An emerging question about putative cancer stem cells in established cell lines—are they true stem cells or a fluctuating cell phenotype?

Pranesh Gunjal, Daniel Pedziwiatr, Ahmed A. Ismail, Sham S. Kakar, and Mariusz Z. Ratajczak

Stem Cell Institute at James Graham Brown Cancer Center, University of Louisville, Louisville, KY 40202, USA.

Abstract: It has been proposed that established cell lines contain populations of cancer stem cells (CSCs), which are responsible for expansion of these cell lines and their metastatic potential. To address this issue better, we employed a human ovarian cancer cell line, A2780, and sorted cells according to the postulated highly metastatic cancer stem cell phenotype, CD24+/CD44-, and the less-metastatic CD24-CD44+ and CD24-CD44- phenotypes. These cells were employed in chemotaxis assays in vitro to migrate in response to conditioned media harvested from bone marrow or liver cells damaged by irradiation and in in vivo assays to grow tumors after injection into immunodeficient mice. We also sorted single cells expressing all three phenotypes by FACS and expanded them to grow clones. We found that the CD24+/CD44- cells are a highly migratory population compared with CD24-CD44+ and CD24-CD44- cells and were seeded in higher numbers in murine bone marrow and liver after intravenous injection. Most importantly, we observed that singly sorted cells efficiently expanded ex vivo into cell populations that represented all phenotypes of the parental cell line. Thus, our data indicate that cells expressing a certain set of markers, e.g., CD24, have at any given moment a higher potential to migrate and metastasize. However, cells that are CD24-negative, if expanded from a singly sorted cell, may give rise to cells containing all of the markers, including CD24. Based on this finding, we propose that the CSC phenotype in cell lines fluctuates with cell expansion.

Keywords: Cancer stem cells, singly sorted cells, changing phenotype, chemotaxis, CD24, CD44.

INTRODUCTION

Mounting evidence indicates that cancer originates from mutated stem or progenitor cells or adult cells that, after mutation, acquire a cancer stem cell phenotype. The presence of cancer stem cells (CSCs) or cancer-initiating cells has been reported among cells isolated from primary tumors, and their presence has also been postulated in established, in vitro-propagated, immortalized cancer cell lines [1–3].

CSCs in established cancer cell lines have been described by the expression of selected cell surface markers that often correspond to phenotypes expressed by normal adult stem cells [1–3]. Putative CSCs have been described by the expression of certain surface receptors (e.g., CD133, c-kit) or adhesion molecules (e.g., CD44, CD24), the exclusion of Hoechst 33342 in FACS analysis in so-called side populations of cells, or the expression of the G subfamily of ATP-binding cassette transporters (ABCG), epithelial-specific antigen (ESA), and aldehyde dehydrogenase (ALDH) [1–5].

In this work, we employed CD24 and CD44 as markers to define CSCs. The CD44 antigen is a receptor for hyaluronic acid and can also interact with osteopontin and the collagens [4–6]. This cell-surface glycoprotein is involved in cell–cell interactions, cell adhesion, and migration. CD44 variant isoforms have been reported to be relevant in the progression of epithelial ovarian cancer and head and neck squamous cell carcinoma [4–6]. Similarly, CD24 is also a cell adhesion molecule [7]. It has been suggested that CD44+/CD24− breast cancer cells are enriched for cells with CSC characteristics [7, 8].

In our experiments, we employed the highly metastatic human ovarian cancer cell line A2780 and sorted cells according to the postulated highly metastatic cancer stem cell phenotype, CD24+CD44+, and the less-metastatic CD24−CD44+ and CD24−CD44− phenotypes. These cells were employed in chemotaxis assays to test their migration in response to conditioned media harvested from bone marrow or liver cells damaged by irradiation and in in vivo inoculation assays to grow tumors in immunodeficient mice. We also sorted single cells...
expressing all three phenotypes by FACS and expanded single cell-derived clones from them.

