tumors following transplantation into immunodeficient mice [1, 12]. Therefore, they can be considered *bona fide* GBM CSCs.

Issues regarding CSC movement are important in

neurosphere biology as cell-cell or cell-environment interactions may have significant impacts on CSC differentiation and contribute to the heterogeneity of the neurosphere. Despite the growing body of literature data on the biology of brain tumor stem cells, floating CSC-derived neurospheres have not been fully characterized from a morphological and functional point of view. Thus, the objective of this study was to characterize the cytoarchitecture of glioma-CSC neurospheres by scanning electron microscopy (SEM), and the behavior of the GBM CSCs within cultures of neurospheres followed in living conditions by time-lapse video microscopy. A cyclic course of synchronous pulsations was found by analyzing with Dynamic Pattern Recognition for temporal data the formation of cell clusters and their oscillatory behavior.

MATERIALS AND METHODS

Cells cultures

CSC line L0627 was obtained from a patient with diagnosis of primary glioblastoma as elsewhere described [7]. GBM specimens were collected from patients with histologic diagnosis of primary GBM (WHO grade 4 glioma) in accordance with the protocol approved by the institutional review board of San Raffaele Scientific Institute (01-CSC07).

Cells were plated in 25 cm² tissue culture flasks at clonal density of 2500-5000 cells/cm² in Dulbecco's modified Eagle's medium/F-12 medium, containing 20 ng/mL of both epidermal growth factor (EGF) and fibroblast growth factor (FGF2, both from Peprotech, Rocky Hill, NY). Population and serial subclonogenic analysis were performed as in [7].

For imaging experiments inserts with 8.0 micron pore-membranes (BD Biosciences, Erembodegem, Belgium) that stood in six-well Costar plates (Corning Inc., Corning, NY) were employed. Cells were harvested and resuspended in serum-free medium in the absence of growth factors at a concentration of 7.5x10⁵ cells/ml. Then, 7.5x10⁵ cells were added to each insert, and 3 ml of complete medium was added to the well underneath the insert.

Western blotting

For western blotting analysis of Wnt 5a expression, cells were separated from neurospheres and suspended in fresh medium. At the indicated times, both cells and supernatants were recovered from each sample.

Cells were subsequently washed in ice-cold Tris-buffered saline (TBS; 20mM Tris-HCl, pH 7.6, 140mM NaCl) and lysed at 4 °C in lysis buffer (10mM Tris-HCl, pH 7.6, 50mM NaCl, 30mM sodium pyrophosphate, 5mM EDTA, 0.5% Nonidet P40, 1% Triton X-100, 50mM NaF, 0.1mM Na₃VO₄, 1mM phenylmethylsulfonyl fluoride, and complete mini proteinase inhibitors). Cell lysates were obtained by centrifugation at 17 000 g for 30 minutes at 4 °C. Supernatants were concentrated by using centrifugal filter devices (Amicon® Centricon® Millipore). Protein concentration was determined by employing the DC Protein Assay (Bio-Rad Laboratories).

Proteins were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes that

were blocked for 1 hour at room temperature with 5% BSA in TTBS. Incubations with primary antibodies and with horseradish peroxidase-conjugated secondary antibodies were performed in blocking solution overnight at 4 °C and for 1 hour at room temperature, respectively. Immunoreactive bands were visualized by employing the ECL kit.

Scanning electron microscopy

For SEM analysis, the membranes containing cells were removed from the inserts. At the indicated times, membranes were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 30 minutes, post-fixed with 1% OsO₄ in the same buffer, dehydrated through a graded ethanol series, critical point dried with CO₂, and gold coated by sputtering. Samples were examined with a Cambridge Stereoscan 360 scanning electron microscope (Cambridge Instruments Ltd, Cambridge, UK).

Time lapse microscopy

Cellular behavior was monitored by phase contrast microscopy using a OKOLab Time lapse video microscopy equipped with a microincubator (37 °C, 5% CO₂) equipped with an x-y-z motorized stage controller and a digital CCD camera. Time-lapse experiments were carried out for up to 96 hours. Images were acquired every 5 minutes and converted into video using OKOLab software.

A global synchronization index was determined by analyzing with Dynamic Pattern Recognition of temporal data the formation of cell clusters and their oscillatory behavior. The whole video was converted in a sequence of grey level images. Once the contrast and the background of each image was normalized, a new sequence of images was generated by the difference between each couple of successive images, in which the static background does not contribute, and only the movement component of the original images is shown. The mean intensity of every such image, plotted against time, is the index we have adopted to evaluate the global oscillations observed in the cell culture.

