Full Length Research Paper

Isolation and propagation of *in vitro* breast cancer stem cells from tumor biopsy in Vietnamese women

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Cancer stem cells are the origin of tumors and have been isolated successfully from different kinds of tumors. Breast cancer stem cells have been recently identified in breast carcinoma with markers CD44+/CD24-/dim. This population can cause tumor and display stem cell-like properties. However, direct evidences that breast cancer stem cells can be propagated *in vitro* is still lacking. This research was carried out to isolate and propagate *in vitro* breast cancer stem cells from tumor biopsy. Breast tumor biopsy was used to isolate breast cancer cells by primary tissue culture. As such, breast cancer stem cells were isolated from breast cancer cells by catcher-tube based cell sorter on flow cytometer machine. These cells were propagated by an *in vitro* culture in a free serum specific medium. The results showed that the CD44+CD24-/dim cell population that were maintained, were capable of self-renewal and extensive proliferation as clonal non-adherent spherical clusters. Interestingly, cultured cells were CD44+CD24-/dim expressed by the putative stem cell marker Oct-4, resistant with verapamil at 50 µg/ml, and gave rise to new tumors when as few as 1000 cells were injected into the mammary fat pad of immune-deficient mice. This population was a suitable *in vitro* model to study breast cancer stem cells and develop therapeutic strategies to treat breast cancer.

Key words: Breast cancer, breast tumor, cancer stem cell, CD44+CD24-/dim.

INTRODUCTION

Cancer is a disease that causes cells to grow unlimitedly, thus, forming tumors in the body. Tumors are groups of cells that contain many kinds of cells with different biological characteristics (Reya et al., 2001; Campbell and Polyak, 2007). Many publications showed evidences for existence of cancer stem cells (CSCs) in malignant tumors. CSCs have been identified in many solid tumors, including brain, prostate, pancreatic, liver, colon, head and neck, lung, and skin tumors (Anton Aparicio et al., 2007; Ceder et al., 2008; Eramo et al., 2008; Ferrandina et al., 2007; Glinsky, 2007; Li et al., 2007; Prince et al., 2007; Seo et al., 2007). Indeed, this idea was first postulated by Rudolph Virchow and Julius Cohnheim in the nineteenth century (Bignold et al., 2006; Huntly et al., 2005). Virchow’s embryo rest hypothesis noted the similarities between fetal tissue and cancer cells (Sell, 2004). Later, Cohnheim and Durante extended this hypothesis that there exist embryonic remnants immature organs, and Beard hypothesized that cancer arises either from germ cells or from placental tissue.

The concept about tumor containing heterogeneous populations of cells was demonstrated firstly by Lapidot and colleagues (Lapidot et al., 1994) in leukemia. They showed that CD34+CD38− cells isolated from acute myeloid leukemia patients developed a tumor when injected into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, while injection of even larger numbers of the more differentiated cells, CD34+CD38−, did not initiate tumor formation. Moreover, the tumors formed by injection of the CD34+CD38− cells were similar in morphology compare to original tumors. And this concept also was demonstrated in solid tumors,
especially breast cancer by Al-Hajj (Al-Haii et al., 2003). Following the results of Al-Hajj et al. (2003), the cells expressed protein CD44 and weakly or unexpressed protein CD24 could established the new tumors when allografted in mice. A lot of other researches demonstrated that 200 cells with this phenotype could cause tumors in NOD/SCID mouse models. While 20,000 cells not been expressed these markers could not cause tumor after transplantation in mice. These tumors contained a lot of different kinds of cells and CSCs. These CSCs derived from this tumor could continuously cause tumor when injected in immunodeficiency mice. This capacity of CSCs demonstrated that they could undergo self-renewal for a long time.

In Vietnam, there are about 20.3 breast cancer patients per 100,000 people. This disease is the most common cancer in Vietnamese women. Up to date, there is not any publication about existing breast CSCs in their tumors. Therefore, this research is aimed at demonstrating the existence of breast CSCs in Vietnam. Moreover, this research further confirmed the hypothesis of breast CSCs in breast cancer.

