

ISOLATION AND CHARACTERIZATION OF GLIAL TUMOR CELLS WITH STEM-LIKE PROPERTIES

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Abstract

Cancer is the most complex disease of modern medicine, a major public health problem of the 21st century, a multifactorial disease that arises from disorder in gene expression involved in the normal metabolism of cell. The statistics state that over half a million people died of cancer in 2012 and more than 1.5 million will be diagnosed with a malignant tumor in the U.S. alone (Ahmadi R. et al., 2009). Compared with other specialties where the mortality rate has dropped dramatically in recent decades, the prevalence of cancer records ascending trends. The standard treatment is generally not changed in the last 20 years, consisting of surgery followed by adjuvant chemotherapy or radiation therapy. Despite progress in the pharmaceutical field, standard therapy for malignant gliomas is merely palliative, but if we cure the patient diagnosed with brain cancer and prolong life not just a few months, a different approach is needed and a better knowledge of tumor cell biology.

Key words: glial tumor cells, isolation, therapy, chemo-radio-resistant.

INTRODUCTION

Stem cells present in adult bodies, in all tissue, are pluripotent cells (they have the ability to give birth to cells belonging to several cell lines derived from the 3 embryonic layers: mesoderm, ectoderm and endoderm).

Characteristics that define stem cells are self-healing ability and extensive proliferation (Smith A. A., 2006). The starting point was the discovery of deeper functional characteristics of embryonic stem cells and embryonic germ cells. It was observed the presence of stem cells in almost any tissue in the brain, liver, and muscles. The physiological status of normal stem cells is the "quiescent", being in G0. This phenomenon of plasticity of the cells means that the cell has the ability to differentiate into multiple tissue types. Following various signals received from the environment, asymmetric division is triggered that generates both an identical stem cell (self-renewal) and a low plasticity progenitor. The progenitor will continue dividing and forming asymmetric intermediate compartment (transit amplifying cell) that will generate ultimately differentiated specialized cells. Some cells, such as the bone marrow, are always active, with rapid division necessary to maintain the massive erythrocytes and white blood cells. In other locations, such as skin or intestinal epithelium, stem cells divide slowly but steadily replacing tissue. This capability has raised another issue that of the target cell for malignant

transformation - the pluripotent stem cells as a potential starting point of carcinogenesis (Giangreco A. et al., 2007). These cells are susceptible to carcinogenic agents, due to their increased proliferative capacity with the development of mutations during the replication of DNA. Stem cells can become tumorigenic only if there is at least one permanent genetic mutation that destabilizes their growth (Le Celso C. et al, 2004). There is also another hypothesis, equally interesting, about the differentiation of adult stem cells in response to tissue damage (Weissman I.L. et al., 2001).

Tumor stem cells can occur in two ways:

- Loss of cell growth regulation of normal stem cell;
 - The dedifferentiation of adult stem cells or progenitor cells.
- Stem-cell activation can take place in three ways:
- Enabling spontaneous division similar to embryonic or hematopoietic cells;
 - Hormonal stimulation (estrogen in breast and ovarian cancers);
 - Stimulation following an inflammatory process, exposure to chemical carcinogens, infection, environmental factors, smoking, UV radiation etc.

MATERIAL AND METHODS

Isolation of these cells was performed by two methods. The tumor fragment was completely immersed in the culture medium, kept at 4°C for 24 hours without affecting cell viability. The processed fragment was transferred to a recess with laminar flow in the Petri dishes. After that several washes with phosphate buffer (PBS) took place. The fragment was reimmersed completely in medium. Mechanic sterile process followed up to obtain as small pieces (2-3 mm) as possible. Cells are released into the environment, and the cell suspension was taken up in a syringe and passed through the Filcons filter type (the network 70µm) to obtain a single cell suspension. The cell suspension is then centrifuged at 1200rpm for 7 minutes at 19°C. The supernatant is discarded and the cell pellet suspended in 7 ml complete medium and seeded into 25cm² Cole flasks, and incubated at 37°C in a 5% CO₂ atmosphere.

The other method used is that of tumor explants. Tumor fragment is washed in medium supplemented with antibiotics and then dissected into 1x1x1 mm³ fragments and then cultured in Petri dishes in 1 ml fetal calf serum (FCS). After about three hours there was added 3 ml of DMEM (Dulbecco's Modified Eagles Medium) supplemented with FCS, glutamine, antibiotics and non-essential amino acid (purchased from Sigma Aldrich, St. Louis, MO, USA). After 3 weeks it was observed a population of cells with fusiform appearance. When it reached about 70-80% subconfluent, the cells

were detached using trypsin 0.25% and addition of EDTA. Cells were resuspended in DMEM/F12 (1:1) supplemented with 15 ng/ml fibroblast growth factor (FGF), 20 ng/ml epidermal growth factor (EGF), 2 mM/L - Glutamine, 4 IU insulin growth factor (IGF) and B27 (1:50). High viability > 80% was confirmed by staining with Trypan Blue. The cells were grown and stored in an incubator at 37°C and 7% CO₂.

