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Enhanced efficacy of chemotherapy for breast cancer stem cells by simultaneous suppression of multidrug resistance and antiapoptotic cellular defense

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ABSTRACT

While chemotherapy is universally recognized as a frontline treatment strategy for breast cancer, it is not always successful; among the leading causes of treatment failure is existing and/or acquired multidrug resistance. Cancer stem cells (CSCs), which constitute a minority of the cells of a tumor, are acknowledged to be responsible for increased resistance to chemo-drugs through a combination of increased expression of ATP-binding cassette transporters (ABC transporters), an increased anti-apoptotic defense, and/or the ability for extensive DNA repair like normal stem cells. Consequently, more effective therapy, especially targeted to CSCs, is urgently required. We studied the characteristics of 231-CSCs (CD44+/CD24−) sorted from human MDA-MB-231 breast cancer cells and demonstrated that 231-CSCs exhibited enhanced capacities for proliferation, migration, tumorigenesis and chemotherapy resistance. To address these multifunctional facets of CSCs, we devised a non-ionic surfactant-based vesicle (niosome) co-delivery system to simultaneously deliver siRNAs, targeted to both the ABC transporter (ABCG2) and the anti-apoptosis defense gene (BCL2), and doxorubicin (DOX) to CSCs. The rationale is to sensitize CSCs to DOX by down regulating the drug-resistance gene ABCG2 and simultaneously induce apoptosis by lowering BCL2 expression. The co-delivery system (CDS) successfully delivered siRNAs and DOX to the cytoplasm and nuclei, respectively, and resulted in a down-regulation of ABCG2− and BCL2 mRNAs in CSCs by 60% and 65%, respectively, compared to the control. A corresponding decrease in protein expression was observed using Western blotting. The IC_{50} of DOX in CSCs concurrently decreased significantly. Our result established CDS as a promising multi-drug delivery platform for cancer treatment.

Statement of Significance

Cancer stem cells (CSCs) are acknowledged to be responsible for increased resistance to chemo-drugs through a combination of increased expression of ABC transporters, an increased anti-apoptotic defense, and/or the ability for extensive DNA repair like normal stem cells. Consequently, effective therapy, especially to CSCs, is urgently required. In current study, we studied the characteristics of 231-CSCs sorted from human MDA-MB-231 breast cancer cells and found that 231-CSCs possessed enhanced proliferation, migration, tumorigenesis, and DOX resistance. We employed a non-ionic surfactant-based vesicle (niosome) delivery system to simultaneously deliver siRNAs targeted to multi-drug resistance genes, and DOX to kill 231-CSCs. The CDS showed an enhanced therapeutic effect by resensitizing 231-CSCs to DOX and may constitute a promising candidate for cancer chemotherapy.

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1. Introduction

Breast cancer is the most common malignant disease among Western women. Current treatment strategies, including chemotherapy and radiotherapy, frequently lead to acquired multiple drug resistance (MDR) and, consequently, treatment failure. In recent years, with the discovery of cancer stem cells (CSCs) in various types of cancers [1–3], the theory of tumorigenesis has been renewed. Al-Hajj et al. [1] successfully isolated the CSC subpopulations from breast cancer based on characteristic stem cell surface markers CD44+/CD24−. As few as 1000 cells with this phenotype could consistently form tumors in NOD/SCID mouse which was not possible with other subsets of breast cancer cells. Hence, CSCs are considered to be the driving force in metastases, and are accountable for such tumor properties as proliferation, aggressiveness, and resistance to treatment [4]. In many studies, the relationship between cancer resistance to conventional treatment and CSCs' intrinsic mechanisms of resistance to conventional chemotherapeutics has been a significant and integral hypothesis in the quest for clinically relevant cancer therapy [5].

The main mechanisms of MDR include pump and non-pump resistance [6–8]. Pump resistance is caused by membrane efflux pumps, which can flush out the chemotherapeutic drugs and therefore decrease intracellular drug concentration and efficacy. ABCG2 is a member of ATP-binding cassette transporters (or drug efflux transporter proteins), also known as breast cancer resistance protein (BCRP), and has been found to play a role in anticancer drug resistance [9]. In addition, CSCs overexpress ABCG2 and contribute to the typical MDR [10,11]. Non-pump resistance is caused by the activation of anti-apoptotic defense with BCL2 protein as an important player [12–17]. Therefore, simultaneous suppression of these two genes by RNA interference (RNAi) may sensitize drug-resistant tumor cells, including CSCs, to chemotherapy. Hence, combining this dual RNAi therapy with a chemotherapeutic agent may produce a more effective treatment for cancer patients than the traditional chemotherapy that fails to eliminate drug-resistant CSCs.

