Response of $CD44^{+}/CD24^{-/low}$ breast cancer stem/progenitor cells to tamoxifen- and doxorubicin-induced autophagy

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Received November 26, 2012; Accepted January 28, 2013

DOI: 10.3892/ijmm.2013.1342

Abstract. The cancer stem cell hypothesis emphasizes that cancers are driven by cells having stem cell properties, and it is believed that cancer stem cells (CSCs) may be responsible for resistance against therapeutic approaches and for recurrent tumors. Since the biology of the normal breast requires large numbers of stem cells, it has been thought that breast stem cells play an important role in initiating breast cancer. A better characterization of breast CSCs appears to be an essential step to improve the understanding of the biology of breast cancer and its management. The scope of this study was to isolate breast CSCs from a breast cancer cell line (MCF-7) using cell surface markers, and to test whether these cells have any resistance to autophagic cell death mechanisms mediated by commonly used chemotherapies and hormonal therapies such as doxorubicin (adriamycin) and tamoxifen (anti-estrogen), respectively. For this purpose, the $CD44^{+}/CD24^{-/low}$ MCF-7 breast cancer stem/progenitor cell population was isolated and treated with doxorubicin or tamoxifen and evaluated for their response to growth, autophagy and apoptosis. Our findings suggest that $CD44^{+}/CD24^{-/low}$ cells were less sensitive to doxorubicin, but did not demonstrate a significant difference towards tamoxifen in regards to the induction of autophagy.

Introduction

Breast cancer is the most common cancer and the second leading cause of cancer-related mortalities among women after lung cancer. Since 1896, clinical and experimental studies have supported the idea that breast cancer is a classical model of a hormone-dependent malignancy and that estrogens are mammary carcinogens. Estrogen plays an important role in the growth and differentiation of the normal mammary gland. The effects of estrogens are mediated by the intracellular estrogen receptors (ERs) and ERα is mostly found in the uterus and mammary gland (1). ER is a transcription activator and binds to the estrogen response element of the target gene activating gene transcription (2). It has been noted that estrogen administration enhances tumor growth while estrogen deprivation reduces tumors (3); therefore, elevated levels of estrogen in the body are a risk factor for breast cancer.

Breast cancer treatments include chemotherapy, hormone therapy as well as surgery and radiotherapy depending on the stage. Doxorubicin and tamoxifen are widely used drugs in chemotherapy and hormonal therapy, respectively. The exact mechanism of how doxorubicin works is complex, but it is thought that it interacts with DNA, prevents the DNA double helix from being resealed and stops the process of replication (4). Previous studies including ours have shown that doxorubicin induces autophagy (5). Tamoxifen is an estrogen receptor antagonist which competes with estrogen to bind to its receptor. The estrogen receptor/tamoxifen complex stops the genes being switched on by estrogen (6,7). Studies have shown that tamoxifen induces autophagy and apoptotic cell death in estrogen-positive breast cancer cells (8,9).

Cancer stem cells (CSCs) are cancer cells that possess the characteristics of normal stem cells (10). The cancer stem cell hypothesis proposes that tumors are initiated and maintained by a small fraction of cells, but the origin of these tumorigenic cells are actually not known. The stem cell theory of cancer proposes two major concepts. One theory claims that CSCs arise from mutated stem cells and then these mutated cells expand in such a manner that the mutation is shared by many of the descendants (11). An alternative theory proposes that transformed and differentiated cells acquire stem cell-like characteristics (12). This hypothesis is also known as epithelial to mesenchymal transition (EMT).

The development of the mammary gland suggests that stem cells play an important role in the biology of the breast. This fact suggests that the mammary gland is also particularly prone to carcinogenesis, and that breast stem cells play a very important role in breast cancer. CSCs also have been described in breast cancers. The CSC hypothesis in breast cancer assumes that CSCs can generate cells with a certain type of aberrant and limited differentiation (13). Conventional therapies used at present such as chemo-

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Key words: breast cancer stem cells, tamoxifen, doxorubicin, autophagy, apoptosis

therapy and radiation kill the growing differentiated cells but fail to kill these cancer-initiating cells (14). The reason is assumed that CSCs share the properties of normal stem cells which have mechanisms that make them relatively resistant to chemotherapy. These properties include resistance to drugs and toxins by expression of several ABC transporters, increased expression of BcL-2 family proteins, increased expression of pumps such as breast cancer resistance protein (BCRP) (15) and P-glycoprotein (PGP) (16) and active DNA repair capacity (13). In addition, stem cells divide infrequently in contrast to differentiated cells, and antimitotic chemotherapies are less effective against stem cells than mature cancer cells (13). Moreover, stem cells survive, grow and form colonies as tumorospheres in serum-free suspension, while more differentiated cells die under these conditions. In solid tumors, it has been shown that only a small proportion of the tumor cells are able to form colonies as revealed by *in vitro* clonogenic assays (17).

