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Different administration strategies with paclitaxel induce distinct phenotypes of multidrug resistance in breast cancer cells

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ABSTRACT

Both dose-dense and dose-escalation chemotherapy are administered in clinic. By approximately imitating the schedules of dose-dense and dose-escalation administration with paclitaxel, two novel multidrug resistant (MDR) cell lines Bads-200 and Bats-72 were successfully developed from drug-sensitive breast cancer cell line BCap37, respectively. Different from Bads-200, Bats-72 exhibited stable MDR and significantly enhanced migratory and invasive properties, indicating that they represented two different MDR phenotypes. Our results showed that distinct phenotypes of MDR could be induced by altered administration strategies with a same drug. Administrating paclitaxel in conventional dose-escalation schedule might induce recrudescent tumor cells with stable MDR and increased metastatic capacity.

1. Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total new cancer cases and 14% of the cancer deaths in 2008 [1]. Although various treatments are currently available, chemotherapy remains one of the most important therapeutic strategies for breast cancer [2–4]. Anthracycline-based regimens and taxanes (paclitaxel and docetaxel) are currently considered as standard first-line chemotherapy for metastatic breast cancer (MBC) [3]. In clinic, most chemotherapeutic treatments are administered over a period of several hours at cycles of every 21–28 days [5]. The US Food and Drug Administration approved dosing schedules for taxanes in MBC are 60–100 mg/m^2 for docetaxel (1-h *i.v.*) and 175 mg/m^2 for paclitaxel (3-h *i.v.*) every 3 weeks, respectively [6].

However, recent novel schedules of chemotherapy administration appear to incrementally improve therapeutic outcome [7]. The concept of dose-dense chemotherapy, administering the drugs with a shortened intertreatment interval, is based on the observation that a given dose of chemotherapy always kills a certain fraction of exponentially growing cancer cells [8]. By administering lower doses more frequently, toxicity to normal tissues may be decreased without compromising the drug's antitumor efficacy [6]. Thus, it is believed that more frequent administrations of cytotoxic agents would be more effective in minimizing residual tumor burden compared to conventional dose-escalation chemotherapy [7,9,10]. Numerous mono and combination chemotherapy trials suggested that weekly administration of paclitaxel has a better therapeutic index than the standard every 3-week regimen and is not associated with increased side effects [9,11–13]. These results are very encouraging for the incorporation of dose-dense chemotherapy in every day practice for breast cancer.

Although anticancer therapies with different schedules of administration will alter tumor growth, the effect is usually not long lasting. Due to multiple factors including drug resistance, tumor recurrences frequently occur months to years after an effective response to initial chemotherapy [14]. Previous studies reported that around 40% of breast cancer patients suffer a recurrence [15,16]. The recurrent tumor cells are usually more aggressive, with different phenotypes of drug resistance as well as formidable migration and invasion. The aim of the present study is to determine whether different administration methods with the same anticancer drug could affect the properties of recrudescent tumor cells.

In this study, two newly MDR cell lines, Bads-200 and Bats-72, were developed from chemo-sensitive human breast cancer cell line BCap37 by using different screening strategies. Bads-200 was induced by conventional continuous exposure to paclitaxel with

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dose-stepwise increments, while Bats-72 was developed by an improved method based on pulsed exposure to paclitaxel with time-stepwise increments. Through a series of comparative studies, we found that Bads-200 and Bats-72 represented two different MDR phenotypes, indicating that different administration strategies with the same anticancer drug could induce distinct phenotypes of MDR in breast cancer.

2. Materials and methods

2.1. Cell lines and mice

Human breast cancer cell lines BCap37, Bads-200 and Bats72, were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Female aged 5–6 weeks of athymic nude (nu/nu) mice were purchased from Shanghai SLAC animal facility. All animal care and experiments were conducted according to Zhejiang University Animal Care Committee guidelines.

2.2. Examination of morphology and cell growth rate in vitro

BCap37, Bats-72 and Bads-200 cells were sub-cultured into 35-mm dishes for 48 h. Giemsa staining was carried out by a commercial kit (Jiangcheng Biotech, Nanjing, China). Stained cells were observed and photographed with a bright field microscope. To determine the cell growth rate *in vitro*, BCap37, Bats-72 and Bads-200 cells were plated into 6-well tissue culture plates at a density of 4×10^4 cells/well. Two cell counts for each cell line were made every 24 h for 10 days. The obtained data were subjected to linear regression analysis, in which the population doubling time (T_d) was calculated based on the formula $T_d = \ln 2/\text{slope}$.

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

As described previously [17], cells were harvested and resuspended to a final concentration of 1×10^4 cells/ml. Aliquots of the cell suspension were evenly distributed into 96-well tissue culture plates. After one night of incubation, the designated columns were treated with drug regimes. Four hours prior to the end of time point, MTT solution was added. The medium containing MTT was replaced with 150 μL of DMSO in each well to dissolve the formazan crystals after 4 h-incubation. The absorbance in individual wells was determined at 560 nM using a microplate reader (Bio-Rad, Sunnyvale, CA).

2.4. Examination of cell growth rate and drug resistance in vivo

To develop the human breast xenografts, *in vitro* growing BCap37, Bads-200 and Bats-72 cells (1×10^6 cells in 0.2 mL PBS) were implanted into the right flanks of the homozygous nude athymic mice (female, 5–6-weeks old). When tumors reached a mean diameter of 0.4–0.6 cm, each type of xenografts were randomly divided into three groups and treated with (i) vehicle; (ii) fulvestrant (2 mg per mouse, *i.m.*), (iii) paclitaxel (20 mg/kg, *i.v.*). The same treatment regimens were repeated every 3 days for total of six cycles. Two perpendicular diameters (width and length) of the tumors and body weight of mice were measured every 3 days until the animals were killed. The curve of tumor growth was drawn based on tumor volume and corresponding time (days) after treatment. When the animals were terminated, the tumor tissues were removed and weighted. The inhibition rate of tumor growth (IR) was calculated according to the following formula: IR = 100% × (mean tumor weight of control group − mean tumor weight of experimental group)/mean tumor weight of control group [18]. Data are representative of two separate experiments.