This study was focused on CSCs, sorted on the basis of the cell surface markers CD44 and CD24 from the human MDA-MB-231 breast cancer cells (231-CSCs) in terms of proliferation and migration *in vitro*. In addition, the tumorigenesis and chemotherapy resistance for the 231-CSCs and the unsorted parental cells was studied *in vivo* and induced tumors were treated by local delivery of doxorubicin (DOX) from scaffolds.

The major hurdle in developing siRNA therapeutics is the development of effective delivery system to transport of siRNAs across cell membrane and endosome into the cytosol to initiate the RNA interference machinery. Although a number of vectors including cationic liposomes and polyplexes have been developed in the past decade, the delivery efficacy, complexity of materials synthesis as well as biocompatibility are still major issues that need to be addressed [18–20]. Niosomes, which are mainly composed of non-ionic surfactant sharing similar vesicular structure to liposomes, are a novel type of drug delivery system. In comparison to liposomes, they are considered to be better candidates as carriers for drug delivery due to such factors as higher stability, loading capacity and biocompatibility [21,22]. Niosomal drug delivery is potentially applicable as a carrier for many pharmacological agents in connection with various diseases including cancer. Rogerson et al. reported that niosomal DOX could increase the life span of mice bearing S-180 tumors and decrease the proliferation rate of sarcoma by effectively prolonging DOX's circulation and metabolism [23]. Methotrexate entrapped in niosomes, administered intravenously to S-180 tumor-bearing mice, resulted in higher plasma level, and slower elimination and total tumor regression [24,25]. Although niosomes have been widely investigated as drug delivery carrier, due to their neutral charge, traditional niosomes are not suitable for gene delivery. In contrast, although cationic lipids, such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), have been widely used for gene delivery, the transfection efficiency is usually not sufficient. In this study, we added DOTAP as helper lipid into Span80-based niosome to render its ability for siRNA loading. More importantly, recent studies showed that cationic niosomes are highly potent gene delivery carriers by promoting endosomal escape and transport of payload to cytoplasm [26,27]. Therefore, our system is a new type of cationic niosome for co-delivery of drug and siRNA.

To achieve enhanced therapeutic effect on 231-CSCs, we devised a novel niosomal co-delivery system (CDS), which contains: (1) niosome as a nano-carrier; (2) DOX as an inductor of apoptosis; (3) siRNA targeted to ABCG2 mRNA (siABCG2) as a suppressor of pump resistance; and (4) siRNA targeted to BCL2 mRNA (siBCL2) as a suppressor of the anti-apoptotic defense (non-pump resistance). As a rational design, we hypothesized that the CDS could sensitize 231-CSCs to the chemotherapeutic drug DOX by down-regulating the drug-resistance gene ABCG2 and the anti-apoptosis defense gene BCL2, and thereby constitute a novel promising therapeutic system in the treatment of breast cancer.

2. Materials and methods

2.1. Cell culture

The human MDA-MB-231 breast cancer cell line was maintained in Eagle's Minimum Essential Medium (EMEM) (ATCC® 30-2003™) with 10% fetal bovine serum (FBS) (ATCC® 30-2020™). Cells were incubated at 37 °C in 5% CO_2 in a humidified incubator.

2.2. Flow cytometry

CSCs were identified based on the cell surface markers CD44 and CD24, using flow cytometry with a fluorescence-activated cell sorter (FACS; Becton Dickinson, Denmark). Briefly, MDA-MB-231 cells (1×10^7 cells in phosphate-buffered saline with 0.5% bovine serum albumin (PBS/BSA)) were incubated with FITC mouse anti-human CD44 (anti-CD44 FITC; cat. 555478, BD Biosystem) and PE mouse anti-human CD24 (anti-CD24 PE; cat. 555428, BD Biosystem) for 30 min at room temperature in total darkness. Unbounded antibodies were washed off with PBS/BSA prior to analysis. Following cell sorting, four subpopulations were collected: CD44+/CD24− cells, CD44+/CD24+ cells, CD44−/CD24+ cells, and CD44−/CD24− cells. These subpopulations were maintained in the same manner as the parental cells.

2.3. Proliferation and migration assays

In order to evaluate proliferation, four subpopulations of MDA-MB-231 cells were seeded in six-well plates containing 50,000 cells/well. Cells in each well were counted one, two, four, six, eight and 10 days after seeding. Growth curves of each subpopulation were drawn according to the number of cells at each time point. Based on the growth curves, two out of four subpopulations (CD44+/CD24− and CD44+/CD24+) were selected for further evaluation of cell migration using a monolayer wound healing assay [28]. Briefly, two parallel lines were drawn at the underside of the well with a marker, which served as fiducial marks for the analysis of the wound areas. The two subpopulations were 100% confluent in the six-well plates prior to the first day of analysis. Two

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