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(Article begins on next page)
Cancer stem cells have recently been isolated from several different solid tumors. In breast cancer, the CD44+CD24−/low population is considered to comprise stem-like cells. The identification of cancer stem cells has provided new targets for the development of therapeutics. Oncolytic herpes simplex viruses (oHSVs) are an effective strategy for killing breast cancer cells and treating breast tumors in preclinical models. Here, we examined the efficacy of the oHSV G47Δ in killing breast cancer stem cells. Human breast cancer cell line SK-BR-3 and human primary breast cancer cells were cultured in suspension under conditions conducive to the growth of stem cells. They generated mammospheres, which had cancer stem cell properties. The proportion of CD44+CD24−/low cells in these mammospheres exceeded 95%, as determined by flow cytometry. The mammospheres were found to be highly tumorigenic when implanted subcutaneously in nude BALB/c mice. G47Δ contains the LacZ gene, and X-gal staining of infected cells in vitro and in vivo showed the replication and spread of the virus. G47Δ was found to be highly cytototoxic to the CD44+CD24−/low population in vitro, even when injected at low multiplicities of infection, and G47Δ treatment in vivo significantly inhibited tumor growth compared with mock treatment. This study demonstrates that oHSV is effective against breast cancer stem cells and could be a beneficial strategy for treating breast cancer patients.

INTRODUCTION

Breast cancer is the most common cancer among women, and although it can be diagnosed and treated at an early stage, it is the second leading cause of cancer-related death among women in the United States.1 The disease recurs, mostly metastasizes, in about 30% of all patients with early-stage disease.2 Conventional chemotherapies are initially effective in disease control for patients with metastatic disease but ultimately, most fail. Cancer stem cells are thought to have an important role in recurrence after treatment and contribute to the incurable nature of metastatic breast cancer.3 These cells have the potential for multilineage differentiation, therapeutic resistance, hypoxic resistance and metastasis, and have high tumorigenicity and strong ability for cell invasion. Al-Hajj et al.4 found that palpable tumors were formed when a few hundred CD44+CD24−/low cells were injected into the mammary fat pad of non-obese diabetic/severe combined immunodeficient mice. In this study, we found that mammospheres are generated when the breast cancer cell line SK-BR-3 and human primary breast cancer cells, of which a high proportion are CD44+CD24−/low cells, are cultured in suspension. An important feature of stem cell-like cells is that unlike general tumor cells, they can initiate tumors even when a very small number are present. The present study also found this result. We also confirmed the stem cell-like features of the CD44+CD24−/low cells from among the SK-BR-3 and human primary breast cancer cells, namely, the expression of the common stem cell markers oct4 and sox2. To further determine the stem cell-like characteristics of the CD44+CD24−/low population, we examined them for a key marker of breast cancer stem cells—aldehyde hydrogenase (ALDH1) activity.5,6 Owing to their slow replication and ability to expel antitumor drugs, these cells are believed to be responsible for the failure of many conventional cancer therapies.7-10

Oncolytic virotherapy is a new therapeutic strategy that is based on the inherent cytolysis of viruses and their ability to replicate and spread in tumors in a selective manner.11 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.12 The second generation oHSV vector G207 is currently under clinical trials for malignant glioma.13,14 G207 contains deletions of both copies of the γ34.5 gene, which is the major determinant of HSV neurovirulence, and has the Escherichia coli LacZ gene in its IPC6 gene (UL39), whereby the ribonucleotide reductase needed for replication in non-dividing cells is inactivated.15 In G47Δ, which is derived from G207, the IPC47 gene and US11 promoter are also deleted, whereby growth and immunogenicity are enhanced and yet safety is maintained.16 G47Δ has been shown to be effective in several preclinical breast cancer models, including human xenografts, a model of metastatic breast cancer in the brain, and spontaneously arising breast...
tumors in transgenic mice. In the present study, we demonstrated the efficacy of G47Δ in suppressing cancer stem cells in a human breast cancer stem cell model.

