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VIROTHERAPY TARGETING BREAST CANCER STEMS CELLS BY USING *ONCOLYTIC HERPS SIMPLEX VIRUS.*

MAYTHAM ABBAS ABBODI

Department of Microbiology, Indian Academy Degree College Bangalore, India

ABSTRACT

In the present study I isolated breast cancer stem cells from cell line MDA-MB231 by used CD44 MicroBeads separator. Cancer stem cells (CSCs) were identified by used PCR machine; here specific primers were designed for CD44 and CD24 genes, whereas CD44 positive for breast CSCs and CD24 negative CSCs. MDA-MB231 cells and breast CSCs treated with G47Δ vector. G47Δ showed highly effect on both kinds of cells by killing over 90% of MDA-MB231 and over 80% of the CSCs in vitro. Virus used as therapy demonstrated that pathogenic microorganisms can be modify genetically and use for targeting diseases and oHSV can be used for treatment breast CSCs that are still not understood. This study demonstrates that oHVS effective against breast cancer stem cells and could be a beneficial method for treating cancer stem cells expressed in breast cancer.

KEYWORDS: **oHSV, virotherapy, cancer stem cells, vector , breast cancer, G47Δ.**

MAYTHAM ABBAS ABBODI
Department of Microbiology, Indian Academy Degree College Bangalore, India

INTRODUCTION

Cancer is the most complex genetic disease that is caused primarily by environmental factors. Breast cancer is the most common cancer among women and although it can be diagnosed and treated earlier before tumor develops, it is the second reason of death caused by cancer among women in the world[1]. CSCs 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 multi lineage differentiation, high tumorigenicity and strong ability for cell invasion. $CD^{44+}CD^{24-/low}$ cells are considered as the stem-like cells in the breast cancer. As a consequence, the identification of these cancer stem cells remains paramount in the development of therapeutics owing to the resistance to conventional anti-cancer drugs[2]. Hence the core focus of research on therapeutically oriented studies to find out newly anti-cancer drug. Many challenges and progresses to understand mechanism of development of cancer[3]. Oncolytic virotherapy is not a new idea – as early as the mid 1950s doctors were noticing that cancer patients who suffered a non-related viral infection, or who had been vaccinated recently, showed signs of improvement[4]. In the 1940s and 1950s, studies were conducted in animal models to evaluate the use of viruses in the treatment of tumors. In the 1940s-50s some of the earliest human clinical trials with oncolytic viruses were started. However, for several years' research in this field was delayed due to the inadequate technology. Research has now started to proceed more quickly in finding ways to use viruses therapeutically. As well as the direct anti-cancer effect, oncolytic viruses are also capable of inducing an anti-tumor immune response[5].

Oncolytic virotherapy is a new therapeutic strategy that is based on the inherent cytotoxicity of viruses and their ability to replicate and spread in tumors in a selective manner[6]. From this short outcome we can define Oncolytic virotherapy as a new therapeutic strategy that is based on the inherent cytotoxicity of viruses and their ability to replicate and spread in tumors in a selective manner. Oncolytic herpes simplex virus (oHSV) vectors have many qualities that make them attractive cancer therapeutic agents: They have the ability to replicate in situ, disseminate within the tumor and transfer therapeutic transgenes. Further, they can induce antitumor immune responses and can be eliminated using anti-viral drugs. They are also minimally toxic to normal tissue, a feature important for clinical translation [6, 7]. Recent advances of Oncolytic virotherapy include preclinical proof of feasibility for a single-shot virotherapy cure and identification of drugs that accelerate intratumoral virus propagation. The primary clinical milestone has been completion of accrual in a phase 3 trial of intratumoral herpes simplex virus therapy using talimogene laherparepvec for metastatic melanoma[8].