We observed that cells expressing CD24 or CD44 markers have a higher potential to migrate and metastasize than CD24- and CD44- cells. However, if expanded, CD24- and CD44- singly sorted cells may give rise to cells that acquire CD24 and CD44 expression. Based on this finding, we propose that the CSC phenotype in cell lines fluctuates, and every cell, even if it does not express CSC markers at a given moment, is able to expand into cells that regain expression of these markers.

**MATERIAL AND METHODS**

**Cells**
The A2780 cell line was maintained in Roswell Park Memorial Institute (RPMI) medium 1640 containing 10% FBS, 100 U/ml penicillin, and 10 µg/ml streptomycin. Cells were cultured in a humidified atmosphere of 5% CO2 at 37°C, and the medium was changed every 48 hours.

**Single-cell clonogenicity and sphere assay**
For assessment of the clonogenic potential of single cells, the A2780 cell line was sorted as single cells for 48 hours. The A2780 cell line was maintained in Roswell Park Memorial Institute (RPMI) medium 1640 containing 10% FBS, 100 U/ml penicillin, and 10 µg/ml streptomycin. Cells were cultured in a humidified atmosphere of 5% CO2 at 37°C, and the medium was changed every 48 hours.

**Chemotaxis assay**
Chemotaxis assays were performed in a modified Boyden’s chamber with 8-µm pore polycarbonate membrane inserts (Costar Transwell; Corning Costar, Lowell, MA, USA) as described previously [10]. In brief, freshly sorted cells (CD24+/CD44-, CD24-/CD44+, and CD24-/CD44+) from the A2780 cell line were seeded into the upper chamber of an insert at a density of 2 × 10^5 in 110 µl. The lower chamber was filled with pre-warmed CM harvested from the different organs from 57BL6 mice irradiated at 1000 cGy. Medium supplemented with 0.5% BSA was used as a negative control, while medium supplemented with 10% FBS was used as a positive control. After 48 hours, the inserts were removed from the Transwell supports. The cells that had not migrated were scraped off with cotton wool from the upper membrane, and the cells that had transmigrated to the lower side of the membrane were fixed and stained with HEMA 3 (Protocol, Fisher Scientific, Pittsburgh, PA) and counted on the lower side of the membrane using an inverted microscope.

**Tumorigenic potential for cell transplantation into SCID mice**
To evaluate the in vivo metastatic behavior of freshly sorted cells, CD24+/CD44-,CD24-/CD44+, and CD24-/CD44- cells from the A2780 cell line were inoculated into the peritoneal cavity of SCID-Beige inbred mice. Three mice were used for each group, which were grouped as i) control (injected with CD24+/CD44-A2780 cells), ii) injected with CD24+/CD44- cells, or iii) injected with CD24-/CD44- cells, with 10,000 cells injected per mouse. The mice were monitored for peritoneal swelling and any adverse effects and then euthanized 3–4 weeks after cell inoculation. Bone marrows and livers were removed after sacrifice of these mice, and the presence of ovarian cells (i.e., murine-human chimeras) was evaluated as the difference in the level of human α-satellite DNA expression. DNA was amplified in the extracts isolated from BM- and liver-derived cells using real-time PCR. Briefly, DNA was isolated using the QIAamp DNA Mini kit (Qiagen). Human satellite and murine β-actin DNA levels were detected using real-time PCR and an
correlates with the metastatic phenotype of ovarian cancer cells. Since CD24 and CD44 antigen expression are those that establish metastases and are also likely to be CSCs. Cancer cells that are resistant to radiochemotherapy are those that establish metastases and are also likely to be CSCs. Since CD24 and CD44 antigen expression correlate with the metastatic phenotype of ovarian cancer cells [4–8, 11], we sorted three cell populations, CD24+/CD44−, CD24−/CD44+ and CD24−/CD44− from the A2780 human ovarian cancer cell line by FACS. These cells comprise ~0.02 ± 0.003%, ~0.2 ± 0.05%, and ~99 ± 0.2% of the total population of A2780 cells respectively.

