Tumor initiating cancer stem cells from human breast cancer cell lines

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Abstract. Breast cancer is composed of heterogeneous cell populations with different biological properties. The capacity to form tumors resides in a small group of cells termed tumor initiating cells or cancer stem cells. Tumor initiating cells have been identified in a variety of cancers by sorting of subpopulations based on cell surface markers and transplantation into animal models. Tumor initiating cells have the important feature of self renewal, which is a property in common with stem cells. We examined established breast cancer lines for cells with tumor initiating properties. A dye efflux side population in MCF7 and T47D lines expressed markers of breast cancer stem cells. The side population represents a distinct morphologic and functional subpopulation within the human breast cancer cell lines MCF7 and T47D. The side population from human breast cancer cell lines was able to initiate tumors *in vivo*. The side population cells from human breast cancer cell lines were more resistant to ionizing radiation than the non-side population. We concluded that tumor initiating cells exist in established human breast cancer cell lines.

Introduction

Human breast epithelial cells with stem cell properties have been previously characterized based on cell surface marker expression (1). These cells exist in a less differentiated state and can proliferate in suspension to form mammospheres (2). These studies culminated in isolation of a single cell which can reconstitute a functional mammary gland (3-5). Currently it is not clear whether these cells with remarkable self renewal properties become transformed and contribute to tumor initiation.

Breast tumors are composed of heterogeneous cell populations with different biological properties (6). The capacity to form tumors resides in a small group of cells termed tumor initiating cells or cancer stem cells (7). Tumor initiating cells have been identified in a variety of cancers by sorting of subpopulations based on cell surface markers and transplantation into animal models. Tumor initiating cells have the important feature of self renewal, which is a property in common with stem cells (8). The stem cell phenotype of tumor initiating cells may account for their ability to resist anti-proliferative therapy. This property may lead to cancer recurrence. These studies highlight the need to develop new treatment strategies which can target cancer stem cells.

In the present study we examined established breast cancer lines for cells with tumor initiating properties. We identified a dye efflux side population which is characteristic of stem cells in the human breast cancer cell lines MCF7 and T47D. The side population represents a distinct morphologic and functional subpopulation within the human breast cancer cell lines MCF7 and T47D. The side population from human breast cancer cell lines could initiate tumors *in vivo*. The side population cells from human breast cancer cell lines were more resistant to ionizing radiation than the non-side population. We concluded that tumor initiating cells exist in established human breast cancer cell lines.

Materials and methods

Hoechst staining and flow cytometry. MCF7 and T47D cells were suspended in culture medium and incubated with 5 μg/ml Hoechst 33342 for 90 min at 37˚C. Cells were sorted into a side population which could efflux Hoechst 33342 and a non-side population by flow cytometry using a FACScan flow cytometer (Becton-Dickinson).

Cell culture, mammosphere formation and ionizing radiation treatment. Sorted MCF7 and T47D human breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum and 40 μg/ml gentamicin in a humidified atmosphere of 5% CO_2 at 37˚C. Cells were photographed using phase contrast microscopy. For mammosphere formation, cells at a density of 1000/ml were cultured in suspension using ultralow attachment plates (Corning) and serum free DMEM, 20 ng/ml epidermal growth factor, and 20 ng/ml basic fibroblast growth factor. Cultures were treated with 3 Gy ionizing radiation from a ^{60}Co source (University of Southern California/Norris Comprehensive Cancer Center) followed by culture for 12 h.

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Cell death analysis. Irradiated cells were fixed in 70% ethanol, washed in PBS, and incubated with terminal deoxynucleotidyl transferase and dUTP-fluorescein (cell death) for 1 h at 37˚C (Roche Applied Science). After washing in PBS, the percentage of fluorescent cells was determined by fluorescence activated cell sorting using a Becton-Dickinson flow cytometer.