It has been proposed that CSCs are able to be isolated from breast cancer cell lines using specific cell markers, $CD44^+/CD24^{-/low}$ (18). CD44 is a stem cell marker that is common to different organs and pathologies. However, the $CD44^+/CD24^{-/low}$ phenotype is probably tissue restricted. Al-Hajj *et al* (19) used these cell-surface markers to isolate a subpopulation of highly tumorigenic breast cancer cells from human breast tumor. In breast cancer, a population of $CD44^+/CD24^{-/low}$ cells is considered to be highly enriched in cancer-initiating cells which are 1,000 times more tumorigenic than other cell populations, and injection of as few as 200 cells was found to lead to tumor formation in SCID mice .

Autophagy is a tightly regulated process involving the degradation of a cell's own components through the lysosomal machinery. Autophagy plays a normal part in cell growth, development, and homeostasis, helping to maintain a balance between the synthesis, degradation, and the subsequent recycling of cellular products. During nutrient starvation, autophagy leads to the breakdown of non-vital components and the release of nutrients to maintain metabolism and ATP levels to ensure cell survival (20). In addition, the autophagy-lysosomal pathway is an important mechanism for regulating the homeostasis of intracellular long-lived proteins and organelles (21). Moreover, autophagy plays a role in innate and adaptive immune responses (22). During apoptosis, induced autophagy can be either a protective mechanism or a process that causes cell death. Recent studies have shown that autophagy delays apoptotic death in noninvasive breast cancer cells following DNA damage (23). On the other hand, in the absence of apoptosis, autophagy can trigger a form of cell death (24). A previous study also demonstrated that prolonged autophagy in the absence apoptotic machinery is a cell survival mechanism that delays cell death in hematopoietic cells when growth factors and nutrients are short in supply (25). Malfunctioning of autophagy is observed in many human diseases such as cancer, neurodegenerative, infectious and inflammatory diseases, heart diseases and diabetes (26,27).

Understanding the role of CSCs during carcinogenesis has become a major focus in stem cell biology and cancer research. Therefore, the aim of this study was to isolate breast CSCs and test whether these cells show resistance to hormonal therapy (tamoxifen) similar to their resistance shown to chemotherapy. For that purpose, CSCs were tested initially for their chemotherapy resistance using doxorubicin. The possible autophagic cell death mechanism was then assessed in breast CSCs after they were treated with tamoxifen.

Materials and methods

Culture of MCF-7 cells. MCF-7 cells were kindly provided by Osmangazi University Medical School and cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 GlutaMAX containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) solution (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were grown in T-25 flasks with 4 ml medium and incubated in a CO_2 incubator at 37˚C in 5% CO_2. The medium of the cells was replaced every other day, and cells were passaged when they reached confluence.

Isolation of $CD44^+/CD24^{-/low}$ breast CSCs. MCF-7 cells were detached from the flask by treatment with trypsin-EDTA solution, and the cells were centrifuged. After cells were washed with PBS (pH 7.4), the cell pellet was resuspended in FITC-conjugated CD44 antibody (BD Pharmingen, USA) and PE-conjugated CD24 antibody (BioLegend, USA). The antibody concentration was 10 μl of antibody solution per $1x10^6$ cells. Cells were incubated with the antibodies for 40 min at room temperature (RT) in the dark. Unbound antibodies were washed off with PBS, and the cell pellet was resuspended in culture medium after centrifugation to be sorted. The cells were sorted by a fluorescence-activated cell sorting device (FACSAria II; BD Pharmingen).

Comparison of the growth rate of $CD44^+/CD24^{-/low}$ and MCF-7 cells. $CD44^+/CD24^{-/low}$ and MCF-7 cells were seeded into 24-well plates. At 24, 48, 72 and 96 h of incubation times, the values of the growth rates were determined by a cell proliferation assay. Culture medium was mixed with MTS One Solution (Promega, USA) at a ratio of 5:1, and 240 μl of this mix was added into each well. Cells were incubated for 2.5 h at 37˚C in a CO_2 incubator. Then, 200 μl of solution from each well was transferred into a 96-well plate. Absorbances were measured at 490 nm.

