Cancer stem cells from glioblastoma multiforme: culturing and phenotype

DS Kyurkchiev*

Abstract

Introduction
Glioblastoma multiforme is the most common and malignant brain tumour. The contemporary concept of its development is based on cancer stem cells which originate from the transformation of the normal neural stem cells present in the brain. Both neural stem cells and cancer stem cells express markers such as Nestin, Sox-2, CD133, CD44 and occasionally glial fibrillary acidic protein. Both cell types have similar signal transduction pathways and a number of properties as self-renewal, differentiation and proliferation capacity. Various approaches to culturing cells isolated from glioblastoma multiforme and the corresponding expression of markers for cancer stem cells are the object of this review.

Methodology
Cells isolated from glioblastoma can be cultured in serum-free media containing epidermal growth factor and fibroblast growth factor or in media with foetal bovine serum. It is possible to culture the cells in media combining these basic approaches.

Discussion
According to the concept that is predominant in the literature, neural stem cells represent the best model of cancer stem cells when cells isolated from glioblastoma are cultured. Cells in neurospheres express Nestin, Sox-2, CD133, CD44; they have genetic alterations similar to the tumour and tumourigenicity. In contrast, adherent glioblastoma cells seem to lose the expression of Nestin and CD133 and start to express glial fibrillary acidic protein; they have weaker tumourigenicity and possess genetic changes different from those in the primary tumour. However, there are plenty of data which prove that this concept is not absolutely true. Semi-adherent cellular growth has been described with simultaneous expression of Nestin and glial fibrillary acidic protein, and quite often the adherent cells have a higher expression of markers for cancer stem cells in comparison with the neurospheres.

Conclusion
It can be stated that the protocol for glioblastoma multiforme cells cultured as neurospheres is not the only one and not necessarily the best model for cancer stem cells.

Methodology

Cancer Stem Cells

In vitro and in vivo study of glioblastoma multiforme cells: a review

Introduction
Glioblastoma multiforme (GBM) is the most malignant and the most common primary brain tumour. Currently generally accepted is the hypothesis that cancer stem cells (CSCs) are the basic pathology factors leading to development of GBM and these cells are the results from the transformation of normal neural stem cells (NSCs)²-⁶.

Neural stem cells
NSCs are described at any stage of the development – from the embryo to the adult organism, and they are located in their specific niches in the subventricular zones, in the subgranular zone and in the dentate gyrus of the hippocampus²-¹⁰. In these niches NSCs are situated around the blood vessels. They are in communication with other cells and the extracellular matrix and the basic factors regulating their proliferation and destiny are epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF)¹¹. The niches are characterised by a relative hypoxia which seems to be very important for the survival and proliferation of NSCs¹². Various cellular types are described in the niches, such as neuroblasts (type A), NSCs (type B) and transitory amplifying progenitors (type C) and all these cells are surrounded by ependymal cells¹³. NSCs are pluripotent cells capable of self-renewal and differentiation as a result of which they lose their ‘stemness’¹⁴. An obligatory condition for this is the presence of blood serum and the absence of EGF and bFGF which are the factors responsible for proliferation and sustaining the NSCs in undifferentiated state²,⁷,⁹,¹⁴. The proliferative capacity of the NSCs and their association with blood vessels renders the cells highly mobile and makes possible their movement to zones with hypoxia⁰,¹².

NSCs express specific phenotypic markers and the most important are the following: Nestin is cytoplasmic microfilament protein correlated with ‘stemness’ and related to the organisation of the cytoskeleton, cellular signalling, organogenesis and cellular metabolism. In the course of differentiation the NSCs lose expression of Nestin and start to express βIII-tubulin and glial fibrillary acidic protein (GFAP)⁵,⁷,⁸,¹². GFAP has been described as a marker of astrocyte differentiation but still it can be co-expressed parallel to Nestin by NSCs⁹,¹¹.

Licensee OA Publishing London 2014. Creative Commons Attribution License (CC-BY)

Sox-2 is transcription factor expressed by NSCs with cytoplasmic localisation which is connected to the process of differentiation\textsuperscript{7,9}. CD44 is a transmembrane molecule with a role in the adhesion between cells and the extracellular matrix which is expressed on the surface of NSCs\textsuperscript{15,16}.