RESULTS AND DISCUSSION

The discovery of neural stem cells (NSCs) and cancer stem cells (CSCs) has stimulated great excitement, as well as heated debates, for both stem cell and cancer biologists. NSCs and CSCs cultured in serum-free medium form aggregates named neurospheres, in both suspension and stationary culture [13-16].

Here we report a morphological and ultrastructural characterization associated with novel functional data on neurospheres generated by CSCs isolated from GBM patient [7] the most malignant among the adult human brain tumors. Since cell movement as well as cell-cell or cell-environment interactions in neurospheres may have significant impacts on the understanding of brain CSC biology, the behaviour of GBM CSCs within cultures of "neurospheres" was followed in living conditions by time-lapse video microscopy.

In order to follow the initial phases of cell organization into neurospheres, mechanically disaggregated CSCs were seeded on uncoated membranes and moni-

tored by video microscopy. After seeding, cells rapidly adhered to the membranes and organized in small spheroid-like structures, which merged subsequently and formed enlarged neurospheres (Electronic Supplementary Material video 1: *please contact the corresponding author*) (Figure 1A). The first morphological characteris-

tic emerging was that, up to five days of culture, CSCs failed to organize in neurospheres with a well-defined spherical shape. Instead, CSCs grouped in cell clusters with uneven external rims. In addition, CSCs clusters revealed a strong propensity to merge.