Determination of the inhibitory concentrations of tamoxifen and doxorubicin. $CD44^+/CD24^{-/low}$ and MCF-7 cells (1,250 cells/well) were seeded into each well of a 96-well plate, and different concentrations of tamoxifen and doxorubicin were applied to the cells one day later by diluting the drugs with culture medium. Following 72 h of drug treatment, the MTS experiment was performed. Culture medium was mixed with MTS One Solution at a ratio of 5:1, and 120 μl of MTS/culture medium was added to each well. The cells were incubated with the solution for 2 h at 37˚C. Then, absorbances were measured at 490 nm.

Analysis of apoptosis by Annexin V staining and flow cytometry. The FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen) was used to detect apoptotic cell death in the tamoxifen- and doxorubicin-treated cells incubated for 48 h. Untreated and drug-treated $CD44^+/CD24^{-/low}$ and

MCF-7 cells were detached from the flasks. Cells were transferred to FACS tubes, washed with PBS and centrifuged. The cell pellets were dissolved in 5 μl Annexin V and 5 μl propidium iodide (PI) solution and incubated at RT for 20 min in the dark. Meanwhile, 1 ml binding buffer was diluted with 9 ml PBS. Then, 400 μl diluted binding buffer was added into each FACS tube and the cells were analyzed by flow cytometry.

Analysis of autophagy by flow cytometry. Tamoxifen- and doxorubicin-treated $CD44^+/CD24^{-/low}$ and MCF-7 cells and untreated cells were detached from flasks following 48 h of treatment and transferred to FACS tubes. Acridine orange (1 μl; Sigma-Aldrich Co., Germany) was mixed with 10 ml culture medium. The mixture (1 ml) was added into each tube after they were centrifuged. The cell pellets were resuspended in the acridine orange staining solution and incubated at 37˚C for 15 min. The cell pellets were then washed with PBS, and finally the cell pellets were resuspended in 300 μl PBS to be analyzed by flow cytometry.

Western blot analysis of the LC3 protein. Both MCF-7 and $CD44^+/CD24^{-/low}$ cells were seeded, and one day later they were treated with tamoxifen and doxorubicin, respectively. At 48 h, treated and control cells were collected by trypsinization, washed with PBS, and the pellets were lysed with 100-150 μl lysis buffer. The protein concentration of the lysates was determined with Coomassie protein assay reagent (Thermo Scientific, USA) following the manufacturer's instructions. Subsequently, 20 μg protein of each sample was loaded onto a 12% separating acrylamide gel. After blotting, the membrane was incubated with LC3 (Axxora Nanotools, Germany) and β-actin (Cell Signaling, Technology, Inc., USA) primary antibodies at a concentration of 1 μg/ml at 4˚C. On the next day, the membrane was then incubated with the secondary antibody at a dilution of 1:10,000 for 1 h at RT with gentle agitation. Finally, the membrane was washed with TBST and then incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 5 min. Images of the proteins were captured with a molecular imager (ChemiDoc XRS+; Bio-Rad, USA) at a suitable time (change with respect to the primary antibody).

Results

Determination of the half maximal inhibitory concentrations of tamoxifen and doxorubicin. The initial step for evaluating the effect of tamoxifen and doxorubicin on isolated breast CSCs was to determine the half maximal inhibitory concentration (IC_{50}) of the drugs. Cell proliferation assay (MTS) was used to determine the IC_{50} of each drug by treating the MCF-7 cells with different concentations. Following 72 h of treatment, the results showed that the IC_{50} of tamoxifen and doxorubicin in MCF-7 cells was ~10 and 0.65 μM, respectively (Fig. 1). Therefore, 10 μM tamoxifen and 0.7 μM doxorubicin were used for the subsequent experiments. Moreover, we found that tamoxifen-induced effects on apoptosis and autophagy were significant at 48 h in the MCF-7 cells. For this reason, both tamoxifen and doxorubicin were incubated with the cells for 48 h.