MATERIALS AND METHODS

Cells and viruses

Human breast cancer tissue was obtained from six breast invasive ductal carcinoma patients who had not received chemotherapy but had undergone modified radical mastectomy (obtained from Breast Cancer Center of the Third Affiliated Hospital of Sun Yat-sen University, China). All samples were washed with phosphate-buffered saline (PBS), treated for fat removal, mechanically disaggregated, and then digested and filtered as described previously. The tumor tissue was minced and digested for about 12–18 h at 37°C with a solution of 100 U ml⁻¹ collagenase I (Gibco), 150 U ml⁻¹ hyaluronidase (Sigma), 10% calf serum (Gibco, Grand Island, NY, USA) and 5 mg l⁻¹ bovine insulin (Sigma, St Louis, MO, USA) in Dulbecco’s modified Eagle’s medium (DMEM; Gibco). The digested tissue was strained with a 40-μm strainer, the cell suspension was washed with PBS and the red blood cells were lysed. The cells treated by the above steps and the cell line SK-BR-3 (obtained from Dr Zeng Mu-sheng, Sun Yat-sen University Cancer Center, China) were cultured in DMEM-F12 medium (Gibco) supplemented with 10 μg ml⁻¹ basic fibroblast growth factor, 20 μg ml⁻¹ epidermal growth factor (both from Peprotech, Grand Island, NY, USA), 5 mg l⁻¹ insulin (Gibco) and 827 (1:50; Gibco) at 37°C in 5% CO₂. Foreskin-derived fibroblasts (obtained from Dr Zhang Qi, Department of Hepatic Surgery, the Third Affiliated Hospital of Sun Yat-sen University, China) that were isolated as previously described were cultured in 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) at 37°C in 5% CO₂. G47Δ was constructed as described previously. The virus was grown and titrated on Vero cells (American Type Culture Collection, Rockville, Maryland, USA) and on SK-BR-3 cells.

Identification of breast cancer stem cells

Vimentin, fibronectin, cytokeratin activity was measured with immunofluorescent staining. Primary human breast cancer cells and foreskin-derived fibroblasts were seeded into 48-well plates at 10,000 cells per well. After overnight incubation, cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Then cells were washed twice with PBS and incubated with 2% BSA and 0.2% Triton X-100 in PBS for 1 h at room temperature. Next, 200 μl of a primary antibody solution was added to each well, cells were incubated overnight at 4°C. Then cells were washed twice with PBS and incubated with phycoerythrin-labeled secondary antibody for 1 h at room temperature. Finally, cells were stained with hoechst 33342 for 10 min. Immunofluorescence was visualized using an immunofluorescence microscope. CD45 and CD31 activity were measured by flow cytometry with allophycocyanin-conjugated antibodies and a two-color immunofluorescence microscope. CD45 and CD31 activity were measured by flow cytometry with allophycocyanin-conjugated antibodies and a two-color immunofluorescence microscope.

RESULTS

Identification of human primary breast cancer cells

The purity of the primary marmospheres was verified by using flow cytometry and immunostaining. We found that no more than 0.5% of the primary marmospheres were positive for CD31 or CD45 by flow cytometry. The primary marmospheres were negative for fibroblastic markers such as vimentin and fibronectin, whereas these cells strongly expressed cytokeratin (Figures 1c and d). Taken together, these observations clarify that these primary marmospheres were prepared with minimal contamination by fibroblast populations, hematopoietic cells or endothelial cell.

Identification of breast cancer stem cells

Currently, marmosphere cultures and flow cytometric sorting for the CD44+/CD24−/low subpopulation are the main methods for enrichment of breast cancer stem/progenitor cells. We used suspension culture of SK-BR-3 and human primary breast cancer mammosphere cells to generate marmospheres (Figure 2b) in which the CD44+/CD24−/low subpopulation was enriched to 99.6 ± 0.07% and 95.3 ± 1.2%, respectively (Figure 2a). The marmosphere cells were then allowed to form adherent cultures, thereby enabling the easy detection of oHSV G47Δ cytopathicity. The adherent cultures (Figure 2d) of SK-BR-3 and human primary breast cancer cells retained a high proportion of CD44+/CD24−/low cells (96.7 ± 1.7%, 92.1 ± 2.5%, respectively; Figure 2c). The difference
in the proportion of CD44\(^+\)CD24\(^-\)/low cells between the
suspended and adherent mammospheres was not found to be
statistically significant (\(P > 0.05\)). Further, under normal conditions,
the proportion of mammospheres formed by the SK-BR-3 cells
(4.1 ± 0.3\%) was significantly higher than that formed by the
human primary breast cancer cells (2.7 ± 1.1\%) (\(P < 0.05\)).