When reaching 70-80% subconfluent cultures, protocol requires passage of cells, followed by freezing them. Posting of cell from growing plate is performed using treatment with 0.25% trypsin EDTA for 5 min at 37°C and then two washes with PBS of adherent cells layer. Trypsin was inactivated in an environment with 10% fetal calf serum, and the detached cells are transferred to centrifuge tubes and centrifuged at 1200 rpm for 5 min. The cell pellet is then resuspended in culture medium and the cells are passed into new culture plates.

Freezing can be made during cells' passage. After the cell suspension is obtained, some cells were resuspended in 1.5 ml of freezing medium: complete medium in which the cells were grown to 30-60% fetal serum and 10% dimethyl sulfur oxide (DMSO). Cells and the freezing medium are transferred into Mr. Frosty cryovials containing isopropanol, which will be subject to a gradual freezing 1°C/ min in the freezer (-80°C). Two days cryovials are immersed in liquid nitrogen at -170°C. Thawing of the cells must be performed as quickly as possible (2-3). Cryovials from liquid nitrogen are immersed in a water bath 37°C, and the cells were pipetted gently, centrifuged at 1200 rpm for 5 minutes at 22°C. After disposal of the supernatant, the cells are resuspended in culture medium supplemented with 20% fetal calf serum and seeded in culture dishes.

The ability to proliferate in culture medium without FCS serum-free.

Tumor cells are grown in a final concentration in serum-free medium 100.000/ml. After 3-5 days some of the cells will initiate the process of apoptosis, but most of them are detached from the flask's surface and form three-dimensional structures similar to spheres. These tumor-spheres are initially formed from 5-10 cells, but in time they will grow to much larger sizes, reaching 100-150 cells. If they are collected and centrifuged at 1000 rpm for 10 minutes, tumor-spheres will be disrupted. However, after replating, the process repeats itself and after 3-5 days the spheres will be observed in culture again (Oermann E. et al., 2010).

Tumor resistance to various chemotherapy (multidrug resistance).

Multidrug resistance can be present from the time of diagnosis or may be acquired later, after a period of clinical and radiographic remission. Different molecular mechanisms are responsible for this clinical observation. The best studied is the mediator MDR1 P-glycoprotein with ABCG2. Measurement of MDR1 in cell cultures was initially facilitated by

the observation that different small molecules with fluorescent properties, such as DiOC2, Rhodamine 123, Calcein AM, are correlated specifically to ABCG2 pump. We have used the test of Rhodamine 123 efflux. At 24 hours after repetitive chemotherapy, tumor-spheres were collected in 1.8 ml Eppendorf tubes, centrifuged at 3000 rpm for 5 minutes and mechanically separable with a syringe needle. After washing with PBS, the cells were counted and 20,000 cells were resuspended in 90 μ l PBS. There was added 10 μ l of the working solution of 10 μ g/ml Rhodamine 123 so that the final concentration of Rhodamine was 1mg/ ml. Eppendorf tubes were incubated for 30 min at 37°C in the dark under 7% CO₂. After expiry of the incubation period the cells were washed twice by centrifugation at 3000 rpm for 5 minutes with cold PBS (4°C) and transferred to 24-well plates for fluorescence intensity measurement.

Immunocytochemical staining - to demonstrate the presence of normal stem cell-specific membrane proteins, and of tumor stem cell.

Immunocytochemical staining protocol is started by preparing the fixing solution, containing 4% paraformaldehyde in PBS (4g paraformaldehyde/ 100ml PBS). For a more rapid dissolution, the solution is heated to 90°C. The block solution and the permeability of the membrane solution can contain bovine serum albumin (BSA) 1%, Triton X-100 0.1% in PBS or BSA 10% in PBS. Setting the samples was performed with 4% paraformaldehyde solution for 20 minutes at room temperature. Fixing was followed by 3 washes with PBS (Cui L. et al., 2010). The cell membrane is permeabilized by exposing cells over 15 minutes to a solution of 0.1% Triton X100 followed by 3 washes with PBS. The primary antibodies are block by nonspecific binding using 10% BSA for 20 minutes at room temperature, followed by 3 washes with PBS, and incubating at 4°C for 1 hour or overnight at 4°C, followed by 3 washes with PBS. Incubation with the secondary antibody labeled with fluorochrome, in the dark and at 37°C for 45 min is followed by washing three times with PBS and by the fitting of the lamella.