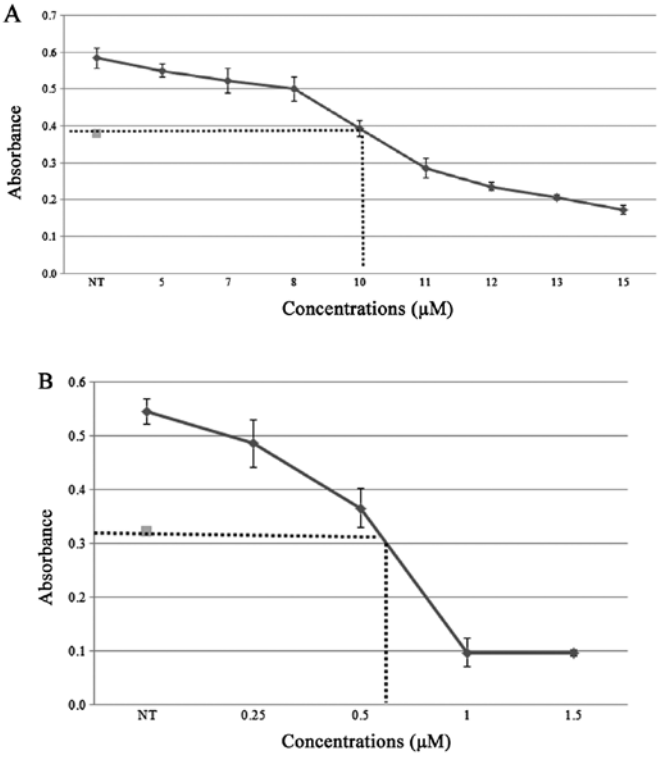

Figure 1. Half maximal inhibitory concentration (IC_{50}) of (A) tamoxifen and (B) doxorubicin in MCF-7 cells as determined by the MTS assay. NT, non-treated.

Isolation of $CD44^+/CD24^{-/low}$ cells. Different techniques for the isolation of the breast CSC population have been used. In this study, breast CSCs were isolated from the breast cancer cell line (MCF-7) using specific cell markers. These isolated cells were positive for CD44 and negative/low for CD24 markers. $CD44^+/CD24^{-/low}$ cells were sorted from the MCF-7 cells using CD44 and CD24 antibodies at the same time. At the end, it was observed that there were ~32% $CD44^+$ cells and 23% $CD24^{-/low}$ cells in the MCF-7 cells. Overall, there was ~1% $CD44^+/CD24^{-/low}$ cells present in the MCF-7 cell line (Fig. 2). $CD44^+/CD24^-$ cells were used immediately following sorting since they start to lose their properties by half in 5 days.

Comparison of the growth rate of $CD44^+/CD24^{-/low}$ cells and MCF-7 cells. CSCs display particular features; one of which is slow cell growth. To test this parameter, cell cycle rates of sorted cells and parental cells were compared. The proliferation assay results demonstrated that $CD44^+/CD24^{-/low}$ cells proliferated slower than the MCF-7 cells when they were allowed to grow for 96 h (Fig. 3).

Effect of tamoxifen and doxorubicin on the cell proliferation of MCF-7 and $CD44^+/CD24^{-/low}$ cells. Previous studies have shown that $CD44^+/CD24^{-/low}$ cells show resistance to chemotherapeutic drugs. Based on these studies, we tested the sorted cells for resistance to chemotherapy using doxorubicin and evaluated their viability by MTS cell proliferation assay. We compared $CD44^+/CD24^{-/low}$ cells to MCF-7 cells after treatment using the same drug. Moreover, the same procedures were also performed to evaluate the effects of tamoxifen in this CSC population.