Reverse transcription-polymerase chain reaction. RNA was extracted using a commercially available kit (Qiagen) and reverse transcribed using SuperScript II reverse transcriptase according to manufacturer's instructions (Invitrogen). cDNA was amplified using specific primers in 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween-20, 1 mM EGTA, 50 μM of each dNTP, and 2.5 U Taq DNA polymerase (Roche Applied Science). Amplification with ß-actin cDNA using primers 5'-ACA GGAAGTCCCTTGCCATC-3' and 5'-ACTGGTCTCAAG TCAGTGTACAGG-3' as the internal control was carried out by real-time PCR (iCycler, Bio-Rad) using cycle parameters 94˚C for 25 sec, 55˚C for 1 min, and 72˚C for 1 min. The following gene specific primers were used: CD44, 5'-ATTGCTTTCCACTGAGGTTG-3' and 5'-ACCCTTTTG TGCCTCTGAC-3'; CD24, 5'-CCCAAATCCAACTAATGC CAC-3' and 5'-AGAGTAGAGATGCAGAAGAGAG-3'; K18, 5'-TTGAGTCAGAGCTGGCACAGAC-3' and 5'-TCA GACACCACTTTGCCATCC-3'.

Nude mouse xenografts. Side population or non-side population (10^4) were suspended in culture medium and injected into the abdominal fat pads of 8-week old virgin female nude mice. The number of resulting tumors reaching 5 mm was recorded followed by dissection and dissociation into single cells for sorting by flow cytometry to determine the presence of the side population.

Results

Stem cells have been shown preferentially to efflux vital dye (9,10). To determine if this population was present in human breast cancer cell lines, we incubated MCF7 and T47D cells with the vital dye Hoechst 33342 and sorted them by flow cytometry. As shown in Fig. 1, both MCF7 and T47D lines contained a dye efflux side population comprising 2-4% of cells. To determine if this population expressed additional stem cell markers, we performed quantitative RT-PCR on sorted cells. As shown in Fig. 2, the side population of cells expressed 10-fold higher levels of the cancer stem cell marker CD44. In contrast, the side population expressed 14-fold lower levels of the mammary epithelial marker CD24. All cells expressed high levels of the simple epithelial keratin 18, a marker of the mammary luminal population. We concluded that the dye efflux side population in MCF7 and T47D lines expressed markers of breast cancer stem cells.

To begin to functionally analyze the side population cells, we grew sorted cells in suspension culture to determine if they could form mammospheres. As shown in Fig. 3A, the side population cells from both MCF7 and T47D lines underwent 12 population doublings during the time course of the experiment. In contrast the non-side population either failed

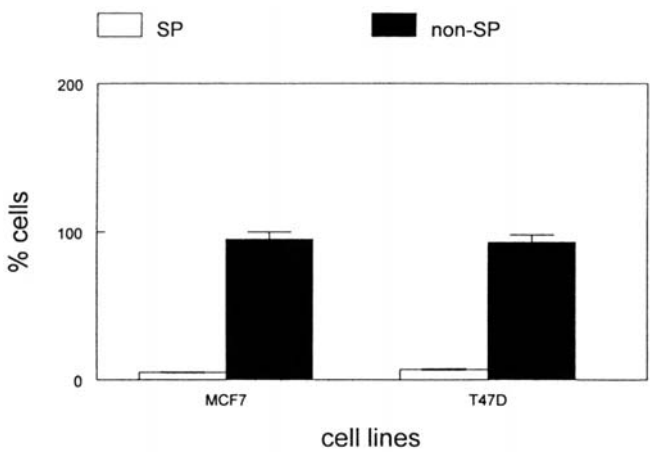


Figure 1. Human breast cancer cell lines contain a side population of cells which can efflux vital dye. The human breast cancer cell lines MCF7 and T47D were incubated with Hoechst 33342 followed by sorting by flow cytometry as described in Materials and methods. The percentages of side population (SP) and non-side population (non-SP) are shown. Error bars indicate standard error measurement of at least three independent experiments.