To further evaluate the stem cell characteristics of the CD44\(^+\)CD24\(^-\)/low population, the expression of Oct4 and Sox2 was
examined. The SK-BR-3 and human primary breast cancer cell
mammospheres expressed Oct4 and Sox2 protein, as detected by
immunofluorescent analysis (Figures 3a and b), and Oct4 and Sox2
mRNA was detected by RT-PCR (Figure 3c). Flow cytometry and the ALDEFLUOR assay for detecting
ALDH activity showed the percentage of ALDEFLUOR-positive
SK-BR-3 and human primary breast cancer mammospheres to be
37.6 ± 1.5\% and 27.3 ± 1.3\% respectively. These values
were over 10-fold higher than those of the SK-BR-3 and
human primary breast cancer cells, which were 2.9 ± 0.5\% and
1.5 ± 0.3\% respectively \((P < 0.001\); Figure 4). To observe the tumor
formation rate of possible stem cells, 1 × 10\(^3\), 1 × 10\(^4\) and 1 × 10\(^5\)
SK-BR-3 mammosphere cells and parental cells were implanted in
the left flank of 6-week-old female nude BALB/c mice. All
mice developed tumors when injected with 1 × 10\(^3\) or 1 × 10\(^4\)
SK-BR-3 mammosphere cells. However, one of the mice developed
mammosphere cells when injected with 1 × 10\(^5\) SK-BR-3 parental cells. This
difference was statistically significant and strongly supports the
conclusion that the tumor formation rate of mammosphere cells is
much higher than that of non-mammosphere cells (Table 1).

**In vitro cytotoxicity**

G47\(\alpha\) was found to be highly cytotoxic to both types of
mammosphere cells in vitro. Over 50\% of the cells were killed
by days 3 and 4 when infected with the virus at an MOI of 0.1 and
0.01, respectively (Figure 5, left); similarly, over 90\% of the non-
mammosphere cells were killed by day 4 when infected with the
virus at an MOI of 0.1 and 0.01 (Figure 5, right). The spread of the
G47\(\alpha\) vector in adherent cultures was visualized by X-gal
histochemical analysis of the infected cells (Figure 6). Most cells
were dead by day 6, because of which the number of X-gal-
positive cells detected decreased.

**Treatment of tumors in vivo**

CD44\(^+\)CD24\(^-\)/low cells from the mammospheres formed tumors
when injected into the left flank of 6-week-old female nude BALB/c
mice. All mice developed tumors when injected with 1 × 10\(^3\) or 1 × 10\(^4\)
SK-BR-3 mammosphere cells. However, one of the mice developed
tumors when injected with 1 × 10\(^5\) SK-BR-3 parental cells. This
difference was statistically significant and strongly supports the
conclusion that the tumor formation rate of mammosphere cells is
much higher than that of non-mammosphere cells (Table 1).

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**Figure 1.** Identification of human primary breast cancer cells. Flow cytometric analysis for endothelial marker CD31 (a) and hematopoietic
marker CD45 (b) on human breast cancer cells. The percentage of CD31- or CD45-positive cells is indicated. Immunofluorescent staining of
vimentin, fibronectin and cytokeratin in primary cancer cells (c) and foreskin-derived fibroblasts (d). Fibroblasts served as a positive control for
vimentin and fibronectin. We found that foreskin-derived fibroblasts strongly expressed fibroblastic markers vimentin and fibronectin, but
these cells were negative for cytokeratin. In contrast, human primary breast cancer cells strongly expressed cytokeratin, whereas these cells
were negative for vimentin or fibronectin. Scale bar, 50 μm.
Cells infected with G47Δ (MOI = 0.1) before implantation did not form tumors (n = 10; followed up until day 58). To evaluate the antitumor efficacy of G47Δ, the established tumors (approximately 5 mm in maximal diameter) were inoculated with the virus (1 × 10⁷ p.f.u.) or PBS (mock) on days 0, 3, 7 and 10. G47Δ treatment significantly inhibited tumor growth, (P < 0.05; Figures 7a and c). No mice had apparent tumors on days 21 and 24 after injection with G47Δ. In a separate experiment, mice were killed and X-gal histochemical analysis was performed to detect infected cells indicative of G47Δ replication. Replication was observed even 13 days after a single virus injection (Figure 7b). When tumors were removed from mice 58 days after implantation, the proportion of
CD44 + CD24−/low cells was reduced to between 3% and 5% in the mock- and oHSV-treated groups, respectively (Figure 8), similar to the level seen initially in non-treated SK-BR-3 cells (data not shown).