RESULTS

In the case of glial progenitors, isolation is much faster compared to other lines isolated in our facility. The early progenitors were observed around the tumor explants after 3-5 days (*Fig. 1*). Also, the phenotype is different. Initially, tumor cells have a neuron-like phenotype with many extension (*Fig. 2 and 3*), but after three consecutive passages, there was selected a subpopulation of cells with fusiform appearance (*Fig. 4 and 5*). The cells have a fusiform appearance, a high proliferation rate and the property of clone expansion (*Fig. 6*). Clone expansion was demonstrated by

the ability of a single cell to divide itself until the appearance of a group of cells identical to the original progenitor.

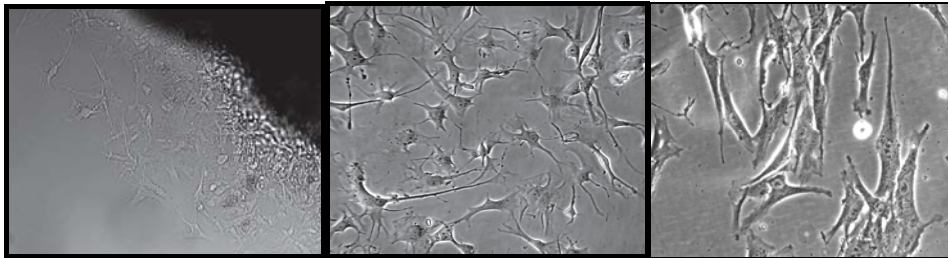
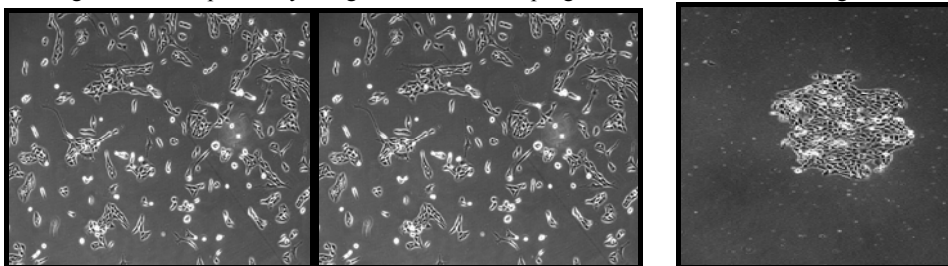


Fig. 1 Tumor explant Day 5 Figure 2 and 3. Glial progenitors. 100x and 200x magnification



Figures 4 and 5. Changing the appearance of glial cells Figure 6. Clonal expansion property

All cells have the property to form sphere-tumors when they are grown in serum-free medium (*Fig. 7 and 8*). The serum-free medium is DMEM/F12 culture medium supplemented with the mentioned growth factors, but without fetal calf serum. Sphere-tumors formation is an acknowledgment of the strain tumor in vitro potential to invade surrounding tissues and even distant metastasis.

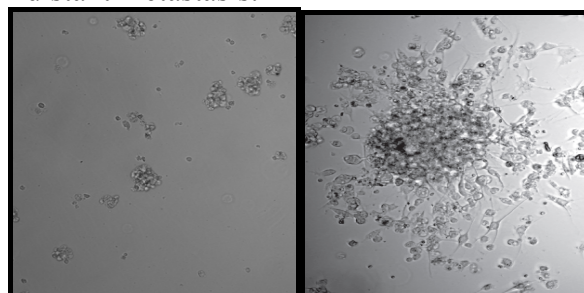
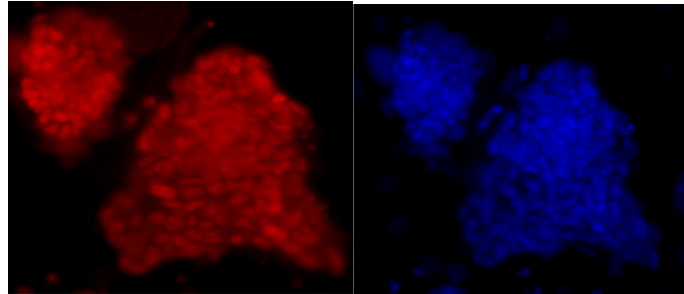


Figure 7 and 8. Sphere-tumors in serum-free medium

But comments on the appearance of the cell using optical microscopy are absolutely insufficient to demonstrate that the isolated populations are indeed tumor stem-like progenitors. A first step to prove the stem-like character is staining immunocytochemistry using monoclonal antibodies to reveal the presence of certain proteins or intracytoplasmic membranes (Lo Celso C. et al., 2004, Patru C. et al., 2010). These proteins are markers of pluripotent, being present only in the early stages of human

ontogenesis. The main pluripotent markers for glial cells are CD90+ and CD133+. (Fig. 9 and 10).



