Oncology and Translational Medicine
DOI 10.1007/s10330-015-0062-3
June 2015, Vol. 1, No. 3, P120–P124
ORIGINAL ARTICLE

Preliminary research on dendritic cells loaded with resistant breast cancer antigens in breast cancer-bearing nude mice*

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Abstract

Objective The aim of the study was to investigate the inhibitory effects of dendritic cells (DCs) loaded with resistant breast cancer antigens on breast cancer in nude mice.

Methods A single-cell suspension was prepared from a primary breast cancer and chemotherapeutic drugs were screened using the ATP-PCA susceptibility testing system. Cancer cells were treated with 1/10 × IC_{50}, 1/5 × IC_{50}, 1/2 × IC_{50}, 1 × IC_{50}, and 2 × IC_{50} medium until their growth became steady in the 2 × IC_{50} medium. Peripheral blood mononuclear cells (PBMCs) were obtained from the peripheral blood of patients with leukapheresis. The obtained adherent cells were induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) to generate DCs, which carried resistant strain cell lysis compounds or non-treated cancer cell lysis compounds. The former mature DCs carried resistant breast tumor antigens. A breast tumor-bearing nude mouse model was established with these resistant strains and the mice were randomly divided in three groups. The mice in the treatment group were injected with DCs loaded with resistant breast cancer antigens. The control group consisted of mice injected with DCs loaded with primary tumor cell antigens and the blank group consisted of mice injected with the same volume of normal saline. Changes in the cancers were observed.

Results After treatment with the effector cells, the cancer volume and weight were significantly different to those before treatment in every group of mice ($P < 0.05$). The tumor volume in the blank group was the largest (3.362 ± 0.068 cm^3) and the tumor weight was 637.50 ± 59.398 mg. Compared to the blank group, the tumor volume in the experimental group was the smallest (1.273 ± 0.071 cm^3) and the tumor weight was 206.81 ± 32.711 mg.

Conclusion DCs loaded with resistant breast cancer antigens demonstrated a significant inhibition effect on the cancers of breast tumor-bearing nude mice.

Key words ATP-TCA; breast cancer; dendritic cell; nude mice; resistance

Received: 16 January 2015
Revised: 10 February 2015
Accepted: 25 March 2015

Breast cancer is a malignant tumor that threatens women's physical and mental health. While chemotherapy is an indispensable mean of treatment, the clinical effect is often not ideal. It is mainly due to the high resistance of breast cancer cells to chemotherapeutic drugs. Tumor multidrug resistance (MDR) has become the main reason for the failure of chemotherapy in patients with cancer.

As the most powerful and dedicated antigen-presenting cells (APC) in the immune system, dendritic cells (DCs) are pivotal in starting, regulating, and maintaining immune responses. The tumor-specific immune response is mainly the result of T cell activation and depends on effective antigen presentation. DCs are the only APC cells capable of activating naive T cells [1]. Over the years, for cancer patients after chemotherapy, DC-based biological treatment modalities have received much attention [2]. By separating the patient tumor cells and loading the split antigens to DCs, patients with cancer may be treated after reinfusion. However, this therapy targeting ordinary cancer cells is rather ineffective for resistant residual tumors. In this study, the drug-resistant strains from primary breast tumor cells were split and loaded to DCs. The

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* Supported by a grant from the Qingdao Science and Technology Plan Project (No.2013-1-3-33-NSH).

Table 1 Drugs concentration used *in vitro* ATP-TCA test

Drugs	100% TDC (ug/mL)
5-Fu	25
MTX	2.75
ADM	1.0
PTX	10.0
DDP	3.6

inhibitory effect of loaded DCs was then observed on tumor-bearing nude mice.

Materials and methods

Materials

Primary tumor cells

Primary tumor cells were obtained from a female patient, aged 41, first diagnosed at the Breast Center of the Affiliated Hospital of Qingdao University in March 2014. Intraoperative frozen section pathology confirmed the tumor as breast cancer. It was later diagnosed as stage x carcinoma *in situ*.