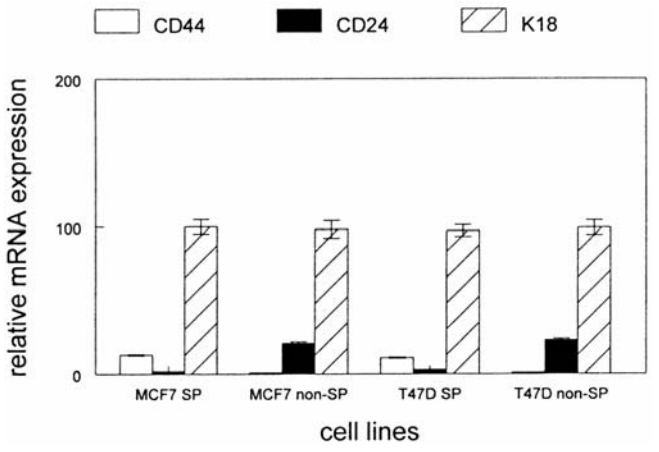
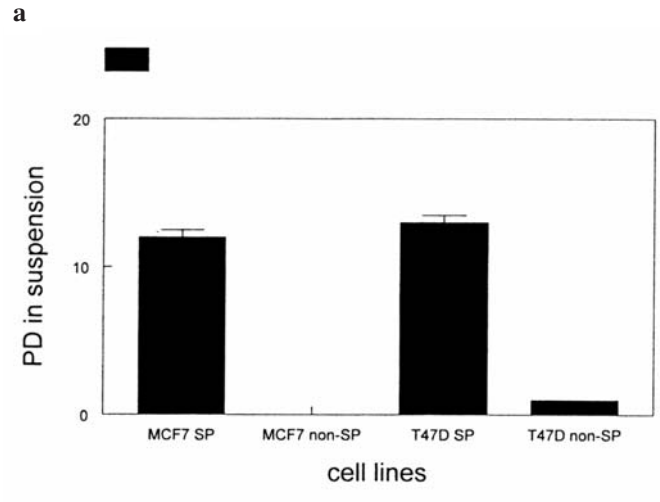


Figure 2. The human breast cancer side population cells express higher levels of CD44 and lower levels of CD24. The side population cells (SP) were sorted by flow cytometry from the non-side population (non-SP) cells in the MCF7 and T47D lines. Relative mRNA expression of CD44, CD24 and K18 were determined as described in Materials and methods. Error bars indicate SEM of at least three independent experiments.

to form mammospheres in suspension culture (MCF7) or underwent only 1-2 population doublings (T47D). We also analyzed these sorted populations in monolayer culture. As shown in Fig. 3B, the side population from both MCF7 and T47D lines consisted of small tightly packed cells with high nuclear/cytoplasmic ratio which formed colonies regular borders (panels A and B). In contrast, the non-side population cells consisted of large cells with low nuclear/cytoplasmic ratio and formed colonies with irregular borders (panel C). These results indicate that the side population represents a distinct morphologic and functional subpopulation within the human breast cancer cell lines MCF7 and T47D.

To determine if the side population was more tumorigenic than the non-side population cells, we injected both groups



into the mammary fat pads of nude mice. As shown in Fig. 4A, the side population cells formed tumors in 20 of 24 injection sites. In contrast the non-side population cells either failed to form tumors (T47D) or only in 2 of 24 injection sites (MCF7). To determine if the side population cells could be isolated from the tumor xenografts, we dissected the tumors and dissociated them into single cells followed by vital dye staining and sorting by flow cytometry. As shown in Fig. 4B, cells from the tumor xenografts contained a side population which comprised 2-4% of sorted cells. This population was detected in xenografts originating from both MCF7 and T47D lines. These results indicate that the side population from human breast cancer cell lines could initiate tumors *in vivo*.

To determine if the side population cells were more resistant to anti-proliferative therapy, we exposed these and the non-side population cells to ionizing radiation. As shown