MATERIALS AND METHODS

Cells and viruses

Established breast cancer metastatic cell lines such as MDA-MB 231 obtained from American Type Culture Collection (Manassas, VA, USA). The cancer cells were maintained in Libovitz's Media supplemented with 10% FBS and 2 mM L-Glutamine at 37ºC with no CO_2. All other normal cells were maintained in DMEM supplemented with 10% FBS and 4 mM L-Glutamine at 37 ºC with 5% CO_2. G47Δ was originally constructed by Todo T, Martuza RL, Rabkin SD & Johnson PA[9]. The virus was grown and tittered on Vero cells (African green monkey kidney; ATCC, Manassas, VA, USA) in DMEM with glucose (4.5 g l_1) supplemented with 10% calf serum at 37ºC in 5% CO2.

Identification of CSCs

To provide a novel tool for the isolation of CD^{44+} cell populations, a monoclonal antibody specific for CD44 was coupled to superparamagnetic MACS MicroBeads. After trypsin-based dissociation, the cells were incubated with CD^{44}

MicroBeads for 15 minutes, washed, separated using an LS Column, and identify using PCR machine by looking for CD44 and CD24 genes.

RT-PCR

Total RNA was extracted from breast cancer stem cells in monolyer using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. cDNA was synthesized in 20 uL reaction containing 5 ug total RNA, 1 ug oligo(dT), 0.2 ug random hexamers, 2 uL of 10 mM dNTP mix (25 mmol/L each of dGTP, dATP, dCTP, and dTTP), and 1 uL of 200 units/uL SuperScript Reverse Transcriptase. PCR was carried out in 50 uL reaction containing 1 uL of the synthesized cDNA in 30 cycles of 30 seconds at 94^0C, 30 seconds at the melting temperature (Tm) of the primers, and 30 to 60 seconds depending on the product size (60 seconds for 1 kb) at 72^0C. Products were resolved on a 2% agarose gel.

In vitro cytotoxicity assessment by MTT Assay

Growth inhibitory or inducing effects of various substrates on cell lines can be determined by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] proliferation assay as described by Mossman *et al.*, 1983[10]. Here medium used DMEM and proceeded protocol for three days as mentioned below. Day one: Trypsinize one T-25 flask and add 5 ml of complete media to trypsinized cells, centrifuge in a sterile 15 ml falcon tube at ~400 x g for 5 min, remove media and resuspend cells to 1.0 ml with complete media, take 10ul of the cell suspension in 10 ul of Trypan blue, count and record cells per ml, DILUTE the cells to $1X10^6$ cells per ml in complete media to dilute cells, Add 5000 total cells into each well and incubate overnight. Day two: Treat cells on day two with G47Δ with following concentrations 1000 pfu/ml, 5000 pfu/ml, 10000 pfu/ml, 50000 pfu/ml, 100000 pfu/ml; then Filter the G47Δ and add 100000 pfu/ ml in the triplicate wells, make dilutions for rest of the concentrations and add to the wells, incubate the plate back to incubator. Day three: Add 20 µl of 5 mg/ml MTS to each well, all should be done aseptically, incubate for 2 hours at 37 ^{o}C in incubator and read absorbance at 490 nm.

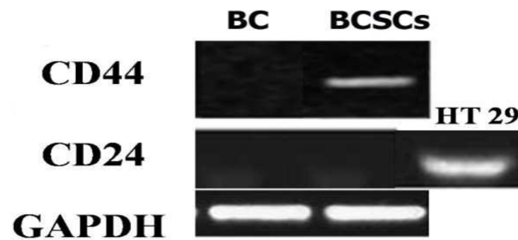
RESULTS AND DISCUSSION

The CSCs isolated from MDA-MB231 cells subjected to molecular studies. From the CSCs and MDA-MB231 cells total RNA was isolated and cDNA was prepared. The cDNA was used for RT-PCR studies. The cDNA was amplified with specific primers (Table-1) for CD44, CD24, and GAPDH (housekeeping gene). The amplified product was run on agarose gel as showed in figure 1.

Table 1
The Primer sequences

CD44	**F**	**5'-GGCCGAATTCIGCACAGACAGAATCCCTGCTACC-3'**
	R	**5'- GGCCGAATTCIGGGGTGGAATGTGTCTTGGTCTC-3'**
CD24	**F**	**5′-GGCACTGCTCCTACCCACGCAG-3**
	R	**5′-GCCACATTGGAATTCCAGACGCC-3′**

Figure 1
Gel electrophoresis picture shown different genes were expressed in 2D breast cancer cells and 2D CSCs.



