Contribution of Autophagy in Acquired Drug Resistance of Human Breast Cancer Cells MCF7 to Doxorubicin

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Abstract

Introduction: At present, autophagy has attracted increased attention as a potential therapeutic target for breast cancer. Many investigators are trying to overcome drug resistance by inhibition of autophagy. The development of in vitro drug-resistant cell lines with well-characterized autophagy-related markers is required to discover new autophagy inhibitors. Therefore, in this study we established resistant human breast cancer cells MCF7 to doxorubicin (DOX) and used flow cytometric assays to study autophagic flux.

Materials and Methods: Resistant MCF7 subline (MCF7.res) was derived from parental MCF7 cells (MCF7.par) by continuous exposure to stepwise increasing concentrations of DOX (30–54 μM). Autophagic flux was assessed by the antibody labeling of the microtubule-associated protein LC3-II and analyzed by flow cytometry. In addition, lysosomal mass was measured flow cytometrically by LysoTracker Green (LTG) staining. The median fluorescence intensity of anti-LC3-II and LTG was compared between parental and resistant MCF7 cells. For detection of apoptotic cell death, Annexin V/propidium iodide staining was performed. After ~6 months, MCF7.res subline was obtained from MCF7.par cells.

Results: DOX resistance was confirmed by measurement of fold resistance and growth curve analysis. Our established MCF7.res subline exhibited 7.1-fold resistance to DOX compared with MCF7.par cells. Flow cytometric analysis revealed that LC3-II level and LTG signal were elevated in MCF7.res compared with MCF7.par, elucidating increased autophagic flux. The results of apoptosis assay showed that chloroquine (an autophagy inhibitor) could reverse drug resistance of MCF7.res by inhibiting autophagy.

Conclusion: Our established DOX-resistant MCF7 cells model is reliable and applicable for investigating new autophagy inhibitors.

Keywords: autophagy, drug resistance, doxorubicin, MCF7 cells

Introduction

According to estimates from the worldwide incidence and mortality of cancer, breast cancer is the common cause of tumor-related death in women.1 Drug therapy remains central to the treatment of breast cancer. However, acquired chemoresistance can occur in the majority of initially responsive patients leading to more aggressive and threatening tumor relapse that could be a big deal in the management of breast cancer.2 So, better knowledge of the molecular mechanisms involved in anticancer drug resistance would enable right selection of treatment regimen and ameliorate patient outcomes.

Acquired resistance to chemotherapy agents presents a major obstacle facing effective cancer treatment.3 Chemoresistance can be attributed to a large number of identified mechanisms such as drug inactivation, alternation in the drug targets, increased drug efflux, and activation of survival mechanisms like autophagy and deregulation of apoptosis.4 Among the mechanisms involved in chemoresistance, our knowledge about the role of autophagy, that is inevitably metabolic adaption pathway of cancer cells,5 is still restricted.

The term autophagy describes a homeostatic recycling process in all cells that provides a housekeeping cellular turnover and taking out damaged organelles and allowing the cells to adapt to physiological and pathological conditions.6 Increasing evidence has shown that autophagy is involved in tumor cell resistance to chemotherapeutic drugs, and application of the autophagy inhibitors in association with conventional chemotherapy might be a promising strategy to overcome anticancer therapeutic resistance.7

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Based on the majority of studies, autophagy is induced in response to chemotherapy and anti-estrogens in breast cancer\(^8\) playing a protecting role against drug therapy-induced apoptosis\(^9\) that can lead to the development of anticancer drug resistance. At present, hydroxychloroquine (HCQ) is the only Food and Drug Administration-approved autophagy inhibitor used in clinical investigation for breast cancer treatment.\(^10\) Meanwhile, pharmacodynamics studies revealed that high doses of HCQ up to 1200 mg/day is required to achieve sufficient autophagy inhibition.\(^11\) Thus, taking HCQ long-term at high doses can cause undesirable side effects such as irreversible retinopathy.\(^12\) In this regard, discovering better and more specific autophagy inhibitors with lower toxicity is urgently needed.