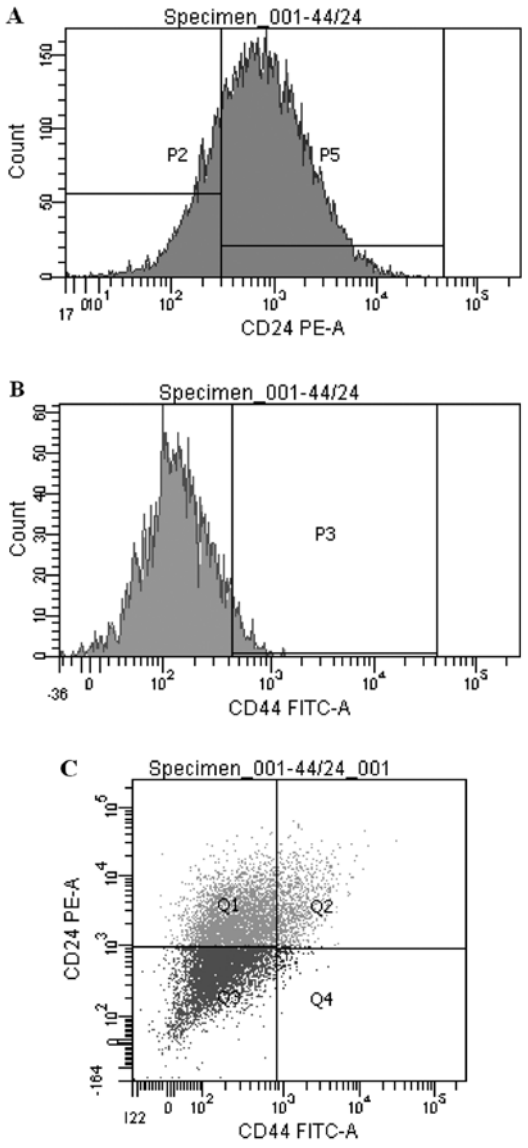

Figure 2. FACS histogram and quadrant data of CD44- and CD24-labeled MCF-7 cells (A) CD24-negative MCF-7 cells in P2 gate (B) CD44-positive and CD24-positive cells in P3 gate. (C) $CD44^{+}/CD24^{-/low}$ cells in the Q4 quadrant.

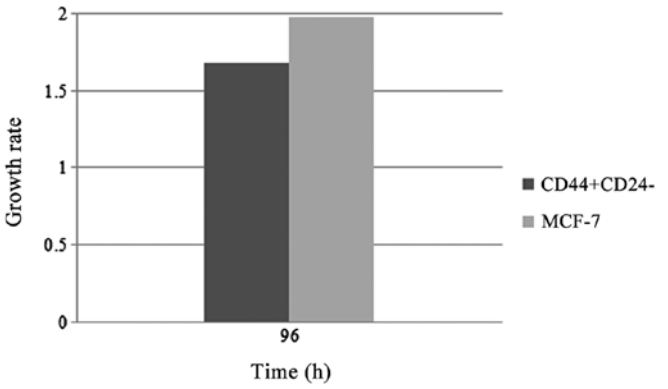

Figure 3. Comparison of the growth rates of MCF-7 and $CD44^{+}/CD24^{-/low}$ cells at 96 h of incubation.

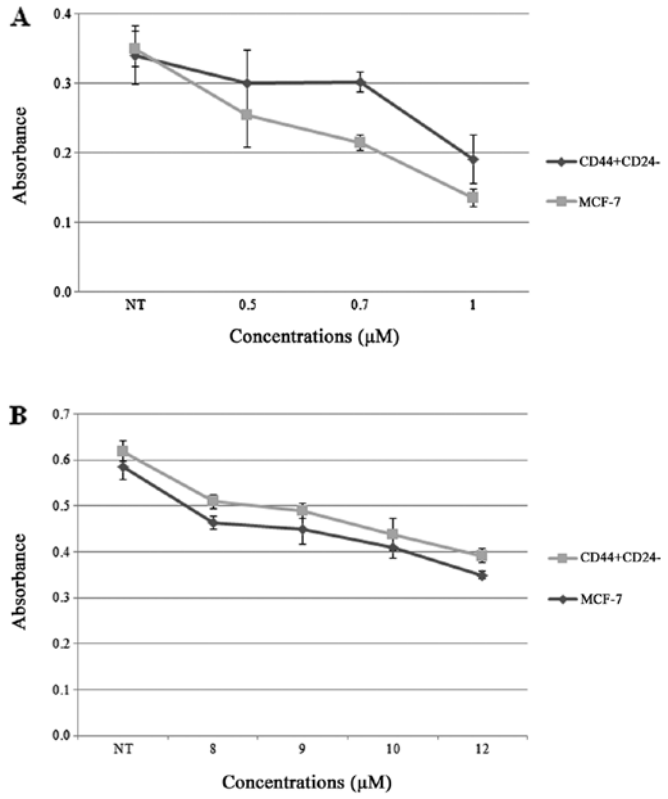

Figure 4. MTS cell proliferation assay of MCF-7 and $CD44^{+}/CD24^{-/low}$ cells following treatment with (A) doxorubicin and (B) tamoxifen.

Both isolated breast CSCs and parental cells were treated with doxorubicin for 48 h. Proliferation assay results demonstrated that the $CD44^{+}/CD24^{-/low}$ cells were less sensitive to doxorubicin when compared to MCF-7 cells when these cells were treated with concentrations at 0.5, 0.7 and 1 μM (Fig. 4A). In contrast, the proliferation assay results of tamoxifen experiments revealed that $CD44^{+}/CD24^{-/low}$ cells did show a slight resistance to tamoxifen when compared to the MCF-7 cells (Fig. 4B).