Three methods are commonly used for the isolation of CSCs. These methods include:

1. The isolation by sorting of a side population (SP) based on Hoechst dye efflux.
2. Sorting on the basis of cell surface marker expression.
3. Sphere culture (mammosphere culture).

CSCs were obtained from three different methods, in degrees of enrichment of CSCs as well as advantages and limitations. Despite the isolation methodology, the cells were named CSCs when they passed some assays, including tumorigenicity, self-renewal, and the ability to histologically recapitulate the tumor of origin.

In this research, breast cancer stem cells were firstly isolated by sorting on the basic of cell surface CD44 and CD24 marker expression and then they were cultured in mammosphere type. The propagation of breast cancer stem cells was carried out by cell suspension techniques in free-serum medium. After that, these CSC candidates were checked about tumorigenicity, self-renewal, and the ability to histologically recapitulate the tumor of origin.

MATERIALS AND METHODS

Primary tissue culture

Isolation and in vitro expansion of stem cells was carried out from breast tumor specimens. Tumor specimens were obtained from consenting patients, whereas tumor biopsy, obtained from a hospital, was transferred to the laboratory. Biopsy was washed 3 to 4 times by PBS (phosphate buffer saline) supplemented with antibiotic-antimycotic to remove blood and then the fat binding in the biopsy as well as in the necrotic part. After that, biopsy was cut into some small fragments of about 1 to 2 mm² in square. These fragments were seeded in a tissue dish that is 35 mm in diameter.

Isolation of candidate breast cancer stem cells

Candidate breast cancer stem cells were isolated by sorting CD44+CD24−/dim cell population by catch-terube based cell sorter in combination with the flow cytometer (Facsscalibur, BD Bioscience). The cells that were obtained from the primary tissue culture were stained with 20 µl anti-body CD44 and 20 µl anti-body CD24 (all bought from BD Bioscience) in 5 ml tube in 10⁻⁷ cells/ml concentration. Tubes had been incubated in the dark, in room temperature for 45 min and more tubes were added to the FACScflow solution (1 ml) in 10⁻⁷ cells/ml concentration. In CellQuest Pro software, CD44+CD24−/dim cell population was identified by quadrant analysis. CD44+CD24−/dim cells were positive with CD44 and negative or weakly positive with CD24. In Figure 1, this population was R1 and it was sorted into a 50 ml tube coated with BSA (bovine serum albumin) 1 mg/ml initially. Candidate cells were harvested by centrifuging at 3,000 rpm for 5 min.

Culture of breast cancer stem cells

After sorting, candidate breast cancer stem cells were plated at 1,000 cells/ml in serum-free DMEM-F12, supplemented with 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF), 5 ng/mL insulin and 0.4% bovine serum albumin (all from Sigma). Cells grown in these conditions as non-adherent spherical clusters of cells (usually named “spheres” or “mammamospheres”) were enzymatically dissociated every 3 days by incubation in a 0.25% trypsin-EDTA solution (Sigma, St Louis, MO) for 2 min at 37°C. Conversely, differentiation was induced by culturing mammamosphere derived cells for 8 days on collagen-coated dishes in DMEM-F12, supplemented with 5% fetal bovine serum (Sigma, St Louis, MO) without growth factors.

Sphere formation assay

Primary spheres were dissociated as previously described and 100 cells per well were plated in 96-well culture dishes in 200 µL growth medium. As such, 25 µL of the medium per well was added every 2 days. However, the number of spheres for each well was evaluated after 7 days of culture.

In vivo injection of mammosphere cells

Spheres were collected, enzymatically dissociated, washed in PBS, and kept at 4°C until they were injected into the layer that is under the skin (subcutaneous) of the 5-week-old SCID mice. Mice received an estradiol supplementation (0.4 mg/kg s.c., Progynon Depot, Schering-Plough, Kenilworth, NY) every 10 days for 40 days after cell injection and were inspected for tumor appearance, by observation and palpation, for 15 weeks following cell injection. After this time interval, all mice were sacrificed by cervical dislocation and the presence of each tumor nodule was confirmed by necropsy.