Main reagents and instruments

Roswell Park Memorial Institute medium (RPMI)-1640 was purchased from Beijing Solarbio Science &Technology Co., Ltd. (Beijing, China). Methyl thiazolyl tetrazolium and dimethyl sulfoxide were procured from Sigma-Aldrich (USA). Fetal bovine serum was purchased from Hangzhou Sijiqing Company (China). Recombinant human macrophage colony-stimulating factor (rh-GM-CSF) and recombinant human interleukin (rh-IL-4) were purchased from Xiamen Amoytop Company (China). Enzyme-linked immunosorbent assay (ELISA) kit was purchased from Shenzhen Jingmei Company (China). ATP-TCA kit, including complete medium, tumor tissue detachment enzymes (collagenase, hyaluronidase, and DNA enzymes), cell extracts, luciferin-luciferase, plates, and test panels, were purchased from Beijing Jin Zijing Biomedical Technology Co., Ltd. (China). Other reagents were of analytical grade and produced in China. The fluorescence analyzer Fluorescence analyzer MPLl was purchased from Berthold (Germany) and the flow cytometer was purchased from Beckman (USA).

Nude mice

Thirty 4–5-weeks-old female nude mice were purchased from Shanghai SLACCAS Experimental Animal Co., Ltd. (China).

Methods

Primary breast tumor tissue detachment

The connective tissue and filaments were removed from the primary tumor tissue. The tissue was detached by using collagenase. The tissue was minced with sterile blade. The tumor fragments were mixed with 300 U/mL of collagenase and 100 U/mL of hyaluronidase. The mixture was pipetted every 2 h and incubated for 12 h. The mixture was then filtered by using a 45 μm nylon mesh, washed twice with RPMI-1640 and phosphate buffered saline (PBS) to obtain a single cell suspension, and stained with trypan blue.

ATP-TCA susceptibility testing

Chemotherapy drugs in clinical use in our hospital were selected for the test. These include doxorubicin hydrochloride (Adriamycin ADM), paclitaxel (PTX), cisplatin (DDP), methotrexate (MTX), fluorouracil (5-Fu). The tested drugs were added into a 96-well culture plate. The drugs used in this study and their 100% TDC (test drug concentrations) (100% TDC referred to the peak plasma concentration of the drug in the human body, PPC) were presented in Table 1. The concentration of each drug combination was the same with that of the drug used alone. For each drug a dose response curve, 100, 50, 25, 12.5, and 6.25% TDC, was used in duplicate. Two rows (8 wells each) of drug-free wells were used as control wells. One row was for drug-free control and the other row was for the maximum inhibition control. The tumor cells were seeded at a concentration of 2×10^4 cells/well and incubated at 37 ℃ and in 5% CO_2 atmosphere for 5 days. The extracted ATP was measured following the manufacturer's instruction and using a fluorescence scanner. The inhibition rates were analyzed for each concentration and the IC_{50} was calculated for each drug.

Resistant breast tumor cell preparation

Chemotherapy drugs with susceptibility test results and their IC_{50} values and primary breast tumor cells. When the cell culture reached 80% confluence, the above-described sensitive chemotherapy drugs were used at a concentration of $1/10 \times IC_{50}$, $1/5 \times IC_{50}$, $1/2 \times IC_{50}$, $1 \times IC_{50}$, and $2 \times IC_{50}$. Breast cancer cells were incubated in the presence of the chemotherapeutic drugs at a concentration of $1/10 \times IC_{50}$ for 24 h. The cultured cells were still alive in the medium with no anti-cancer drugs. When the cells reached a density of 10^7, they were incubated with chemotherapeutic drugs at a concentration of $1/5 \times IC_{50}$. This process was repeated until breast tumor cells stably grew in $2 \times IC_{50}$ medium. The cells that steadily grew in medium containing $1/10 \times IC_{50}$, $1/5 \times IC_{50}$, $1/2 \times IC_{50}$, $1 \times IC_{50}$, and $2 \times IC_{50}$ were named R1, R2, R3, R4, and R5, respectively. The primary cells were named R0. The resistant cells were then grown in medium without chemotherapeutics for another 6 weeks.

DC in vitro culture

DC [3] were isolated from the peripheral blood mononuclear cells (PBMCs) from the patient and cultured. PBMCs were obtained from a patient with leukapheresis. PBMCs were incubated for 2 h at 37 ℃ in a 5% CO_2 atmosphere after washing. The non-adherent cells were

removed and the adherent cells were cultured in RPMI-1640. Rh-GM-CSF (125 ng/mL) and rh-IL-4) (125 ng/mL were added and the cells were cultured for 5 days to obtain DCs.