Induction of apoptosis and autophagy in $CD44^{+}/CD24^{-/low}$ cells by doxorubicin and tamoxifen. In addition to the proliferation assay, we carried out experiments to ascertain whether $CD44^{+}/CD24^{-/low}$ breast CSCs exhibit resistance to apoptosis or autophagy in response to chemotherapy and hormonal therapy. Both sorted and parental cells were treated with doxorubicin and tamoxifen, and then stained by Annexin V and PI for detection of apoptosis and acridine orange for the autophagy studies. The cells were then analyzed by flow cytometry. Flow cytometric results demonstrated that $CD44^{+}/CD24^{-/low}$ cells underwent ~10% less apoptotic cell death comparing to the MCF-7 parental cells following treatment with doxorubicin (Fig. 5A), supporting the results of the cell proliferation assay. Therefore, the results of the doxorubicin studies revealed that breast CSCs exhibit slight resistance to apoptosis following treatment with chemotherapy drugs. With respect to autophagy, on the other hand, $CD44^{+}/CD24^{-/low}$ cells did not show any resistance to doxorubicin as indicated by acridine orange stain and flow cytometric analysis when compared to the MCF-7 cells (Fig. 5B).

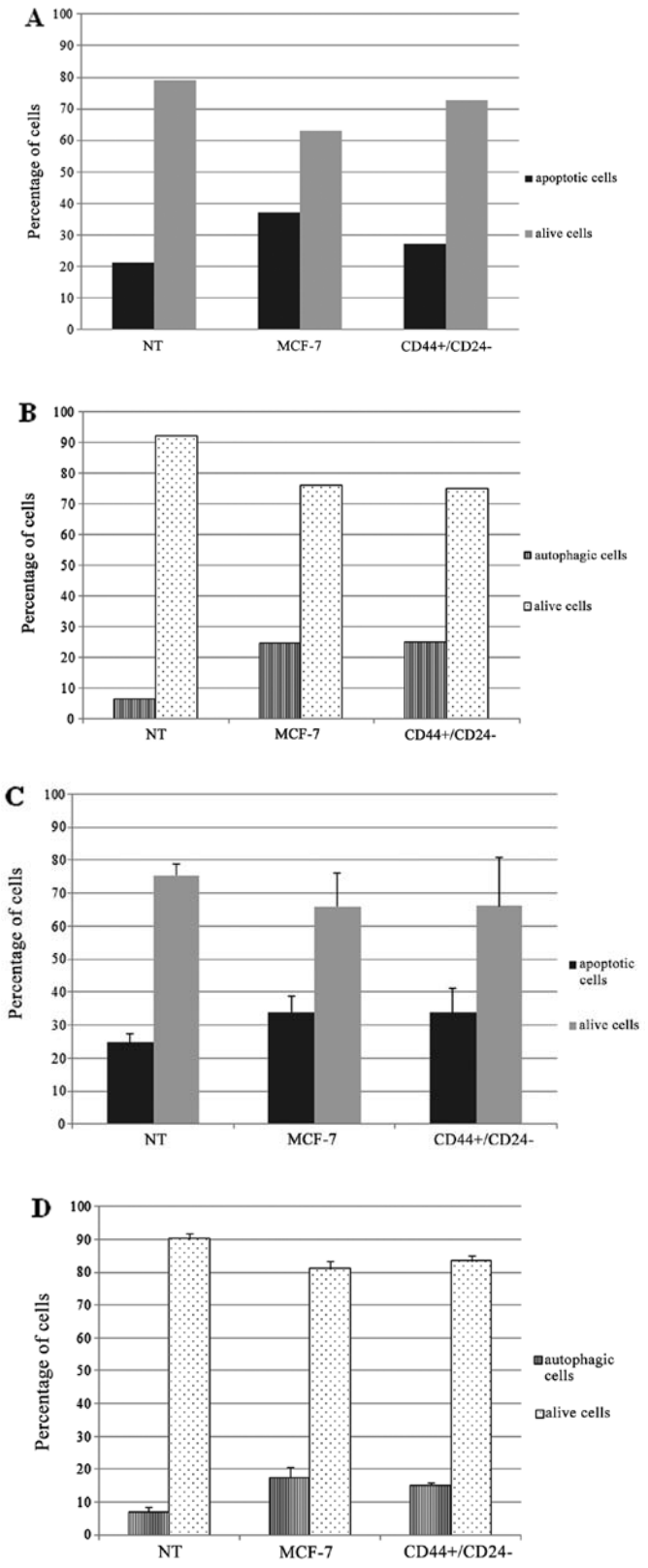

Figure 5. Flow cytometric results of the effects of doxorubicin and tamoxifen on the apoptosis and autophagy of MCF-7 and CD44^{+}/CD24$^{-/low}$ cells after 48 h of treatment. (A) Doxorubicin-induced apoptosis and (B) autophagy; (C) tamoxifen-induced apoptosis and (D) autophagy.

In the case of tamoxifen, CD44^{+}/CD24$^{-/low}$ cells did not show any difference in terms of resistance to tamoxifen when compared to the parental MCF-7 cells (Fig. 5C and D). The apoptosis and autophagy results of the CD44^{+}/CD24$^{-/low}$ cells and MCF-7 cells were similar.

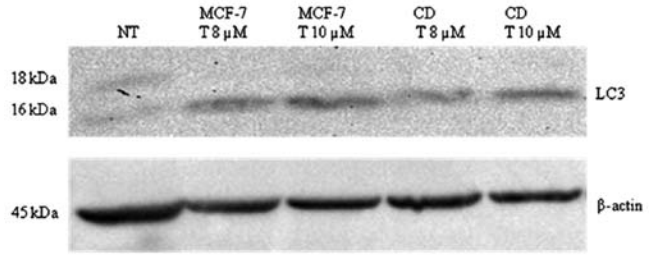