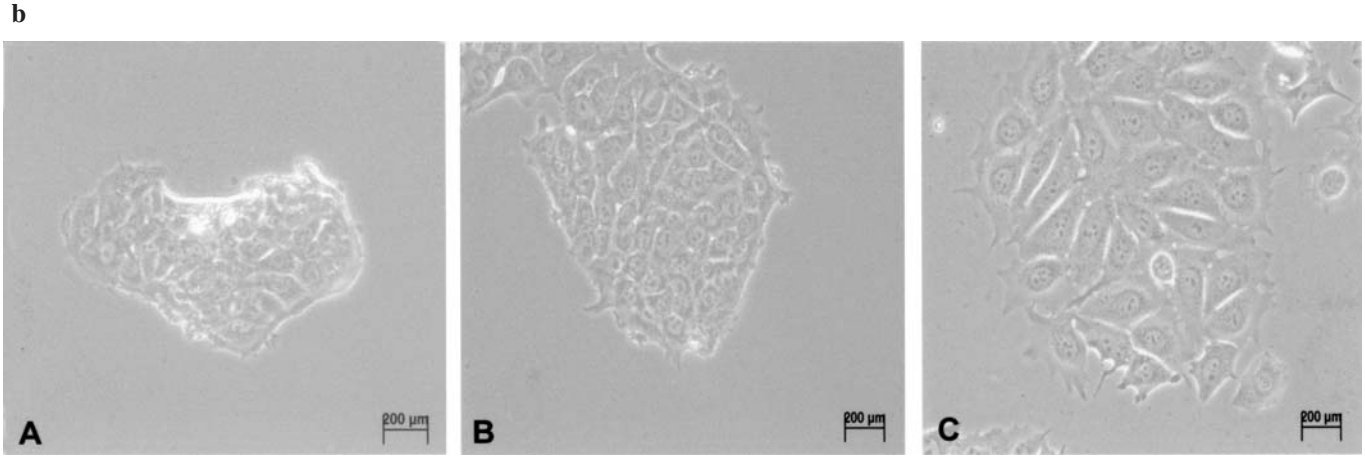


Figure 3. The human breast cancer side population of cells is highly proliferative in suspension cultures. (a) The side population cells (SP) were sorted by flow cytometry from the non-side population (non-SP) cells in the MCF7 and T47D lines. Cells were grown in suspension culture as described in Materials and methods. Error bars indicate SEM of at least three independent experiments. (b) The side population forms colonies of small tightly packed cells compared to those derived from non-side population cells. Panels A and B show colonies from side population cells of the MCF7 and T47D lines respectively. Panel C shows a typical colony derived from non-side population cells. Note smaller size of side population cells. Scale bar = 200 μm.

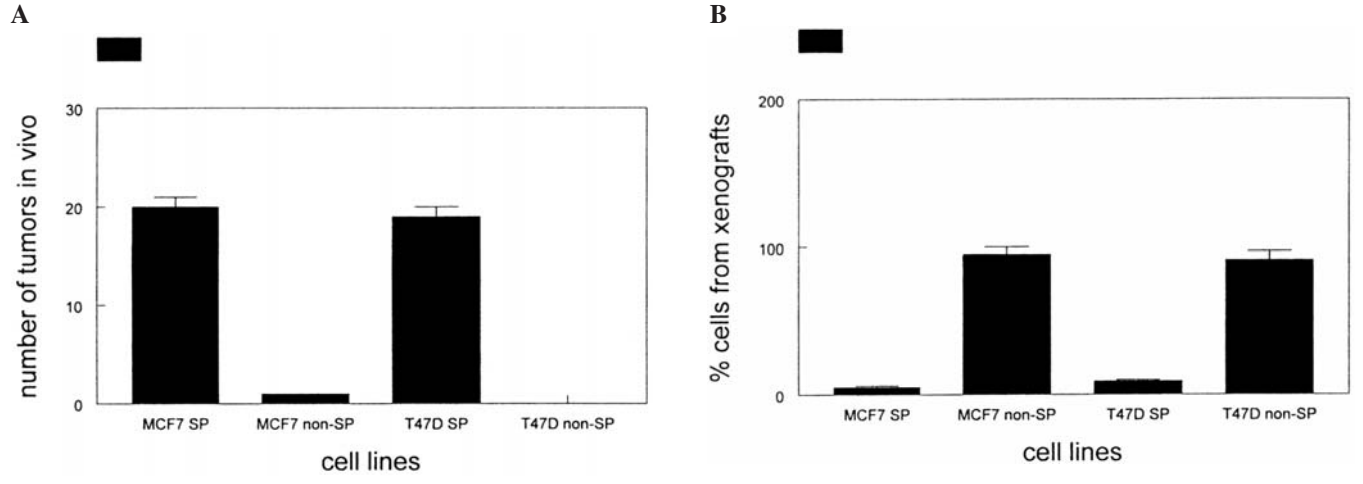


Figure 4. Side population breast cancer cells are tumorigenic in nude mice. (A) The side population cells (SP) were sorted by flow cytometry from the non-side population (non-SP) cells in the MCF7 and T47D lines. Cells were injected into nude mice as described in Materials and methods. The number of tumors which developed from each cell group is shown. (B) Side population breast cancer cells are present in tumor xenografts recovered from nude mice. Xenografts were derived from side population (SP) or non-side population (non-SP) cells from MCF7 or T47D human breast cancer cell lines. Error bars indicate SEM of at least three independent experiments.