Oct-4 gene expression assay

Oct-4 gene expression was evaluated by RT realtime PCR. In each
Figure 1. Cell population with suitable size was gated in the R1 region (a). Breast cancer stem cells were identified by expression of CD44 and an un-expression or weak expression of CD24 in quadrant analysis (b).

Anti-cancer drug assay

Breast cancer stem cell (CD44+CD24\(^-\)/dim) and breast cancer cells were at a density of 0.4 and \(10^4\) cells per well in 24-well plate (Nunc), respectively in DMEM/F12/10% FBS. After 24 h culture for confluence, the cells were treated with 50 \(\mu\)g/ml verapamil (Sigma). The cells were observed under inverted phase contrast microscope (Carl-Zeiss, Gemany) after 24, 48 and 72 h treatment. The apoptosis of two populations were investigated by flow cytometry using Annexin-V and PI (BD Bioscience, USA).

RESULTS AND DISCUSSION

Existence of breast cancer stem cells in primary tissue culture

Establishment of primary cultures of mammary gland precursors

The study was carried out to culture primarily 21 tumors from 21 different patients. About 15 samples of the 21 tumors had grown out with a lot of single cells surrounding the tissues. The cells from the 15 samples were propagated until they reach the 80% cell confluence. In almost all the samples, single cells appeared around the fifth day, with the earliest on the third day (Figure 2). Then cells proliferated rapidly and got clonal combination on the fifteenth day. There were two kinds of cell shape in the primary culture. These are epithelial cells with bean shape and big nucleus; and stromal cells like fibroblast having small nucleus and long shape.

Existence of CD44+CD24\(^-\)/dim cell sub-population in primary cell lines

When analyzing 15 primary cell samples with two markers, CD44 and CD24, all of them had a small population of cells that was positive with CD44 and negative or weakly positive with CD24. This population got 3.59 ± 1.65% in average in the total number of cells derived from the primary culture with the lowest ratio at 1.25% and the highest at 7.12%. The result also showed that there were about 50% of the cells that were positive with marker CD24. However, there were more than 90% of the cells that were negative with marker CD44. By flow cytometry analysis, almost all the cells that were positive with CD44 were negative or weakly positive with CD24. As such, CD44+CD24\(^-\)/dim cell population have characteristics of cancer stem cells after culture.

Expression of breast cancer stem cell markers

Following the results of Clarke et al. (2006), xenotransplanta-
Figure 2. Results of the primary tissue culture. (A) Migration of cells out of the tissue after 7 days, (B) Two kinds of cells appearing in the primary culture (epithelial cells and stromal cells).

Figure 3. Expression of CD44 (A) and CD24 (B) was identified by immunohistochemistry. Candidate cancer stem cells were stained with anti CD44-FITC and anti CD24-FITC, and were counterstained with Hoescht 33342 (stained nucleus).

tation was used to isolate the population of cells that have tumorigenicity potential in NOD/SCID mice (Al-Hajj et al., 2003). This cell population expressed CD44 protein, but unexpressed or weakly expressed protein CD24 in the cell surface. This result was confirmed by flow cytometry and immunohistochemistry (Figure 3 and 4. They demonstrated that 200 cells with these phenotype could cause tumors when injected into NOD/SCID mice, while 20,000 cells without these phenotype could not cause tumors in vivo. However, the population that was isolated by this research expressed CD44+CD24-/dim phenotype.

