Original Article

Isolation, identification, and spheroids formation of breast cancer stem cells, therapeutics implications

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ABSTRACT

Aims: Cancer stem cells (CSCs) are population of cells present in tumors, which can undergo self-renewal and differentiation. Three-dimensional (3D) *in vitro* models mimic features of the *in vivo* environment and provide unique perspectives on the behavior of stem cells. **Materials and Methods:** In this study, MDA-MB 231 cells were grown in two-dimensional (2D) monolayers and 3D spheroid formats and CSCs were isolated and grown as spheroids. The isolated CSCs were subjected to molecular studies for detection of CD44, CD24, MMP1, ABCG2, ALDH1, and GAPDH markers. **Results:** The monolayer of CSCs grown as spheroids showed better growth rate than the MDA-MB 231 cells, which shows the efficacy of 3D spheroid format of growing CSCs. CD44 show increased expression in spheroids compared to 2D culture of MDA-MB 231. ALDH1 a key marker of breast stem cells was highly expressed in BCSCs and MDA-MB 231 grown in 3D, while being absent in CSCs and MDA-MB 231 cells grown in 2D. **Conclusions:** The CSCs grown as spheroids showed better growth rate, which showed the efficacy of 3D spheroid format for CSCs culture. Since the association between BCSCs prevalence and clinical outcome and the evidence presented in this study support key roles of CSCs in breast cancer metastasis and drug resistance, it has been proposed that new therapies must target these cells.

Key words: Cancer, cancer stem cells, MDA-MB 231, reverse transcriptase-polymerase chain reaction, spheroid

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in women, encompassing 16% of all female cancers. It is estimated that 1.4 million new cases of breast cancer occurs worldwide annually. However, breast cancer has been thought to be a disease of the developed countries, about 69% of all breast cancer mortality occurs in developing countries.[1] The breast cancer, which might be invasive, is heterogeneous with respect to histology, gene expression, and clinical outcome. The deepening of our understanding of normal biology has made it clear that stem cells have a critical role not only in the generation of complex multicellular organisms, but also in the development of tumors. Previous findings support the concept that cells with the properties of stem cells are integral to the development and perpetuation of several forms of human cancer.[2,3] The discovery of cancer stem cells (CSCs) in solid tumors has changed our view of carcinogenesis and chemotherapy. One of the unique features of the bone-marrow stem cells that are required for normal hematopoiesis is their capacity for self-renewal. In the hematopoietic system, there are three different populations of multipotent progenitors stem cells with a capacity for long-term renewal, stem cells with a capacity for short-term renewal, and multipotent progenitors that cannot renew, but differentiate into the varied lineages in the bone-marrow.[3,4] Cancer stem cells play an important role in the cancer recurrence after the treatment, the development of metastasizes and therapeutic resistance. This is owing to its potential for multilineage differentiation, high tumorigenicity and strong ability for cell invasion.[5]

Hence, the main focus of research is on the therapeutically oriented studies involving the evaluation of newly developed

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anti-cancer drugs. The conventional two-dimensional (2D) analysis of monolayers of tumor cells resulted in the loss of structure and tissue function. To understand the complex biology of breast cancer and improve the clinical management of the disease, three-dimensional (3D) experimental model system that recapitulates the *in vivo* functions, interactions and architecture of the mammary gland and breast tumors is needed.[6] Several progresses were made in understanding the mechanisms of cancer development.[7] Cancer stem cells (CSCs) are tumor cells with similar fundamental attributes to normal adult stem cells which are capable of dividing asymmetrically to produce stem cell for self-renewal, and progenitor cell to produce phenotypically diverse cancer cells that constitute tumors.[8] CSCs are thought to play a role in recurrence of cancer after treatment and contribute to metastatic breast cancer.[9] The CSC hypothesis considers that only CSCs will initiate and sustain tumor growth, but also are responsible for the metastatic dissemination and therapeutic resistance of tumors.[10] In this study, CSCs were isolated and grown as 3D spheroids. The CSCs were then analyzed for molecular markers such as CD44, CD24, MMP1, ABCG2, ALDH1, and GAPDH (housekeeping gene). Here, we report that the monolayer of breast cancer stem cells grown as spheroids showed better growth rate than the MDA-MB 231 cells and thus, it shows the efficacy of spheroid format of growing CSCs.

MATERIALS AND METHODS

Cell culture

Established breast cancer metastatic cell lines (MDA-MB 231) obtained from American Type Culture Collection (Manassas, VA, USA). The cancer cells were maintained in Libovitz's Media supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37°C with no CO_2.

Isolation of stem cells

To provide a novel tool for the isolation of $CD44^+$ cell populations, a monoclonal antibody specific for CD44 was coupled to superparamagnetic MACS micro-beads. After trypsin-based dissociation, the cells were incubated with CD44 micro-beads for 15 min, washed, separated using an LS column, and identify using polymerase chain reaction (PCR) machine by looking for CD44 gene.