**DISCUSSION**

Cancer stem cells have been proposed as initiators of cancer and may be responsible for cancer recurrence, development of metastases and resistance to therapy.3,28,29 Breast cancer stem cells have been isolated using various techniques, including sorting for cell surface markers, serum-free suspension culture, sorting for side population cells and sorting for ALDH.6,21,26,29–31 We used serum-free suspension culture to enrich stem cells, which we then detected as the CD44 + CD24−/low population and ALDH activity by flow cytometry. The percentage of ALDEFLUOR-positive SK-BR-3 and human primary breast cancer mammospheres are 37.6 ± 1.5% and 27.3 ± 1.3%, respectively, which is significantly higher than those of the SK-BR-3 and human primary breast cancer cells, (P < 0.001) and the proportion of CD44+CD24−/low cells in the non-mammosphere cells and mammosphere cells was about 4% and 99%, respectively. The purity of the primary mammospheres is also an important factor, which was verified with minimal contamination by fibroblast populations, hematopoietic cells or endothelial cell by using flow cytometry and immunostaining. The tumor formation rate after implantation of 1 × 10³ mammosphere cells in 6-week-old female nude BALB/c mice was up to 50%. Although all the mice developed tumors when injected with 1 × 10⁴ or 1 × 10⁵ SK-BR-3 mammosphere cells, only one of the six mice developed tumors when injected 1 × 10³ non-mammosphere cells. Thus, it is clear that mammosphere cells were almost 100-fold more tumorigenic than non-mammosphere cells, indicating that our serum-free suspension culture enriched cancer stem cells. Similar results for SK-BR-3 cells have been reported previously.32

In this study, we found that suspended mammosphere cells tended toward adherent growth in the course of passage, though the reason for this phenomenon is not very clear at present. However, the in vitro cytotoxicity test is easy to conduct because of the phenomenon of adherent mammosphere growth. When mammospheres were cultured in the serum medium, the frequency at which the characteristics of stem cells was observed

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**Table 1.** The tumor formation rate of SK-BR-3 mammosphere cells and SK-BR-3 cells in BALB/c mice

<table>
<thead>
<tr>
<th>Number of cells implanted</th>
<th>1 × 10³</th>
<th>1 × 10⁴</th>
<th>1 × 10⁵</th>
</tr>
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<tbody>
<tr>
<td>SK-BR-3 mammosphere cells</td>
<td>2/4</td>
<td>6/6*</td>
<td>6/6*</td>
</tr>
<tr>
<td>SK-BR-3 cells</td>
<td>0/4</td>
<td>0/6</td>
<td>1/6</td>
</tr>
</tbody>
</table>

*P < 0.05, χ² test.
decreased significantly, proving that the nature of stem cells changes with differentiation. The mammospheres also expressed the stem cell markers Oct4 and Sox2, which supported their stem-like phenotype.

Oncolytic virotherapy has been translated to the clinic, with numerous oncolytic viruses being tested in patients with different cancers.\(^3^3\) This includes studies on oHSV in patients with subcutaneous nodules of recurrent breast cancer.\(^3^4,3^5\) Although the breast cancer stem cells were susceptible to G47\(\Delta\), it took about 6 days to reduce viability by over 90% when the virus was injected at an MOI of 0.01. Over 90% of the non-stem cells were killed by day 4 when injected at an MOI of 0.1 or 0.01. Thus, the cytotoxicity of G47\(\Delta\) to CD44\(^+\)CD24\(^-\)/low cells was weaker than that to breast cancer cells cultured under standard conditions. Infection of mammospheres with G47\(\Delta\) at a low MOI blocked their ability to form tumors in vivo. When the tumors were removed from the mice 58 days after implantation, the proportion of CD44\(^+\)CD24\(^-\)/low was reduced almost 20-fold, indicating that cancer stem cells differentiate during tumor growth, as has been reported previously.\(^4\) Therefore, the bulk of tumor cells are not stem cell-like, and these

Figure 5. In vitro killing of mammosphere cells and parental cells of SK-BR-3 by G47\(\Delta\). Adherent cultured mammosphere cells and parental cells of SK-BR-3 in six-well dishes were infected with G47\(\Delta\) at an MOI of 0.01 or 0.1 or with PBS (mock), and the average number of cells from duplicate wells is plotted as the percent in the mock wells.

Figure 6. X-gal staining of human primary breast cancer mammospheres infected with G47\(\Delta\). Adherent human primary breast cancer mammospheres cultures in six-well dishes were infected with G47\(\Delta\) at an MOI of 0.01 or 0.1 or with PBS (mock), and the cells were stained with X-gal to identify infected cells (blue).
cells may be more susceptible to oHSV. Under in vivo conditions, G47Δ inhibited the growth of established tumors.

To our knowledge, this is the first report of the treatment of breast cancer stem cells with oHSV. The oHSV vector G47Δ was effective in killing human breast cancer stem cells in vitro and treating tumors in an animal model. The ability of oHSV to target breast cancer stem cells efficiently is an important attribute that supports the possibility of successful clinical translation.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

REFERENCES


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