CD133 is a specific surface marker for NSCs\textsuperscript{9,17} and its significance will be discussed in detail further.

Cancer stem cells

The CSFs hypothesis postulates that GBM formation is due to transformation of NSCs into CSCs\textsuperscript{4,12}. This might be due to mutations in the NSCs or to genetic changes in partially differentiated progenitor cells originating from the NSCs\textsuperscript{7,14}. It has been shown that partially differentiated cell can be transformed to CSCs after treatment with EGF and bFGF\textsuperscript{18} and these changes can be observed with type B cells (NSCs), with type C cells and even with differentiated cells\textsuperscript{9,10}.

Transformation of NSCs or progenitors in CSCs is considered to be related to normal cellular mechanisms but in abnormal order, at abnormal time and intensity\textsuperscript{19}. It is accepted that the regulatory Hedgehog systems which are related to genes for stemness and self-renewal are affected, as well as EGF signal pathway affecting the differentiation. Mutations in PTET (tumour suppressor gene) are also described. As a result, a maturation arrest is observed leading to intensive cell divisions and lack of differentiation\textsuperscript{19}.

The resultant CSCs are described as a small subset of slowly dividing cells with cell renewal capacity, tumourigenicity, association with genes specific to NSCs, capability for migration and differentiation in cell populations similar to the primary tumour\textsuperscript{4,8,19}. CSCs are cells at the end of a process of dedifferentiation and they have common signal pathways with NSCs, vigorous proliferative potential regulated by EGF and bFGF and ability to differentiate as neurons and glial cells in the absence of these factors but in the presence of blood serum\textsuperscript{2,7}.

The cellular composition of GBM is described as ‘organised chaos’ because pleomorphic tumour cells, proliferating blood vessels, infiltrating inflammatory cells and necrosis are observed. The CSCs which are about 1%–3% of the cellular composition are in fact the ‘organiser’ of the tumour\textsuperscript{4,14}. These cells seem to be at the top of the hierarchy of the tumour cells and they can by themselves form a GBM\textsuperscript{20}. Various types of CSCs are described in reference to their tumourigenic potential and genetic anomalies depending on their location in different areas of the tumour. These facts raise the question whether CSCs are transformed cell types or just specific functional state of cells\textsuperscript{2,12}.

As far as the expression of markers is concerned CSCs similar to NSCs express Nestin either alone or together with expression of GFAP\textsuperscript{4,7,8}. Expression of Sox-2 is observed but quite often as aberrant expression as a sign of disorganisation of the process of differentiation\textsuperscript{19}. In contrast to the cytoplasmic expression of Sox-2 specific to NSCs, Sox-2 in CSCs is expressed in the cell nucleus\textsuperscript{7}. Another marker used for phenotypic characterisation of CSCs is CD44 which is with a role for tumour invasion and is detected on cells from the invasive part of the tumour\textsuperscript{7}.

CD133 is a marker which is believed to be expressed under hypoxic conditions\textsuperscript{2,21} and its significance is quite contradictory in relation to CSCs. Some authors claim that CD133 is the marker which identifies the CSCs\textsuperscript{1,14,16,20}, its expression is a poor prognostic sign\textsuperscript{2} and only the CD133+ cells have tumourigenic properties\textsuperscript{14,22}. A hierarchy is described as the CD133+ cells at the top

**Table 1** Comparison between NSCs and CSCs in reference to their major features and expression of specific phenotype markers

<table>
<thead>
<tr>
<th></th>
<th>NSCs</th>
<th>CSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Self-renewal</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Differentiation</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Multipotency</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Signal pathways</strong></td>
<td>Similar</td>
<td></td>
</tr>
<tr>
<td><strong>Genetic alterations</strong></td>
<td>Similar</td>
<td></td>
</tr>
<tr>
<td>Nestin</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GFAP</td>
<td>Yes or no</td>
<td>Yes or no</td>
</tr>
<tr>
<td>Sox-2</td>
<td>Yes (predominantly cytoplasmic)</td>
<td>Yes (predominantly nuclear)</td>
</tr>
<tr>
<td>CD133</td>
<td>Yes</td>
<td>Yes (disputable)</td>
</tr>
<tr>
<td>CD44</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

CSCs, cancer stem cells; GFAP, glial fibrillary acidic protein; NSCs, neural stem cells.