Developing in vitro models for drug resistance are one of the most useful tools to describe the interaction between tumor cells and anticancer agents in clinic.\(^13\) Of most publications in this research field, there is a very little emphasis and commonality in the methodology of developing drug-resistant cell lines.\(^14\) So, in the present research, we aimed to develop a simple and effective method to generate a doxorubicin (DOX)-resistant cell line, using human breast cancer cell (MCF7) as an in vitro model to determine the role of autophagy in resistant breast tumor cells.

**Materials and Methods**

**Materials**

LC3B Antibody Kit for Autophagy (containing LC3B rabbit polyclonal antibody, chloroquine [CQ] diphosphate), Alexa Fluor 647 goat antirabbit immunoglobulin G (IgG) and LysoTracker Green (LTG) were from Invitrogen, Ltd. DOX hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). All other materials used in this research were purchased from Sigma, Biowest, and Merck.

**Cell culture**

Human breast adenocarcinoma cell lines (MCF7) was obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran) and were maintained as a monolayer in Dulbecco’s modified Eagle’s medium supplemented with 10% heat inactivated fetal bovine serum and without antibiotics. Cells were incubated at 37°C with 5% CO\(_2\) gas.

**Induction of DOX-resistant MCF7 cells**

Determination of initial dose and treatment duration. To optimize the initial dose, DOX dose–response curve for parental MCF7 cells was generated using MTT assay. MCF7 (10\(^5\) cells) were cultured in 96-well plates and incubated for 24 hours before addition of DOX. After this time, cells were exposed to different concentrations of DOX (5–200 \(\mu\)M). Drug-free control was included in the assay. Plates were incubated for 72 hours. After the incubation time, MTT solution was added at a final concentration of 0.5 mg/mL and incubated for 4 hours until intracellular purple formazan was visible. Then, MTT-containing media were removed and solubilizing solution (200 \(\mu\)L) was added to each well. After dissolving, the absorbance was measured at 570 nm with plate reader. The concentration of DOX, which resulted in 20% growth inhibition over 3-day exposure, was the commencement of treatment.

The development of DOX-resistant MCF cell line. To develop a DOX-resistant MCF7 cell line, a continuous stepwise increase of DOX was selected. Parental MCF7 cells were seeded into two 25 cm\(^2\) flasks. One of them was left untreated and allowed to grow alongside the drug-treated flask. This flask was named MCF7-par. In the other flask, the cells treated with chronic IC\(_{20}\) of DOX (30 \(\mu\)M) obtained from MTT assay and were named MCF7-res. The cells maintained in DOX-containing medium and the media replenished every 3 days. As the cells reached 70%–80% confluency, they subcultured in the usual manner. After 2 weeks of treatment, in the initial steps, DOX concentration was increased 1.5-fold (45 \(\mu\)M) and the above process was repeated over the next 2 months. At this point, DOX concentration was increased 1.25-fold (56 \(\mu\)M) as a final concentration for a further 2 months.

**Confirmation of drug resistance**

Measurement of fold resistance. The sensitivity of parental cell line (MCF7-par) and the established resistant subline (MCF7-res) was determined using MTT assay. For cytotoxicity assay, MCF7-res cells were grown in drug-free medium 1 week before the experiment. The cells were treated with different concentrations of DOX for 48 hours and the IC\(_{50}\) values were calculated in the exponential phase of growth inhibition over 3-day exposure, was the commencement of treatment.