Figures 9 and 10. Coloration anti-CD133 and anti-CD90 stem-like cells isolated from glioma

A further step in characterization of the tumor stem cell function is efflux of Rhodamine 123 test. These cell lines were compared to stabilized HFL (fibroblasts isolated from the normal lung parenchyma) and MLS (differentiated tumor cells isolated from ovarian adenocarcinoma). Glial tumor stems have a more intense level of dye efflux compared to normal differentiated human cells (HFL) or differentiated tumor cells (MLS) (Fig. 11).

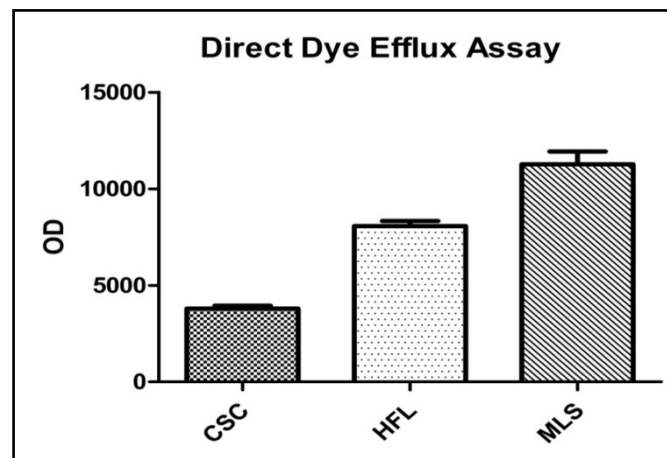


Figure 11. Rhodamine 123 efflux test for glial tumor progenitors

DISCUSSIONS

Heterogeneous nature of cancer was inferred by pathologists for more than a century through Rudolf Virchows' publications (Ikushima T. et al., 2009), but the fact that immature tumor cells can differentiate in vivo in less pluripotent progenitors was demonstrated with the development of modern techniques of molecular biology in the 1980's. First functional tests

for tumor cell progenitors are published decades ago by Schlag and Flentje. These tests involved agar gel in the development of tumor cell colonies derived from cancers (Rahman M. et al., 2011). The modern concept of chemotherapy-resistant tumor cell progenitor is relatively new in the literature and provides a plausible explanation for failure of malignant tumors. Tumor stem cells can withstand conventional therapy due to the presence on the surface of the cell membrane of an efflux pump that is part of the ABC proteins family.

The structure is composed of 5 domains of ABCG2 submembrane (*Fig. 8*) (Bosch T.M., 2008). Its work is based on the formation of the intermediate cycle of ATP reaction that underlies the ATP degradation in ADP and subsequent energy used in the transport of various organic or inorganic compounds outside the cell. Over-expression of the ABCG2 is observed in several tumor cell lines, and confers such a phenotype resistant to polychemotherapy. This resistance is conferred compared to multiple compounds such as Mitoxantrone, methotrexate, Topotecan, platinum, Flavopiridol or different anthracyclines. ABCG2 pump interact with drugs recently approved by the FDA as tyrosine kinase inhibitors Gleevec / Imatinib and Iressa (Brendel C. et al., 2007, Ivnitski-Steele I. et al., 2008, Kourti M. et al., 2007).

CONCLUSIONS

Hypothesis of tumor stem cells represents a turning point in medical oncology. This hypothesis unifies clinical observations and laboratory data into a single coherent model of carcinogenesis.

Compared to the classic model where all cells have equal potential to invade surrounding tissue and distant metastasis, the modern concept launches the idea of the existence of a small population of tumor cells with intrinsic tumorigenicity properties.

Conventional chemotherapy can eliminate differentiated cells in active division, but is completely ineffective if stem cells, in G0 stage.

Under the influence of tumor niche, this population has a clear evolutionary advantage compared to its equivalent more differentiated, avoids normal cell cycle checkpoints proliferation and ultimately leads to tumor relapse and metastasis to other organs.

In conclusion, it is necessary to isolate and correctly identify CST so they could be studied properly and to develop future effective therapy in oncology and radiotherapy.

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