Resistant antigen loading

Resistant breast tumor cells were heat-shocked in a water bath at 42 ℃ for 2 h. The cells were frozen in liquid nitrogen and thawed five times at room temperature (25 ℃). The cells were observed with an inverted microscope until they were completely broken down. The resulting lysate solution was centrifuged at 600 rpm for 1 min. The supernatant was collected and filtered using a 0.45 μm membrane filter. The resulting filtrate contains the antigens from the resistant breast tumor cells. It was kept at -80°C for future use. DCs were then loaded with the resistant breast tumor cell antigens and incubated at 37 ℃ in a 5% CO_2 atmosphere for 4 h. The mature antigen-loaded dendritic cells were harvested by centrifugation at 1000 rpm for 10 min, washed with saline, resuspended at a concentration of 1×10^7 cells/mL, and kept at 4 ℃. Similarly, antigen-loaded DCs were prepared from primary breast tumor cells, without drug resistance treatment, and stored for later use. The phenotype of the DCs was examined by flow cytometry.

Establishment of the tumor-bearing nude mouse model

The tumor-bearing nude mouse model was prepared to test resistant breast tumor antigens [4]. DC cells were collected during the logarithmic growth phase, washed with saline, centrifuged, and counted. A suspension at a concentration of 1×10^8 cells/ mL was prepared and 1 mL of the cell suspension was injected in the right armpit fat pads of each mouse until the tumor diameter reached 0.5 cm.

Effect of the resistant breast tumor antigens on tumors in nude mice

The tumor-bearing mice were randomly divided in three groups (n = 10 for each group). Mice in the experimental group were injected with DCs that carried resistant breast tumor antigens. Mice in the control group were injected with DCs that carried resistant breast tumor antigen without drug resistance treatment. Mice in the blank group were injected with the same volume of normal saline. Briefly, the tumor-bearing nude mice in the experimental and control group were subcutaneously injected with 0.5 mL of 2×10^6 mL of DCs on day 0. The same dose was injected a second time, 15 days later. Mice in the blank group received saline instead. The size of the tumors was measured on day 0, 7, 14, 28, and 35. The tumor growth curve and the mice survival time were analyzed.

Statistical analysis

The data were analyzed by using SPSS16.0 statistical software (SPSS, Chicago, IL, USA). The data are presented as mean ± standard deviation. The different groups were compared by using analysis of variance. $P < 0.05$ was considered statistically significant.

Results

Susceptibility testing criteria

According to Kurbacher *et al* [5], high sensitivity (SS) is observed when $IC_{90} \leq 100\%$ TDC and $IC_{50} > 25\%$ TDC. Partial sensitivity (PS) was observed when $IC_{90} > 100\%$ TDC and $IC_{50} \leq 25\%$ TDC. Low sensitivity (WS) was observed when $IC_{90} \leq 100\%$ TDC and $IC_{50} > 25\%$ TDC. Resistance (R) was observed when $IC_{90} > 100$ % TDC and $IC_{50} > 25\%$ TDC. Drugs that were effective *in vitro* present either a SS or a PS, while drugs that were ineffective present a WS or R. *In vitro* efficiency was defined as: (SS + PS) / (SS + PS + WS + R) × 100% (Fig. 1).

DC morphologic observation and identification

A typical dendritic cell presents dendritic or burr-like protrusions. DCs, loaded with breast cancer cell antigens after resistance screening, were stained with CD86-FITC, CD80-FITC, CD83-PE, and CD11C-Percp. Phenotypic changes were examined after loading by flow cytometry. The positive rate of CD11 C/CD83, CD11 C/CD86, and CD11 C/CD80 improved (Fig. 2).

Tumor size and tumor growth curves

In each group, after treatment, the tumor volume and weight were significantly different than those before treatment ($P < 0.05$). The lethal effect on cancer cells and the life-prolonging effect on tumor-bearing mice were also significant ($P < 0.05$). The tumor size in the control and the experimental group decreased ($P < 0.05$), while that in the blank group increased ($P < 0.05$). The tumor volume in the blank group was the largest (3.362 ± 0.068 cm^3). Compared to the blank group, the tumor volume in the experimental group was the smallest (1.273 ± 0.071 cm^3). There were obvious differences in the tumor weight. Tumor growth was greatly inhibited in the experimental group and the tumor-inhibiting rate was the highest (Table 2 and Fig. 3).