CSCs in motion tended to aggregate immediately af-

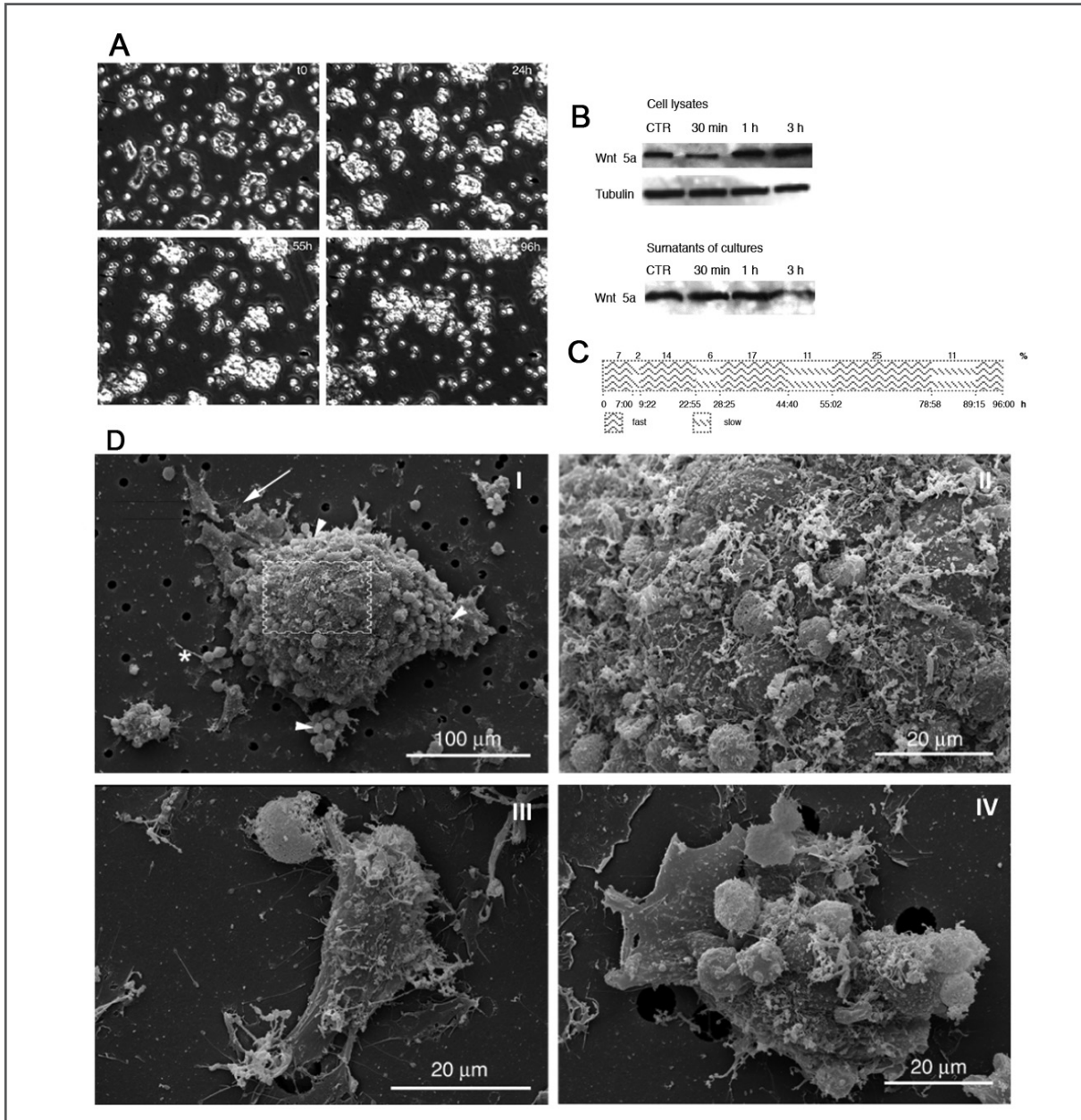


Figure 1

A) Frames from time-lapse video microscopy. Images at t0, 24, 55 and 96 h. B) Wnt 5a expression in cell lysates and in supernatants of cultures. Level of expression of Wnt 5a in CSCs cell lysates has been evaluated before (CTR) and after disaggregation of neurospheres, and culturing in fresh medium for 30 minutes, 1h and 3h. Secreted Wnt 5a has been determined in the supernatants recovered from cultures before (CTR) and after disaggregation of neurospheres, and culturing in fresh medium at 30 minutes, 1h and 3h. C) Time-lapse study. Graphic representation of cyclic course (0-96 h) of synchronous pulsation characterized by a cyclic course made of "fast" and "slow" alternate phases. D) Scanning electron microscopy imaging. (I) GBM CSC-derived neurosphere of about 200 micron after 48 h of culture. CSC-derived neurospheres appear ultrastructurally heterogeneous. GBM CSCs cells closely interact each other in the centre of the spheres, giving as result a very compact structure. (II) Area delimited by the white rectangular box in (I). A fibrous material secreted by cells themselves appeared to cover the cluster. Two cell subpopulations were identified: the first population display a large cell size and a surface covered by short microvilli and small blebs (arrows). Cells of the second population are smaller, rounded in shape and displayed a rough surface (arrow heads). Large cells extended lamellar and filopodial protrusions (asterisk), as they were "hunting" cells to add to the colony (III, IV).

ter seeding. Additionally, highly motile cells (HMCs) were noted to move and lightly strike other stationary and static cells. Some of HCMs flipped and run away, whereas others joined to and increased the neurosphere size. After five days, neurospheres merged into each other and formed a single large neurosphere.

Noteworthy, live imaging showed that, starting few minutes after cell seeding on uncoated membranes, cell clusters began to synchronously pulsate. Synchronous pulsations of CSCs followed a cyclic course made of “fast” and “slow” pulsing phases (Electronic Supplementary Material video1: *please contact the corresponding author*) (Figure 1C). After 7 hours, the cells stopped fast pulsing and entered in a slow phase long about 2 hours. Alternation of “slow” phases with “fast” pulsation phases was observed. During 96 h of observation, the length of “fast” phase triplicate, whereas that of “slow” phases quintuplicate. “Fast” pulsation phase was characterized by short-time distinct phases of contraction and stretching. On the other hand, in “slow” pulsation phase, cell clusters preferentially tended to stretch and increase their volume. Interestingly, in both phases neurospheres behave as a sort of multicellular organisms.

It is interesting to note that CSCs aggregation seemed not to originate from serendipitous cell encountering. Thus the engagement of autocrine/paracrine signalling could be hypothesized. In order to test this hypothesis, the involvement of the Wnt/frizzled signalling, known to play a role in the regulation of cancer cell aggressiveness [17] was explored by analysing the expression of both canonical (Wnt3a) and non canonical (Wnt5a) pathways. Wnt3a protein was apparently absent in CSCs, as demonstrated by Western blotting analysis of both total cell lysates and supernatants of cultures (data not shown). On the contrary, Wnt 5a protein was expressed at high levels in CSCs (Figure 1B). Interestingly, the level of expression of Wnt 5a appeared to be correlated with the state of association of cells in neurospheres (Figure 1B). On the contrary, Wnt 5a was released by CSCs in culture medium since the first minutes of cell aggregation (Figure 1B).