Figure 6. Western blot analysis for the LC3 protein demonstrating the autophagic effect of tamoxifen in MCF-7 and CD44^{+}/CD24$^{-/low}$ (CD) cells at 48 h. Lane 1, non-treated cells (NT); lanes 2 and 3, cell lysates of MCF-7 cells treated with 8 and 10 μM tamoxifen (T); lanes 4 and 5, cell lysates of CD44^{+}/CD24$^{-/low}$ cells treated with 8 and 10 μM tamoxifen.

Table I. Relative density of the LC3-II bands of the drug-treated cells compared to the untreated cells.

Samples	Relative density
NT	1
MCF-7 (8 μM tamoxifen)	2.70
MCF-7 (10 μM tamoxifen)	3.30
CD44^{+}/CD24$^{-/low}$ (8 μM tamoxifen)	2.80
CD44^{+}/CD24$^{-/low}$ (10 μM tamoxifen)	3.19

Effect of tamoxifen in CD44^{+}/CD24$^{-/low}$ and MCF-7 cells in respect to the autophagy marker LC3. In order to observe whether tamoxifen has a differential effect on autophagy in sorted CD44^{+}/CD24$^{-/low}$ breast CSCs compared to the parental MCF-7 cells, we analyzed LC3-I and II protein by western blotting. Tamoxifen induced autophagy in both CD44^{+}/CD24$^{-/low}$ breast CSCs and MCF-7 cells at 48 h (Fig. 6). LC3-I (18 kDa) to LC3-II (16 kDa) conversion was clearly visualized in the treated cells. Densitometric analysis of the LC3-II bands of the samples was carried out by ImageJ program (Table I). According to these results, 10 μM tamoxifen induced more extensive autophagic cell death in both the sorted and parental cells when compared to the untreated and 8 μM drug-treated cells. However, the breast CSCs did not show a significant difference in the extent of autophagic cell death when compared to the MCF-7 cells.

Discussion

CSCs were first described by the evidence that the growth and propagation of leukemia were driven by a small population of leukemia cells that have the ability for continual self-renewal, and these cells were termed as CSCs (13). Later, it was proposed that inhibition of tumor stem cells could prevent the recurrence of leukemia (28). Since that time, CSCs have been recognized as important components in carcinogenesis and have been isolated from many types of cancers including breast, brain, skin, head and neck and thyroid (13).