**Detection of autophagy**

Indirect immunofluorescence LC3B labeling. Indirect intracellular labeling with rabbit polyclonal anti-LC3-II was carried out by incubation with fixed and permeabilized parental and resistant MCF7 cell lines. In brief, cells were fixed and permeabilized in 100% methanol at −20°C for 10 minutes. After three times washing in phosphate-buffered saline (PBS), anti-LC3B polyclonal or rabbit immunoglobulin (as isotype control) was added and incubated for 30 minutes at room temperature. After this step, cells were washed in PBS and labeled with secondary fluorescent conjugate Alexa Fluor 647 goat anti-rabbit IgG for 30 minutes. Cells were then washed and resuspended in PBS. The resulting cells were then analyzed on a flow cytometer with a red laser. Median fluorescent intensity (MFI) of LC3-II-AF-647 of the 660/20 nm signal of the cells was compared to determine relative increase in the LC3-II autophagic signal above resting control cells (n = 3).\(^15\)

LTG labeling. To determine the autophagic flux, parental and resistant MCF7 cells were loaded LTG at a final concentration of 50 nM and incubated for 1 hour at 37°C. After incubation, cells were washed in PBS buffer and...
resuspended in 400 μL of PBS. The resulting cells were analyzed on a flow cytometer with excitation at 488 nm and LTG emission collected at 530/30 nm. LTG signals from samples were then compared by histogram analysis of MFI (n = 3).16

Detection of apoptosis by Annexin V labeling

Quantitative assessment of apoptosis was performed using apoptotic detection kit (Annexin V apoptosis detection kit FITC, eboiscience). MCF7.res cells treated with DOX at a concentration of IC50 value in combination with 50 μM CQ (autophagy inhibitor) for 24 hours. Then, cells were detached by trypsinization and washed with PBS. Cells were then suspended in 200 μL binding buffer containing Annexin V, and incubated for 10 minutes at room temperature. After centrifugation (1500 g, 1 minutes), cell pellet was resuspended in 200 μL binding buffer containing 5 μg/mL propidium iodide. Apoptotic cells were detected as Annexin V positive/propidium iodide negative using a partec PAS flow cytometer and data were analyzed by FlowJo 10.5.3 software.

Statistical analysis

Data were analyzed using the GraphPad Prism 8 software and presented as mean ± standard error of mean. Comparisons between more than two groups were analyzed using one-way ANOVA followed by Newman post hoc. Significance of differences were indicated as *p < 0.05 in all comparisons.

Results

Dose–response curve of DOX, the starting dose and selection strategy

As given in Figure 1, dose–response curve for cell viability was determined in human adenocarcinoma cells (MCF7) exposed for 72 hours to different concentrations of DOX. Chronic IC20 value of DOX was measured at 30 μM and used as the first dose.

As explained in Materials and Methods section, our procedure used to develop acquired DOX resistance in MCF7 cell lines, started out with a continuous treatment of low dose of DOX and then the dose escalated upward to 54 μM to achieve stable resistant subline for studying the potential role of autophagy in this model.

Confirmation of DOX-resistant subline

To define our conditioned subline as resistant, the fold resistance and the proliferation ability of the established cells in the presence of DOX was evaluated.

The effect of different concentrations of DOX on viability of parental and resistant MCF7 cells was investigated by MTT assay and the data were used to construct the survival curves as given in Figure 2. The IC50 value of MCF7.res (321 ± 8.37 μM) was 7.1-fold that of MCF7.par (45.22 ± 2.08 μM).

Figure 3 presents the ability of MCF7.res subline to grow in the presence of DOX at the concentration used to condition the cells (54 μM). We observed that our established resistant subline has a usual growth curve; however, the rate of growth and cell population at plateau state were significantly decreased in MCF7.res subline compared with MCF7.par cells (Table 1).