In vitro self renewal

When cultured in the free serum medium, CD44+CD24-/dim cells could not adhere into the flask surface, but floated in the medium with mammosphere style (Figure 5a). Mammosphere creating capacity demonstrated that sorted cells had been self renewed. This characteristic was similiar to stem cells which were isolated from the mammary gland. It was demonstrated that cells derived from the mamary gland which could form mammosphere in vitro could form de novo human mamary gland in mice after xenotransplantation (Dontu et al., 2003), in that sorted cells had been cultured in the free serum medium. After 15 days, a lot of colony were formed in the so called mammosphere and were found floating in the medium. As such, the size of the mammosphere grew bigger and bigger, owing to time. However, the mammospheres were rather condensed when observed through an inver-ted microscope.

In vivo tumor formation

Tumorigenicity is an important characteristic of cancer stem cells. Many researches showed that new tumors could be formed by injecting a little cancer stem cells into immunodeficient mice. In this research, 1 thousand breast cancer stem cells were used to create tumor in mice (Figure 5b). The number of cells needed for tumorigenicity was relatively big when compared to some
The breast cancer stem cells were established with CD44 positive expression and CD24 negative/dim expression.

Mammosphere formed in the serum free medium after 10 culture days (A). The tumor formed subcutaneously after injection of 106 breast cancer stem cells (B).

Breast cancer stem cells expressed by Oct-4

Oct-4 gene is a specific gene marker for embryonic stem cells and the expression of its pluripotent potential is related to stem cells. However, its expression was also found in cancer cells. Previous researches have confirmed that cells or stem cells, expressing Oct-4 gene, could cause tumors after they have been injected in immunodeficient mice. Oct-4 expression in the candidate breast cancer stem cells demonstrated that the confirmed cells isolated from this procedure were breast cancer stem cells (Figure 6).

CD44+CD24-/dim cell population are resistant with anti-cancer drugs

Verapamil was known as an anti-cancer drug for inhibition of drug efflux pump proteins such as P-glycoprotein. They were used in clinical treatment as well as in the research for growth inhibition of tumor or induction to apoptosis. In this research, verapamil was used to test the resistance of the anti-cancer drug of two subpopulations (CD44+CD24-/dim) and breast cancer cell population. The cells were seeded at 6.10^4 cells/cm^2 density at the first day in serum DMEM media. When the cells got to confluence, 50 µg/ml verapamil was supplied with media for culture in a 2 day experiment. The cell numbers of breast cancer cells significantly decreased after 48 h of verapamil treatment. Verapamil possessed strong effect to these cells which led to cell apoptosis, whereas breast cancer stem cells had growth normally under verapamil effect. The data indicated that breast
cancer stem cell population has extreme resistance of anti cancer drug when compared to breast cancer cells (Figure 7). As such, this property remains a serious situation in cancer treatment clinically. This result was also similar with the apoptosis analysis results.

Our results are a 100% similar to that of Al-Haji and colleagues, who showed that breast cancer tumors at stage II and III contained a small population of CD44+/CD24-/dim. Contrary to these results, the results of Honeth et al. (2008) showed that only 31% of their tumors contained breast cancer stem cells. This discordance could depend on our study involving mainly metastatic tissues, while they used both metastatic and primary tumors. However, another study demonstrated 59% of CD44+/CD24- cells in human breast tumors (Mylona et al., 2008).
CONCLUSION

In this study, breast cancer stem cells can be isolated from malignant tumors in Vietnamese women. We retrospectively confirmed that CD44+/CD24dim/ breast cancer cells, which have been prospectively identified as tumorigenic cells, display stem/progenitor cell properties. They are positive with CD44 protein and negative or weakly positive with CD24. Also, they have self-renewal capacity via mammosphere assay and a tumor causing capacity in vivo.

To our knowledge, for the first time, we showed that breast tumorigenic cells with stem/progenitor cell properties can be propagated in vitro as non-adherent mammospheres from breast cancer tissue in Vietnamese women, in keeping with similar findings obtained by normal mammary stem/progenitor cells. This experimental system may represent a suitable in vitro model to study breast cancer-initiating cells and to challenge them with molecularly targeted agents specifically interfering with the self-renewal and survival of breast cancer-initiating cells.

REFERENCES