Licensee OA Publishing London 2014. Creative Commons Attribution License (CC-BY)

of it and they are the real CSCs. Other authors claim that CD133 cannot be used as a universal marker for CSCs. The hierarchical connection between CD133+ and CD133− cells is not accepted and it is claimed that CD133− cell are tumourigenic with self-renewal capacity and proliferation equal to the CD133+ cells. CD133 expression is detected in 60% of GBMs tested, and it is believed that this molecule can migrate from the cytoplasm to the cell membrane. Some GBMs express ‘processed’ form of the CD133 which is not recognised by many of the monoclonal antibodies used. Some additional markers, except the abovementioned, are described both in NSCs and CSCs such as Musashi-1, CXCR4 and CD15.

It is obvious from the data presented above that CSCs have a lot of similarities with the NSCs (Table 1) and this is the basis for the CSCs hypothesis for development of GBM. The aim of this article was to discuss culturing and the phenotype of CSCs from GBM.

Methodology

The author has referenced some of its own studies in this methodology. These referenced studies have been conducted in accordance with the Declaration of Helsinki (1964) and the protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed. All human subjects, in these referenced studies, gave informed consent to participate in these studies.

Additionally, some other protocols for GBM cells cultures are used with the aim to obtain some ‘intermediate’ forms of cell cultures:

1. Culture medium supplemented with EGF, bFGF and B27 with the aim to obtain adherent cells (ACs) in the culture.
2. Medium supplemented with 10% foetal bovine serum (FBS) with the aim to obtain adherent cells (ACs) in the culture.

Discussion

Neurospheres

As mentioned above one of the forms of growth of GBM cells is the NSs as serum-free medium with EGF and bFGF are needed for their formation. This is cell growth type which is common for both NSCs and CSCs. The NSs are heterogeneous aggregates (Figure 1a) and it is believed that they originate from a single cell and its progeny in the NSs are cells from type B or type C. NSs have self-renewal capacity, clonal origin and after dissociation of the structure they form secondary spheres. It is assumed that the NSs represent the most malignant zones of GBM, they are formed from CSCs and have the same genetic and phenotypic characteristics of the primary tumour. The phenotypic changes are manifested by expression of ‘stem-markers’ typical for NSCs such as Nestin, Sox-2, CD44, CD133 and lack of GFAP. Some authors presume that the CD133+ cells form the NSs and in the same time they express Nestin and GFAP simultaneously, while others claim that CD133− cells form the NSs as serum spheres.
can form NSs too\textsuperscript{13}. Data are reported that CD133 is expressed rarely and its expression is quite weak in NSs\textsuperscript{26} and another study demonstrates that from 11 GBM samples growing as NSs only four of them are CD133 positive, whereas in seven samples from this series there is very weak expression or lack of this marker. All samples show tumourigenicity and form NSs when cultured in vitro\textsuperscript{21}.

Adherent cells

Cells isolated from GBM grow as ACs when cultured in the presence of FBS but without growth factors\textsuperscript{26}.

They form a monolayer characterised initially by its heterogeneity which later turns into a homogeneous monolayer of fibroblast-like cells (Figure 1b). The ACs are genetically and phenotypically different from the primary tumour and are capable of self-renewal\textsuperscript{24,9,26}. It is assumed that the seed is due to irreversibly irreversibly irreversible alterations associated with partial differentiation of the GBM cells and this is reflected on the markers expression\textsuperscript{29}. A lower expression of Nestin and Sox-2 is observed compared with the NSs, but a higher expression of GFAP and βIII-tubulin is observed\textsuperscript{28,18,28}. Still, simultaneous expression of Nestin and GFAP has been reported with AC\textsuperscript{7}. Cultures with serum lead to loss of the CD133 expression\textsuperscript{29,26} and as a whole it is assumed that ACs are characterised with ‘partial stemness’\textsuperscript{2}, loss of GBM characteristics, lower capacity of self-renewal and questionable tumourigenicity\textsuperscript{6,29}.