2.5. Quantitative real-time PCR (RT-PCR)

Total RNAs from BCap37, Bats-72 and Bads-200 cells were isolated with TRIzol, purified and dissolved in RNase-free water. RT-PCR was performed to determine the expression of *ABCB1*, *ABCC1* and *ABCG2* in BCap37, Bats-72 and Bads-200 respectively. The primers were designed as follows: ABCB1, forward: 5′-ATGCCTTCATCGAGTCACTGC-3′ and reverse: 5′-ACGAGCTATGGCAA-TGCGTT-3′; ABCC1, forward: 5′-AGCGCTTCCTTCCTGTCGA-3′ and reverse: 5′-TGTTCCGACGTGTCCTCCTT-3′; ABCG2, forward: 5′-AGGATTGAAGCC-AAAGGCAGA-3′ and reverse: 5′-GACCTGCTGCTATGGCCAGT-3′; 18S rRNA, forward: 5′-CGGCTACCACATCCAAGGAA-3′ and reverse: 5′-GCTGGAATTA-CCGCGGCT-3′; 18S rRNA served as an internal control. All assays were set up for a relative quantitative method, in which mean Ct values from BCap37 samples were used as calibrators for data analysis for both Bats-72 and Bads-200 samples. The relative fold changes (FC) of gene expression were calculated by using the standard $2^{-\Delta\Delta ct}$ method.

2.6. Western blotting

Cellular proteins were isolated with a protein extraction buffer (Beyotime, Haimen, China). Equal amounts (40 μg/lane) of proteins were fractionated on 6–10% SDS–PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were incubated with anti-MDR, anti-MRP1, anti-BCRP primary antibodies (Santa Cruz, CA), respectively. After washing with PBS containing 0.1% (v/v) Tween 20, the membranes were incubated with peroxidase-conjugated goat antimouse secondary antibodies followed by enhanced chemiluminescent staining using the ECL system. β-actin was used for normalization of protein loading [19].

2.7. Rhodamine 123 efflux assay

Cells were incubated by incubation medium (Hanks' balanced salt solution supplemented with 10% FBS) with or without verapamil. Followed by incubation for 30 min, Rhodamine 123 was added into the incubation medium (10 μg/mL). The cells were then incubated for additional 1 h. After washed with ice-cold PBS, the cells were trypsinized and resuspended in 500 μL PBS. Intracellular Rhodamine 123 fluorescence intensity was determined with Coulter Epics V instrument (Beckman Coulter, CA).

2.8. In vitro migration and invasion assay

Migration assays were performed in a 24-well Transwell chamber (Corning, MA). Briefly, 5×10^4 cells were seeded to upper chamber in 200 μL of serum-free medium. The lower parts of the chambers were filled with 600 μL of RPMI 1640 medium containing 10% FBS. After incubation for 12 h, the migration cells were then fixed, stained and enumerated. The same procedures were followed for the invasion assay except each Transwell chamber was coated with 30 μg Matrigel and incubation for 24 h.

2.9. In vivo tumor metastasis assay

According to method described previously [20], a total of 0.5 million cells were injected into each nude mouse (female, 5 weeks old) through tail veins. After 4 weeks, the animals were killed, and lungs were harvested and fixed in 10% neutral formalin, embedded in paraffin. Slides were prepared and stained with hematoxylin and eosin (H&E) followed by examination and photography under microscopy.

2.10. Statistical analysis

Data are presented as mean ± standard error of three independent experiments. Two-sided Student's *t* test was used to determine the statistical difference between various experimental and control groups. Differences were considered statistically significant at a level of $P < 0.05$.

3. Results

3.1. Establishment and morphological characterizations of drug resistant cell lines

BCap37 is an ER-negative and Her2-positive human breast cancer cell line (Supplementary Fig. S1). Bads-200 cells were selected based on continuous exposure to paclitaxel using a dose-stepwise incremental strategy from BCap37 (Fig. 1A). In the adaptation stage, BCap37 cells were exposed to paclitaxel for 72 h in stepwise increments of concentrations ranging from 5 nM to 100 nM (5, 10, 20, 50, 100 nM). Following each dose-induced step, surviving cells were amplified in paclitaxel-free medium. After repeating 3 times (for each dose), cells were exposed to the next higher concentration of paclitaxel. In the consolidation stage, previously selected cells were continuously cultured in medium containing 200 nM paclitaxel, until they multiplied normally in medium containing 200 nM paclitaxel. The resulting resistant cell line (Bads-200) was maintained in medium containing 200 nM paclitaxel.

Bats-72 cells were produced based on a strategy of pulsed exposure to paclitaxel with time-stepwise increments (Fig. 1B). Briefly, BCap37 cells were exposed to 200 nM paclitaxel in time increments ranging from 0.5 h to 48 h (0.5, 1, 2, 4, 12, 24, 48 h) in the adaptation stage. The surviving cells were amplified in paclitaxel-free medium. Each pulse treatment was repeated 3 times, followed by exposure to the next longer time-course. In the consolidation stage, previously selected cells were exposed to ten pulses of treat-