Spheroid formation

Breast CSCs spheroids and breast cancer cells spheroids were grown by inoculating 1×10^5 breast cancer cells and 1×10^4 BCSCs and distributed equally on wells in two nonadherent 96-well plates in 500 ml DMEM in each plate bottom by 200 µL of 1% agarose and supplemented with antibiotics, epidermal growth factor, basic fibroblast growth factor, bovine serum albumin, and 10 mM HEPES in addition to 1 × B27 in case of BCSCs.[11,12,13] Clusters of cells were observed after 24 h of initiation. However, it took nearly 4 days for these clusters to form spheroids. The medium was changed on alternative days tilll spheroids were 21 days old.[14] Tumor-sphere numbers are counted under a phase-contrast microscope using the ×40 magnification lens. Process and quantify the digital images (300 pixels inch-1) using ImageJ software (National Institutes of Health, USA).[15]

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from MDA-MB 231 cells, BCSCs in monolayer and 3D module and HT29 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. cDNA was synthesized in 20 µL reaction containing 5 µg total RNA, 1 µg oligo (dT), 0.2 µg random hexamers, 2 µL of 10 mM deoxynucleotide triphosphates mix (25 mmol/L each of deoxyguanosine triphosphate, deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate), and 1 µL of 200 units/µL superscript reverse transcriptase (RT). PCR was carried out in 50 µL reaction containing 1 µL of the synthesized cDNA in 30 cycles of 30 s at 94°C, 30 s at the melting temperature of the primers, and 30-60 s depending on the product size (60 s for 1 kb) at 72°C. Products were resolved on a 2% agarose gel.

RESULTS

MDA-MB 231 cells was grown in 2D monolayers and 3D spheroid formats. The CSCs were isolated using CD44-laden micro-beads and grown as 3D spheroids. The growth curve of monolayer cancer cells and CSCs grown in 3D culture was showed in Figure 1. The results showed that CSCs isolated from cancer cells grow efficiently in 3D format for 21 days and afterword the cells depleted.

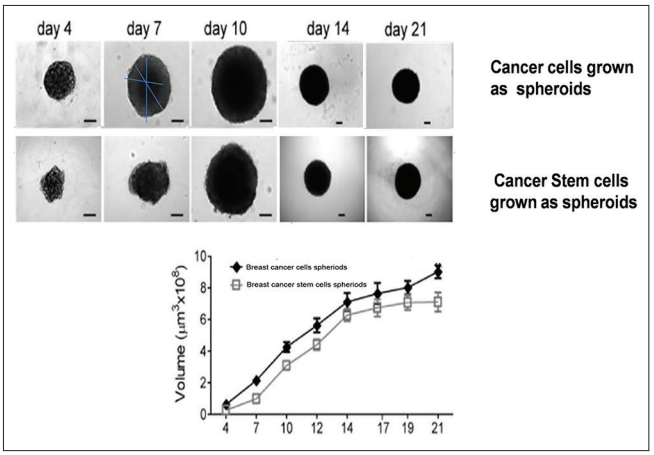

Figure 1: Growth of tumor-spheroid in agar-coated 96 wells flat-bottomed plated with corresponding growth curve. Scale bar: 100 µm

The monolayer of CSCs grown as spheroids in a 96-wells plate showed better growth rate than the MDA-MB 231 cells grown as spheroids, as shown in Figure 2. The 3D CSCs formed spheroids in 50 wells in 96 wells plate compared to cancer cells that formed only 32 spheroids in 96 wells plate.

MDA-MB 231 cells and the CSCs isolated from MDA-MB 231 cells grown in 2D and 3D formats were subjected to molecular studies. From the MDA-MB231, CSCs grown in monolayer and 3D cultures as well as HT29 cells total RNA was isolated and cDNA was prepared and used for RT-PCR studies. The cDNA was amplified with specific primers as showed in Table 1 for CD44, CD24, MMP1, ABCG2, ALDH1 and GAPDH (housekeeping gene). The amplified product was run on agarose gel as shown in Figure 3. HT29 cells amplified with CD24 and GAPDH primers to evaluated validity of CD24 primer since it not observed in other cells.

In the molecular study, CD44 showed higher expression in CSCs in 3D compared to MDA-MB 231 grown in 3D. However, the CD44 expression was appreciably lower in monolayer MDA-MB 231 compared to monolayer CSCs. CD24 expression was not observed in any culture. MMP1 which is shown to support cancer metastasis was expressed in 3D culture only. Furthermore, ALDH1 a key marker of BCSCs was expressed 3D culture only. ABCG2 expression was observed in all cultures except 2D culture of MDA-MB 231. GAPDH a house keeping gene was used as control, which was expressed in all culture formats.