The CSCs marker genes (CD44/ CD24) were verified using RT-PCR. In the molecular study CD44 gene expression was positive in CSCs compared MDA-MB231 cells was negative. Whereas CD24 was negative in both CSCs and MDA-MB231 cells grown as 2D. GAPDH a house keeping gene was used as control which was expressed in all cultures. The G47Δ showed dose-depended cytotocoxity. G47Δ was found to be highly cytotoxic to both types of cells MDA-MB231 and CSCs. Over 90% of the MDA-MB231 cells and over 80% of the CSCs were killed after 2 days when treated with the virus as shown figures 2 - 3.

Figure 2
Percentage Viability of G47Δ treated Cancer Monolayer cells.

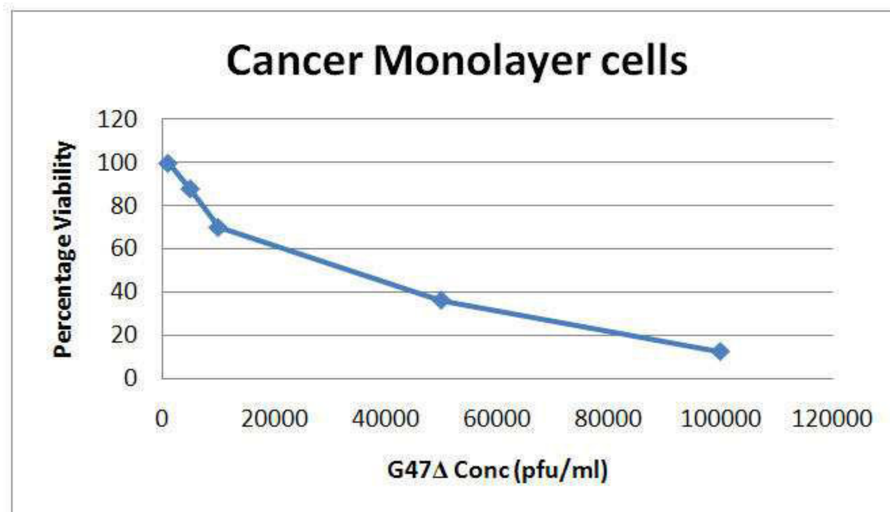
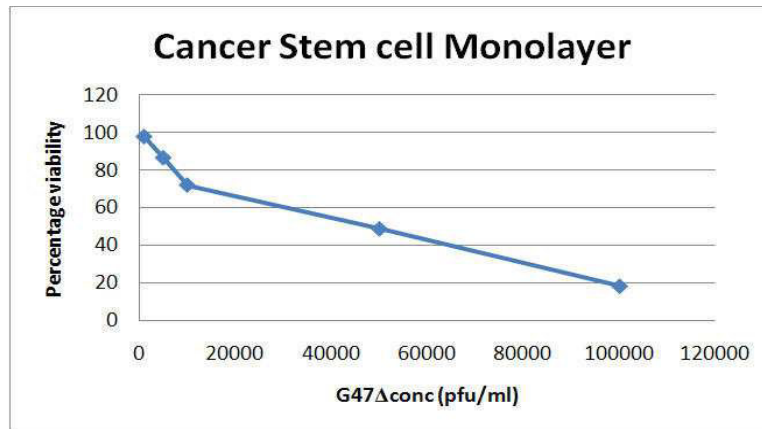


Figure 3
Percentage Viability of G47Δ treated Cancer Stem Cells Monolayer.



The oncolytic effect of G47Δ (100000 pfu) on breast CSCs and cancer cells was much more impressive as more than 90 % breast CSCs and cancer cells were killed. It is interesting from this study that the treatment of metastatic breast cancer cells with oHSV yielded significant oncolysis in both cancer cells as well as CSCs. The oHSV vector G47Δ was effective in killing human breast cancer stem cells and cancer cells in vitro to demonstrating that oHSV is efficient to treatment human breast cancer.

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