All three A2780 cell populations were evaluated for their chemotactic response to conditioned medium (CM) harvested from cells isolated from irradiated bone marrow (BM) and liver and their response to 10% FBS as positive control. In our previous studies, we established that CM from irradiated BM and liver are enriched for several chemotactic attractants for cancer cells [10, 12]. Figure 1 panel A shows that CD24+/CD44− cells sorted from the parental cell line show increased chemotactic responsiveness in Transwell chambers to CM from irradiated BM or liver cells or FBS compared with CD24−/CD44+ and CD24−/CD44− cells.

Next, based on the assumption that CSCs are more resistant to radio- and chemotherapy, we repeated this experiment with all three FACs-sorted cell populations, with the difference that sorted cells were γ-irradiated (1000 cGy) before chemotaxis and pre-incubated with cisplatin (10 μM for 48 hours). Figure 1 panel B demonstrates again that CD24+/CD44− cells as well as CD24−/CD44+ cells, even if exposed to irradiation and cisplatin, retain higher chemotactic responsiveness to BM-derived CM and 10% FBS compared with cells that do not express CD24 or CD44.

RESULTS

A2780 ovarian cancer cell line-derived CD24+CD44− cells are resistant to irradiation and chemotherapy and represent highly migratory cells

Cancer cells that are resistant to radiochemotherapy are those that establish metastases and are also likely to be CSCs. Since CD24 and CD44 antigen expression correlate with the metastatic phenotype of ovarian cancer cells [4–8, 11], we sorted three cell populations, CD24+/CD44−, CD24−/CD44+ and CD24−/CD44− from the A2780 human ovarian cancer cell line by FACS. These cells comprise ~0.02 ± 0.003%, ~0.2 ± 0.05%, and ~99 ± 0.2% of the total population of A2780 cells respectively.

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A2780 ovarian cancer cell line-derived CD24+CD44− cells are highly metastatic in vivo in immunodeficient mice

Based on in vitro studies showing the high migratory potential of CD24+CD44− cells toward CM from irradiated BM and liver, we evaluated the seeding efficiency of all three populations of sorted A2780 cells after intraperitoneal injection into immunodeficient mice. Mice were sacrificed 30 days after cell injection, and the presence of human cells was evaluated by employing quantitative PCR to detect human α-satellite sequences in DNA extracts prepared from murine BM and liver. The number of human cells in the murine organs was calculated by comparing the expression of human Alu sequences with standard curves prepared by mixing different numbers of human and murine cells.

Figure 2 shows the increased seeding efficiency of human ovarian cancer cells in BM and liver in mice injected with CD24+CD44− cells compared with...
mice injected with CD24⁺CD44⁻ or control parental cells.

**Fluctuating phenotype of singly sorted and expanded A2870 human ovarian cancer cells**

Finally, after confirming that expression of CD24 or to a lesser extent, expression of CD44 on A2780 cells corresponds to a highly metastatic potential, we became interested in whether A2780 ovarian cancer cells that do not express CD24 and CD44 antigens may acquire these antigens in culture, and whether less-metastatic cells become highly metastatic over time. In other words, we tested whether the CD24- or CD44-negative phenotype is transient and whether cells expanded from these cells acquire expression of these antigens in expanded progeny.

To address this question, from the parental cell line we sorted single cells expressing three different phenotypes, CD24⁺CD44⁻, CD24⁻CD44⁺, and CD24⁻CD44⁻. These cells, sorted into 96-wells plates under conditions of limiting dilution combined with microscopic control to confirm that each well contained a single cell, were subsequently expanded to grow single cell-derived clones. Figure 3 shows a representative cytogram of the parental cell line and clones expanded from singly sorted cells. As shown in all these cases, singly sorted CD24⁺CD44⁻, CD24⁻CD44⁺, and CD24⁻CD44⁻ cells were able to reestablish all three cell populations that were initially present in the parental cell line. Finally, we confirmed that CD24⁺ cells sorted from cultures initiated by singly sorted CD24⁺CD44⁺ cells became more resistant to radiochemotherapy and migrated better in response to CM from irradiated BM cells than CD24-negative cells (data not shown).