Detection of autophagy by flow cytometric analysis of LC3-II

There are two forms of the microtubule-associated protein LC3 in mammalian cells: soluble cytoplasmic LC3-I and membrane-bound LC3-II. With autophagy activation, LC3-
I is esterified by the binding of phosphatidyl ethanolamine, and becomes modified to the membrane-bound form of LC3-II, which is a preautophagy and autophagy molecular marker. Indirect intracellular labeling with rabbit polyclonal anti-LC3-II was carried out by incubation with fixed and permeabilized parental and resistant MCF7 cell lines. MFI values of LC3-II antibody in parental and resistant MCF7 cells were determined flow cytometrically and expressed as fold increase above parental cells. As given in Figure 4, parental MCF7 cells had a detectable level of LC3-II compared with isotype control. Moreover, a significant increase (6.7-fold) in the LC3-II amount was observed in resistant MCF7 subline compared with that in MCF7.par cells indicating activation of autophagy in resistant MCF7 subline.

Detection of autophagy by flow cytometric analysis of LTG

LysoTracker dyes label acidic spherical granules inside cells. An increased lysosomal mass could be observed during autophagic process and can be measured by the use of LysoTracker dyes. As given in Figure 5 and in accordance with LC3-II responses, a threefold increase in LTG MFI was observed in MCF7.res above MCF7.par level of LTG. This observation indicates activation of autophagy in resistant MCF7 subline.

Table 1. Growth Characteristics of Parental and Established Resistant MCF7 Cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Population doubling time (hours) ± SEM</th>
<th>Plateau saturation density (cell number/mL)</th>
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<tbody>
<tr>
<td>MCF7.par</td>
<td>17.87 ± 1.292</td>
<td>4.6 × 10^6</td>
</tr>
<tr>
<td>MCF7.res</td>
<td>23.04 ± 0.672</td>
<td>2.84 × 10^6</td>
</tr>
</tbody>
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Inhibition of autophagy promotes apoptosis in resistant MCF7 subline

CQ is an autophagy inhibitor and it has been indicated that inhibition of autophagy can promote apoptosis and reverse drug resistance. When 50 μM CQ was combined with DOX, the apoptotic cell death of MCF7.res was increased significantly compared with control group (DOX-resistant MCF7 cells treated with DOX alone) (Figure 6). This observation indicates that inhibition of autophagy can reverse the drug resistance of MCF7.res cells.

Discussion

Cancer cell line models with acquired chemoresistance have been long established with the aim of helping us to clarify mechanisms underlying cytotoxicity and resistance to anticancer drugs. In vitro models for anticancer drug resistance can be divided into two main categories: Clinically relevant and high-level laboratory. In clinically relevant model, low doses of drug and pulsed treatment are used to condition the cells. The established cells often present unstable and very low resistance and the molecular changes cannot be detected or analyzed. Besides, many investigators who intend to study mechanisms of resistance, attempt to develop a high level laboratory model. According to many studies, the resistant sublines derived by high-level laboratory method are more stable, level of resistance is higher, and identification of molecular pathways involved in resistant are easier. Therefore, our selection strategy was to expose MCF7 cells to continuous stepwise increasing of DOX.

The prodeath or prosurvival role of autophagy in cancer still remains controversial. Recent evidence indicates that autophagy prevents cancer development by removing and degradation of damaged organelles and misfolded protein

FIG. 4. Histogram overlay presentation of LC3-II-Alexa-Flour647 in parental and resistant MCF7 cells (A). MFI values of LC3-II antibody in parental and resistant MCF7 cells were determined flow cytometrically and expressed as fold increase above parental cells (B). * T test, **p < 0.01, color bars indicates average fold increase and each individual data point are also shown, n = 3. MFI, median fluorescence intensity.
aggregates. Several studies have reported autophagy defects in many human tumors. On the contrary, once cancer is established, increased autophagic flux may promote survival of tumor cells under conditions of metabolic and genotoxic stresses such as nutritional deficiency, hypoxia, and the presence of chemo and targeted therapies that might cause resistance to anticancer agents.