Discussion

Breast cancer has become one of the world's most common malignancies [6]. The global incidence rate is growing by 2% annually. Chemotherapy plays an important role in the comprehensive treatment, together with surgery [7]. Although various anti-cancer chemotherapy drugs continue to emerge, drug resistance and the high rate of recurrence and metastasis after chemotherapy are still an issue. MDR in breast cancer treatment results in poor effectiveness, high transfer rate, and high mortality. As a

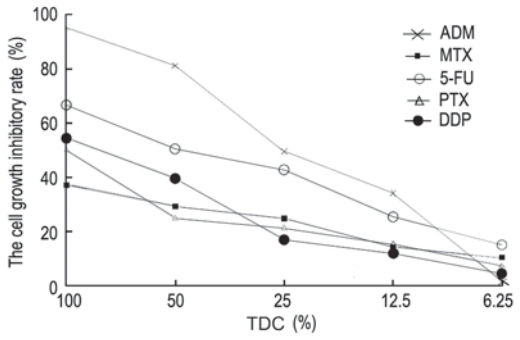

Fig. 1 L Dose-response curves of the different drugs detected using the ATP-TCA method

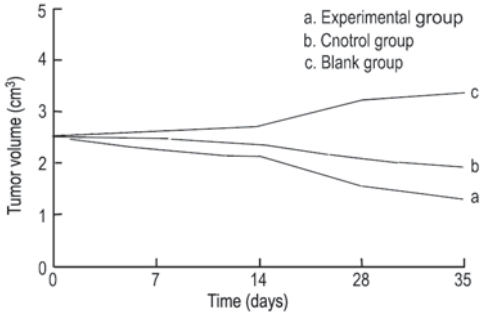

Fig. 3 Tumor growth curves

result of the tumor heterogeneity, the effect of chemotherapy varies widely from patient to patient with the same pathology and even at different stages for the same patient.

There are few DCs in the human body, representing 1% or less of the human PBMCs, which makes it difficult to gain direct access to a large number of DCs. In this study, DCs were isolated from PBMCs. rhGM-CSF is the most fundamental cytokine for the generation of DCs and to maintain their development and differentiation. rhlL-4 inhibits the generation of neutrophils and macrophages, promotes the conversion of mononuclear cells into DCs, and maintains DC maturation. In tumor immunology, cellular immunity mediated by T cell response plays a dominant role. For unexposed antigen *in vivo*, the number of response-specific T-cell precursors is rather low and they are all naive T cells without killing or proliferative capacity. As one of the most powerful and dedicated APC, DC is the only APC that activates naive T cells. DCs are also important immune accessory cells. DCs through Thl and CTL responses remove malignant cells expressing dissent ingredients. DCs initiate the body's immune response.

DC-based tumor vaccine technology is widely applied in the clinic. Now, as a mature biological treatment technology, it has been proven to be quite effective for many patients with cancer [8]. However, the ordinary therapy, targeting ordinary tumor cells, is rather ineffective for resistant residual tumors. In studying tumor MDR, researchers have found that, in the ABC transporter protein family, the p-glycoprotein expressed by the resistance