Previous embryogenetic studies demonstrated that pulsation and stabilization are generated by contractile forces that underlie morphogenesis [18]. Acto-myosin cytoskeleton plays a central role in most of the morphogenetic movements. The acto-myosin cytoskeleton generates contractile and tensile forces in individual cells. These tensile forces can be transmitted between cells or tissue through cell-cell junction or through extracellular matrix [19]. One possibility is that CSCs pulsation results from mechanical interactions between cells. When cell cluster contract, contractile forces generated by the acto-myosin cytoskeleton of one cell could be transmitted to an adjacent cell and vice-versa. However, contraction pulses are observed even when mesoderm cells have lost adhesion with one another [20]. Similarly, CSCs underwent contractions and cytoskeletal movements. Thus, pulsed contractions might not require mechanical cell-cell interactions but could reflect a dynamic property of cellular actomyosin networks [21, 22]. Noteworthy, non canonical Wnt signaling (Wnt 5a) targets cytoskeletal elements (actomyosin) [23]. Wang *et al.* showed the ability of the NSCs to move freely within the neurospheres: the “free trip” of cells within the clusters

could trigger a sort of “pulsation” but this phenomenon could not explain the cyclic course [24].

Despite the growing body of literature data on the biology of brain tumor stem cells, floating neurospheres have not been fully characterized from an ultrastructural point of view. Thus, GBM CSC-derived neurospheres were also characterized by SEM.

Bez and colleagues found that different kinds of cells could be identified at the ultrastructural level (transmission electron microscopy) in human neurospheres based on size, morphology of the cytoplasm and distribution of the mitochondria [13]. In the present study, analysis of GBM CSCs by SEM observations were performed, in order to fill gap in the knowledge of cyto-architecture of GBM CSC-derived neurospheres. It is important to stress the concept that SEM imaging allowed to make a snapshot of a highly dynamic structure, as clearly showed by time-lapse observations in living conditions.

In Figure 1D, panel I, a GBM CSC-derived neurosphere of about 200 micron is shown after 48 h of culture. Near the main body, small cell clusters are also visible. SEM observations highlighted that CSC-derived neurospheres or clusters displayed an irregular morphology and were characterized by the presence of heterogeneous cells. In particular, GBM CSC cells closely interacted each other in the centre of the spheres, giving as result a very compact structure in which it was difficult to distinguish cell boundaries (Figure 1D, panels I and II). A fibrous material secreted by cells themselves appeared to entrap the cluster even if a fibrous capsule was not revealed. Within the neurospheres, the coexistence of two subpopulations was identified: the first population had a large cell size and a surface covered by short microvilli and small blebs. Cells of the second population were smaller than those belonging to the first population, appeared rounded in shape and displayed a rough surface. These cells were also distributed within neurospheres differently from the cells of the first population. Indeed, large cells both constituted the body of the neurosphere and protruded from the structures to contact surrounding cells (Figure 1D, panels I and II). On the contrary, small rounded cells appeared preferentially localized at the periphery (Figure 1D, panel I, arrow heads), and could play different roles within the cluster. In fact, time-lapse video microscopy observations clearly showed that these small rounded cells are fast running. These cells, here named as “HMCs”, rebounded between clusters like a pinball in a flipper. On the other hand, the large cells from clusters extended lamellar and filopodial protrusions (Figure 1D, panels III and IV), to contact the other cells as demonstrated by time-lapse video microscopy observations.

Results herein reported indicate that GBM CSC-derived neurospheres are heterogeneous and are constituted by distinct cell populations capable of forming highly dynamic structures regulated by not serendipitous cell-cell interactions. Such observation performed on a CSC line seems to be confirmed by data obtained on two other CSC lines (personal communication). Although the heterogeneity of CSCs has already been observed [25, 26], this CSC “dance” has never been described before. What causes this kind of “dancing” of CSCs neurospheres? Results obtained in this study,

even if not conclusive strongly suggest the involvement of autocrine/paracrine non canonical Wnt signalling, that will be further characterized. Synchronized pulsations detected by movies performed in living cultures, indicate that GBM CSCs can behave as a "social" cell highly interactive with other members of its species to the point of acting as a multicellular organism that listen to external signals. This findings may contribute to the understanding of neurosphere biology and further studies are now in progress in order to eavesdrop by a multidisciplinary approach on mechanisms underlying

cell motion and the complex scenario of molecular and physical signals by which CSCs talk across each other.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

Received on 1 April 2014.

Accepted on 19 June 2014.

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