At present, targeting autophagy in anticancer drug resistance in breast cancer has become an area of interest. Numerous studies have revealed the contribution of autophagy process in drug resistance development in different subtypes of breast cancer. It has been reported that in HER2-positive breast cancers, treatment with trastuzumab (an antibody against HER2 receptors) or lapatinib (a tyrosine kinase inhibitor) induce autophagy in parental and resistant cells and pharmacologic or genetic inhibition of autophagy could restore the sensitivity of resistant cells. Chemotherapies such as, anthracyclines, taxanes, cyclophosphamide, and platinum agents are usually used in the treatment of triple-negative breast cancer (TNBC) cells. Inhibition of autophagy induced by an insulin-like growth factor 1 receptor (IGF-1R) inhibitor (NVP-AEW541) enhanced apoptotic cell death. Of particular importance is the role of autophagy in the development of resistance to anti-estrogen or chemotherapeutic drugs in MCF7 cells. Autophagy contributes to tamoxifen resistance in MCF7 cells. In addition, it has been indicated that epirubicin resistance in MCF7 cells is owing to induced autophagy and inhibition of autophagy resulted in enhanced apoptosis.

DOX, an anticancer drug belongs to anthracycline group, has a broad-spectrum antineoplastic effect and is used for the treatment of various types of cancers and TNBC and in combination with endocrine therapy for luminal breast cancer. It has been reported that DOX resistance mainly occurs through increased ABC transporters, epithelial mesenchymal transition, changes in target proteins (topoisomerase II and ERK1/2), and overexpression of detoxifying enzymes. Moreover, some studies elucidated that DOX exposure induces autophagic flux that is involved in DOX resistance in a variety of cancer types including osteosarcoma, hepatocellular carcinoma, neuroblastoma, and breast cancer. There is a significant variation in the procedure used to develop DOX resistance in breast cancer cell lines. For instance, the concentration of DOX, the time required to achieve resistance, the definition and level of resistance, and even the measured molecular mechanisms involved in resistance varied from study to study. In a study investigating the effect of autophagy on drug resistance of multiple myeloma (MM) to DOX, DOX-resistant MM cell line was developed by continuously increasing the DOX concentration over 10 months. In this study, the fold resistance index of MM cells was 10.8 and activation of autophagy was observed in these resistant cells. In another study, after 18 months treatment with DOX and paclitaxel, resistant cell lines were obtained from breast cancer cells (MDA-MB-231 and MCF7). Ten DOX-resistant MCF7 cells were generated in this study with an average fold resistance of 10.25. The advantage of our established model of acquired DOX resistance in MCF7 cells in comparison with previous studies is that in a short time, extended treatment with DOX at IC20 resulted in significant DOX resistance (fold resistance of 7.1).

Furthermore, to study the autophagic flux we performed flow cytometric assay that is a fast, valid, and reliable technique and could be an alternative method for costly and time-consuming immunoblotting assays. In summary, in this study we established a DOX-resistant subline for

**FIG. 5.** Histogram overlays of MCF7 LTG expression of parental and resistant cells (A). MFI value of LTG expression for parental and resistant MCF7 cells were determined flow cytometrically and expressed as fold increase above parental cells (B). T test, **p < 0.01, color bars indicates average fold increase and each individual data point are also shown, n = 3. LTG, LysoTracker Green.
breast cancer. The mechanism of resistance was investigated in this model using flow cytometric analysis of LC3-II and LTG. This resistant subline might be applicable to find out new autophagy inhibitors in breast cancer therapy research.

Author Contributions
M.S. designed the study and contributed to data interpretation; N.A. performed in vitro experiments, analyzed data, and wrote the article; and F.S. contributed to financial support and data interpretation.

FIG. 6. Effect of autophagy inhibitor CQ on apoptosis in resistant MCF7 cells. Representative dot plot of Annexin V and propidium iodide staining of resistant MCF7 cells treated with CQ for 48 hours (A). Quantitative analysis of the apoptotic cells (B). ***p < 0.001 versus DOX treated. Columns present mean ± SEM of three experiments. CQ, chloroquine.
Author Disclosure Statement

No competing financial interests exist.

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References


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