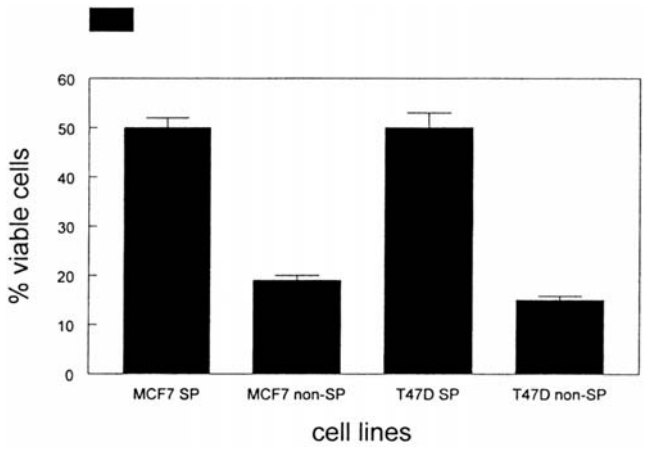


Figure 5. Side population breast cancer cells are more resistant to ionizing radiation. The side population cells (SP) were sorted by flow cytometry from the non-side population (non-SP) cells in the MCF7 and T47D lines. Cells were exposed to ionizing radiation as described in Materials and methods. Cell death was determined by TUNEL analysis. Error bars indicate SEM of at least three independent experiments.

in Fig. 5, 50% of side population cells were viable following ionizing radiation exposure. In contrast, <20% of non-side population cells were viable following ionizing radiation exposure as determined by TUNEL analysis. We concluded that the side population cells from human breast cancer cell lines were more resistant to ionizing radiation than the non-side population.

Discussion

Our results indicate that human breast cancer cell lines contain tumorigenic stem cells which are capable of enhanced population doublings *in vitro* and initiating tumors in nude mouse xenograft models. In human breast cancer tissue, CD44 expressing cells were shown to form tumors in mice (6,11). The CD44 low expressing cells were not tumorigenic in this model. The tumorigenic population could be serially passaged and regenerated themselves and non-tumorigenic populations. The $CD44^+$ population contains rare basal-like tumorigenic cells which have the capacity to generate the majority of luminal phenotype cells (12). In a separate study, breast tissues from women with BRCA1 mutations contained increased numbers of stem cells (13). BRCA1 was required for differentiation of breast progenitor cells to estrogen receptor positive luminal cells. Inhibition of BRCA1 expression increased the stem cell population *in vitro*. These studies indicate that breast cancers contain tumor initiating cells which are regulated by key tumor suppressor pathways.

Our results indicate that breast cancer stem cells are more resistant to ionizing radiation treatment. A previous study showed enrichment of normal mouse mammary progenitor cells following ionizing radiation (14). These progenitor cells demonstrated upregulation of the Wnt signaling pathway which has been implicated in stem cell renewal. Wnt over-expression has been shown to increase the mammary stem cell population in mice (15), which gives rise to transformed luminal progenitors (16). Basal and luminal cells in human breast tissue have differential responses to ionizing radiation (17). Basal cells undergo rapid but short-lived cell cycle arrest in response to ionizing radiation while luminal cells experience longer cell cycle arrest. Spontaneous BRCA1 and p53 deficient mammary tumors in mice initially showed variable resistance to chemotherapy but eventually became resistant (18). The resistant tumors appear to grow from a small fraction of surviving cells. This resistance may be due to upregulated drug transporters. A subsequent study identified increased stem cells present in these resistant tumors (19). These studies indicate that tumor initiating cells in breast cancer are more resistant to conventional anti-proliferative therapies. In future experiments, it will be important to characterize the gene expression changes that are responsible for the stem-like phenotype in breast cancer cells so that more effective therapies can be developed which target these cells.

Acknowledgements

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