DISCUSSION

The existence of CSCs is a subject of debate within medical research, because many studies have not been successful in discovering the similarities and differences between normal tissue stem cells and cancer (stem) cells[16] and the discovery of leukemia stem cells has suggested distinct cell subpopulations.[17] However, 3D culture systems allow cells to organize into structures and mimic their *in vivo* architecture. So far the *in vivo* like properties provided by the 3D model systems that have been implemented for the study of various tissues and cells, including skin, prostate, muscle, colon, bile duct, esophagus, adipocytes, fibroblasts, embryonic stem cells, and mammary cells.[6] In the present study, although of less BCSCs, it shown high efficacy in spheroids formation, whereas formed spheroids more than MDA-MB 231 cells due to self-renewal capacity of CSCs led to high multiplicity.[3,4] However, the insight into the biology of the normal and malignant breast, and to create *in vivo* like models for therapeutic approaches in humans is still lacking. As a result, the 3D model involved in the present study might be useful in testing the drug and validating the target.

CD44, CD24, ALDH genes are key markers for BCSCs $ALDH^+/CD44^+/CD24^-$ subpopulations.[18] In our study, CD44 and MMP1 show highly expression in 3D format of BCSCs and MDA-MB 231 cells and this interpreter breast cancer metastasis behavior since spheroids mimic tissue *in vivo*.[6,19,20] ABCG2 gene shown highly expression in BCSCs in both formats (2D and 3D cultures) as well as 3D format

Table 1: The primer sequences

Gene	Direction	Sequences
CD44	F	5'-GGCCGAATTCIGCACAGACAGAATCCCTGCTACC-3'
	R	5'-GGCCGAATTCIGGGGTGGAATGTGTCTTGGTCTC-3'
CD24	F	5'-GGCACTGCTCCTACCCACGCAG-3'
	R	5'-GCCACATTGGAATTCCAGACGCC-3'
MMP1	F	5'-CTGGCCACAACTGCCAAATG-3'
	R	5'-CTGTCCCTGAACAGCCCAGTACTTA-3'
ABCG2	F	5'GCATTACATGCGGCCGCGATCCTGAG CCTTTGGTTAAGACC-3'
	R	5'CAGGAGTTTCCAGAATTCAATTCTCC-3'
ALDH1	F	5'-TTGGAATTTCCCGTTGGTTA-3'
	R	5'-CTGTAGGCCCATAACCAGGA-3'
GAPDH	F	5'-AGGGCTGCTTTTAACTCTGGT-3'
	R	5'-CCCCACTTGATTTTGGAGGGA-3'

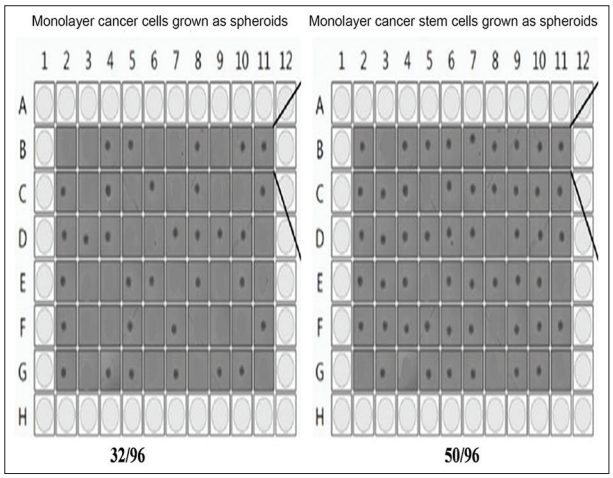

Figure 2: The growth rate in number of spheroids formed by monolayer breast cancer cells and monolayer breast cancer stem cells

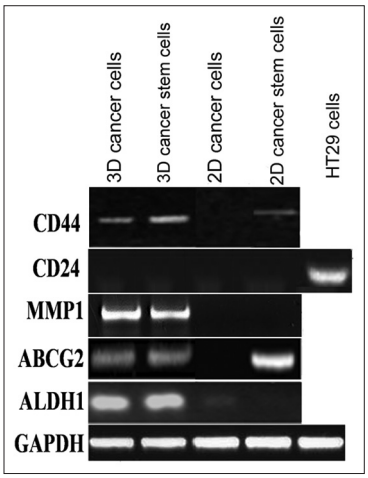

Figure 3: Six genes expressed in three-dimensional and two-dimensional modules of breast cancer cells and breast cancer stem cells

of MDA-MB 231 cells gave rise to understand breast cancer drug resistance.[21]

Currently, breast surgery and irradiation are the local therapies of choice and chemo-hormonal and antihuman epidermal growth factor receptor 2 (HER2, ERBB2) therapies are commonly used as a systemic treatment to prevent outgrowth of distant metastases.[22] For this goal, different strategies have been evaluated, including targeting of membrane markers and transporters, interruption of intracellular signaling pathways and alteration of the BCSCs microenvironment. Thereby 3D cultures of human breast tumors have great potential in providing a better understanding of the pro- and anti-tumorigenic effects of the host-stroma interactions before proceeding into clinical trials on humans.

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