The objective of this study was to isolate breast CSCs from the MCF-7 breast cancer cell line and ascertain whether these isolated cells exhibit a difference in the autophagic response to tamoxifen-based hormonal therapy, which is used to treat ~50-60% of breast cancer patients (8). For that purpose, breast CSCs were isolated from MCF-7 cells by using CD44 and CD24 markers. Sorting results showed that only 1% of MCF-7 cells had $CD44^+/CD24^{-/low}$ breast cancer stem cell markers. This finding corroborated the results of Al-Hajj *et al* (19) who also reported the presence of ~1% of $CD44^+/CD24^{-/low}$ cells in the MCF-7 cell line. Moreover, we observed that the sorted cells started to lose their $CD44^+/CD24^{-/low}$ properties by half in ~5 days. Wright *et al* (29) also demonstrated that these sorted cells did not exhibit the $CD44^+$ and $CD24^-$ stem cell markers and lost chemotherapy drug resistance compared to parental cells when they were passaged four times as a monolayer.

Slow growth rate is a well-known parameter of breast CSCs (13). To ensure that our isolated cells carry stem cell features, we tested and demonstrated that sorted breast CSCs proliferated slower than the parental cells, as supported by Fillmore and Kuperwasser (30). Moreover, Kim *et al* (31) sorted $CD24^{-/low}$ cells from MCF-7 cells and compared the proliferation rate of these cells with the parental and the growth of $CD24^+$ cells by growing them for 72 h in DMEM. Their result revealed that the numbers of cultured $CD24^+$ and parental cells were higher than that in the $CD24^{-/low}$ cells. These results reveal that $CD24^+$ and MCF-7 cells have a higher proliferative capacity than $CD24^{-/low}$ cells and suggest that the expression of CD24 may enhance the growth and proliferation of MCF-7 cells.

It is known that both chemotherapy and radiation kill growing differentiated cells. Conventional chemotherapies are initially effective in controlling tumor growth. However, many patients relapse over time. One explanation for relapse is that cells which have a high tumorigenic potential are resistant to therapy (32). Resistance of CSCs, including breast CSCs toward chemotherapy drugs and radiation has been shown (14). Specifically, a previous study showed that $CD44^+/CD24^{-/low}$ breast CSCs were more resistant to chemotherapy drugs than the parental cells when the cells were treated for 48 h (29). Therefore, we tested the effect of doxorubicin on our sorted $CD44^+/CD24^{-/low}$ cells which were described as a highly tumorigenic subpopulation of breast cancer cells. Proliferation results demonstrated that sorted cells consisted of more viable cells comparing to the MCF-7 cells when these cells were treated with 0.5, 0.7 and 1 μM doxorubicin. In addition, flow cytometric results revealed that apoptotic cell death was ~10% less in the $CD44^+/CD24^{-/low}$ cells than that in the MCF7 cells when treated with 0.7 μM doxorubicin for 48 h, while the autophagic cell death ratio remained the same.

Tamoxifen is an FDA approved drug for the prevention and the treatment of breast cancer. For many years, it has been used as endocrine therapy for the treatment of both early and advanced breast cancer for patients with hormone receptor-positive breast cancer. Studies have shown that tamoxifen induces autophagy and apoptotic cell death in estrogen-positive breast cancer cells (8,9). In addition, we desired to ascertain how tamoxifen affects breast CSCs and whether or not these sorted cells display any resistance to hormonal therapy drugs. Therefore, we initially optimized and identified the effective dose and treatment time of tamoxifen. We then set out to study the effects of tamoxifen on isolated breast CSCs and compared the results with the MCF-7 parental cells. In contrast to doxorubicin, the tamoxifen experimentation results obtained by flow cytometric assays, western blot analysis and cell proliferation assay showed that both apoptotic and autophagic cell death ratios were similar in the $CD44^+/CD24^{-/low}$ cells and MCF7 cells. Overall, our studies demonstrated that isolated $CD44^+/CD24^{-/low}$ breast CSCs do not show significant resistance to tamoxifen which is the front-line therapy in most hormonal therapies for breast cancer patients. In conclusion, previous studies have demonstrated that CSCs show resistance to chemotherapy and radiation. This resistance is also thought to be the reason for the reoccurrence of tumor formation after chemotherapy or radiation therapy. In the present study, breast CSCs were isolated and studied to observe whether they were resistant to hormonal therapy. This study supported the results of previous studies showing that isolated breast CSCs from the MCF-7 breast cancer cell line show slight resistance to undergo apoptosis in response to doxorubicin. In the case of the hormonal therapy drug, tamoxifen, our studies demonstrated that tamoxifen-induced apoptotic and or autophagic cell death in these isolated cells were similar to that noted in MCF-7 cells and did not show a significant difference between the isolated breast CSCs and the parental cells.

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