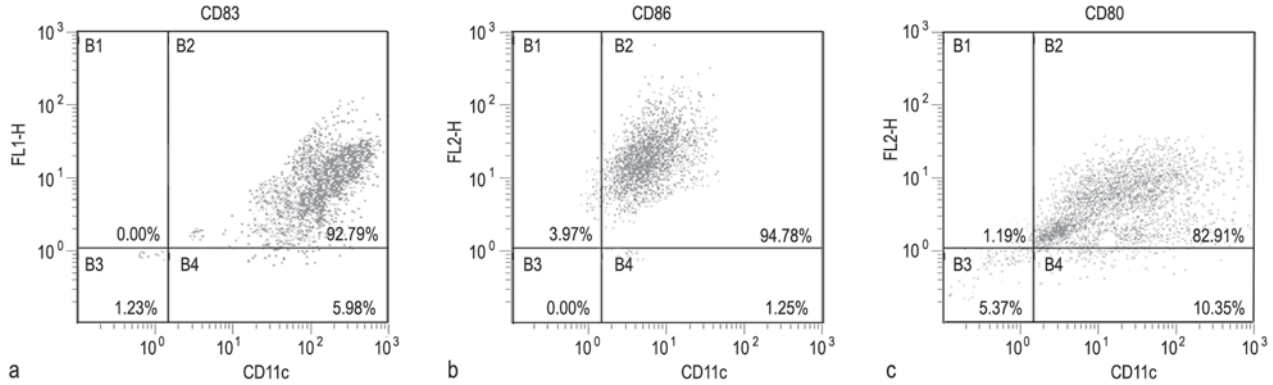

Fig. 2 By using McAb-Double fluorescence labeling, the positive cells expressed CD11c/CD83, CD11c/CD86 and CD11c/CD80 postoperatively increased obviously. (a) The percentage of the DCs with the expression of CD11c+/CD83+ reached to 92.79%; B1: CD83+/CD11c-, B2: CD83+/CD11c+, B3: CD83-/CD11c-, B4: CD83-/CD11c+. (b) The percentage of the DCs with the expression of CD11c+/CD86+ reached to 94.78%. B1: CD83+/CD11c-, B2: CD83+/CD11c+, B3: CD83-/CD11c-, B4: CD83-/CD11c+. (c): The percentage of the DCs with the expression of CD11c+/CD80+ reached to 82.91%. B1: CD83+/CD11c-, B2: CD83+/CD11c+, B3: CD83-/CD11c-, B4: CD83-/CD11c+.

Table 2 Tumor size and tumor weight in tumor-bearing nude mice before and after treatment ($\bar{\chi} \pm s$, $P < 0.05$)

Groups ($n = 10$)	Tumor volume after treatment of N days (cm^3)					Tumor weight (mg)
	0	7	14	28	35	
Experimental group	2.521 ± 0.131	2.311 ± 0 .201	2.109 ± 0.121	1.532 ± 0.213	1.273 ± 0.071	206.81 ± 32.711
Control group	2.507 ± 0.143	2.431 ± 0.331	2.305 ± 0.107	2.116 ± 0.230	1.914 ± 0.051	327.40 ± 31.823
Blank group	2.518 ± 0.149	2.597 ± 0.341	2.705 ± 0.201	3.197 ± 0.199	3.362 ± 0.068	637.50 ± 59.398

gene MDR1 is one of the important causes of resistance [9]. The cancer resistance protein isolated by Doyle *et al* [10] is a transmembrane transporter protein with a molecular weight of 72.6 kDa, also known as breast cancer resistance protein. Its expression has some reference value for breast cancer chemotherapy [11].

Cancer stem cells (CSC/TSC) hypothesis holds that tumor tissue contains a very small amount of cells capable of self-renewal and indefinite proliferation, which drive tumor formation, growth, and metastasis. CSC/TSC working mechanism in treating leukemia as well as solid tumors is currently a hot research field. Moreover, breast cancer cells expressing $CD44^+/CD24^-$/low have been detected, indicating the existence of breast cancer stem cells. These cells can rapidly proliferate and differentiate into a variety of cells. They demonstrate high tumorigenesis abilities in mice [12] and are significantly resistant to chemotherapy and radiotherapy [13]. It has been reported that the more $CD44^+$ $CD24^-$ /low cells the patient has, the more prone the tumor is to metastasis [14].

In this study, resistant cell lines were established by an *in vitro* anticancer drug inducing method. Compared to the resistance gene transfection method, the *in vitro* drug inducing method is more effective in simulating tumor resistance to chemotherapy [15]. ATP-TCA fluorescence sensitivity was used for *in vitro* susceptibility testing. The bioluminescence feature of the fluorescence analyzer is also used to screen breast cancer chemotherapy drugs in clinical application [16]. The present study demonstrates that DCs loaded with breast tumor antigens, obtained after anticancer drug treatment of breast cancer cells by combining heat shock treatment with repeated freezing and thawing lysis, perform far better than those of the control group that did not received drug treatment. The tumor growth inhibition was higher in the treatment group, which provides technical support regarding individualized treatments and for the design of new and combined chemotherapy [17]. This study explores the problems associated with the existing DC treatment targeting ordinary tumor cells such as drug resistance, high rate of recurrence, and metastasis after chemotherapy.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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DOI 10.1007/s10330-015-0062-3
Cite this article as: Zhuang W, Lun LM. Preliminary research on dendritic cells loaded with resistant breast tumor antigens in breast cancer-bearing nude mice. Oncol Transl Med, 2015, 1: 120–124.