**DISCUSSION**

The salient observation of this report is that, while CD24 antigen correlates with the cancer stem cell behavior of A2780 ovarian cancer cells, CD24-negative cells, if expanded ex vivo, give rise again to CD24-positive cells. Therefore, the cancer stem cell phenotype in this cell line seems to fluctuate, and every cell, regardless of whether it expresses stem cell-associated antigens or not, was able to expand into a population of cells that represents all cells present in the parental cell line.

Epithelial ovarian cancer is the most lethal of gynecological cancers and frequently develops in postmenopausal women [1]. Current evidence suggests that the presence of tumor-initiating CSCs has a role in its chemoresistance and relapse. Experiments in vivo have demonstrated that the CD24-positive subpopulation of ovarian cancer cells possesses stem cell-like characteristics [5–8]. This has been confirmed in elegant in vitro experiments where these cells were demonstrated to be relatively quiescent and more chemoresistant than CD24-negative cells [8]. Most importantly, injection of 5 × 10⁵ CD24⁺ ovarian cancer cells into nude mice led to tumor formation, in contrast to injection of CD24⁻ cells, which did not give rise to tumor xenografts [7]. This finding corroborates our finding here that a population of CD24⁺ cells from the A2780 human ovarian cancer cell line is highly resistant to radiochemotherapy and exhibits high in vitro migration in response to CM from irradiated BM and liver cells and is endowed with higher seeding efficiency to various organs after intraperitoneal injection into immunodeficient recipients [9].

We are aware, however, that the phenotype of cancer-initiating cells for ovarian carcinomas is still disputed [1–9, 11]. There have been several markers besides CD24 described in the literature, including CD44, CD117, CD133, the G subfamily of ATP-binding cassette transporters (ABCG), epithelial-specific antigen (ESA), and aldehyde dehydrogenase (ALDH), to identify and investigate human epithelial cancer stem cells [1–9, 11]. While some authors propose that the best markers are CD133 and ALDH and have demonstrated their significance in sphere formation assays [4], others have shown that cells in growing spheroids express low levels of CD24, CD44, and CD177 and only moderate levels of CD133 [6]. These differences may relate to different culture conditions or differences between patient samples and established ovarian cancer cell lines. In our hands, expression of CD24 antigens endows A2780 cells with metastatic potential.
Since CD24 is correlated with a pro-metastatic phenotype, we became interested in whether ovarian cancer cells that do not express this marker may reacquire CD24 expression during expansion. By employing singly sorted cell expansions, we demonstrated that CD24 antigen becomes expressed in the progeny of cells initiated from a population of CD24-negative A2780 cells. Similarly, we observed that singly sorted CD44-negative cells may acquire expression of CD44 during expansion.

Thus, our data provide the important observation that even if cancer cells do not express CSC cell surface markers at a given moment, they may acquire expression of these traits during expansion to become cancer-initiating cells. These findings demonstrate that cells with less potential may give rise to more metastatic cells during expansion. Further studies are needed to elucidate the mechanisms that facilitate expression of these antigens. It is likely that some autocrine/paracrine factors secreted by growing cells, cell density, or cell–cell contacts lead to emergence of newly metastatic cells in a cancer cell line.

CONCLUSION
Stem cell phenotype for cells in established cancer cell lines fluctuates, and cancer cells that do not express CSC markers may acquire expression of these markers and regain increased metastatic potential over time in expansion cultures. Therefore, the definition of CSCs in cancer cell lines should be revisited, as every cell in a cell line may establish cells that express stem cell markers and are more metastatic in behavior.

CONFLICT OF INTEREST STATEMENT
Authors do not have any conflict of interest.

REFERENCES