Comparison between neurospheres and adherent cells and ‘intermediate models’

Published data predominantly claim that the NSs are a better model of CSCs\textsuperscript{23} with higher expression of Nestin, Sox-2 and CD133 and lower expression of differentiation markers such as GFAP\textsuperscript{26}. The NSs have the same genetic alterations as the primary tumour; they are with higher tumourigenicity and higher capacity of self-renewal compared with the ACs. However, it should be stated that the differences between NSs and ACs are not so salient.

Some ‘intermediate’ forms between NSs and ACs have been described and most commonly defined as ‘semi-adherent’ cultures (Figure 1c) in which both NSs and ACs are observed\textsuperscript{18,28}. Moreover, ACs are described which in the presence of serum form aggregates on the bottom of the wells which strongly resemble NSs and when transferred to serum-free medium grow as NSs\textsuperscript{26}.

Although the NSs are accepted as a better model for CSCs as compared with ACs\textsuperscript{26}, there are data which cast doubt on the strict distinction between both cell types as far as the fraction of CSCs are concerned. When NSs are transferred to medium with serum they grow as ACs and vice versa ACs grow as NSs in serum-free medium with EGF and bFGF. This raises the question about some crucial changes in the cell behaviour\textsuperscript{2} and because of that models for cultures are designed which combine the conditions for NSs and ACs. When GBM cells are cultured in serum-free medium with EGF and bFGF in tissue plate coated with laminin, the NSs adhere and grow as ACs and this way the EGF and bFGF get access to all cells which are not accessible otherwise. Almost all cells were shown to be positive for Nestin, Sox-2, CD133 and CD44 which accepted as proof of this assumption\textsuperscript{26}.

Another ‘intermediate model’ uses plates coated with polyHEMA which prevents the adhesion and the GBM cells are cultured with FBS. ‘Serum spheres’ are obtained and the cells show stronger expression of Nestin, Sox-2 and CD44 compared with ACs and NSs. The ‘serum spheres’ do not express CD133 and show an increased migration activity and formation of cell colonies\textsuperscript{26}.

The ‘intermediate model’ that we have developed and used is characterised with culturing of GBM isolated cells in medium supplemented simultaneously with EGF, bFGF and 10% FBS. In all cases we observed growth of ACs and just one case the growth was defined as ‘semi-adherent’. Confocal microscopy and flow cytometry studies of the expression of CSCs markers showed that in GBM samples about 97.1% of the cultured cells expressed Nestin, 79.3% Sox-2, 78.8% CD44 and 71.3% GFAP\textsuperscript{26}. Expression of CD133 was detected just in two GBM samples\textsuperscript{26}. These results show that the model used is closer to the other ‘intermediate models’ than the classical models, such as NSs and ACs. In spite of serum presence the cultured cells expressed in higher percentages CSCs markers with exception of CD133. Although EGF and bFGF were present in the culture medium, the cells grew as ACs and just in a single case they were ‘semi-adherent’ cells but we never observed NSs under these culture conditions (Table 2).

Conclusion

It can be stated that the protocol for GBM cells cultured as NSs is not the only one and not necessarily the best model for CSCs. Plenty of data have been reported pointing to the assumption that the NSs cells expressing specific markers cannot explain completely the concept of CSCs. The reasons are that NSs do not always express higher levels of markers for CSCs compared with ACs and ‘intermediate models’, however ACs and ‘intermediate models’ cells have many properties of CSCs.

Abbreviations list

ACs, adherent cells; bFGF, basic fibroblast growth factor; CSCs, cancer stem cells; EGF, epidermal growth factor; FBS, foetal bovine serum; GBM, glioblastoma multiforme; GFAP, glial fibrillary acidic protein; NSCs, neural stem cells; NSs, neurospheres; polyHEMA, poly-2-hydroxyethyl methacrylate.

Licensee OA Publishing London 2014. Creative Commons Attribution License (CC-BY)

References

Licensee OA Publishing London 2014. Creative Commons Attribution License (CC-BY)