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Effects of two different immunotherapies on triple negative breast cancer in animal model

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ABSTRACT

The ability of immune system to react specifically against tumors inspirited the study of triple negative breast cancer (TNBC) immunotherapies. Sixty spontaneous breast cancer TA2 mice were randomly divided into three groups: GM-CSF group, with therapy of granulocyte–macrophage colony-stimulating factor (GM-CSF) combined with breast cancer stem cells associated antigens and cytosine–phosphorothioate–guanine oligodeoxynucleotides (CpG-ODNs); DC–CIK group, with infusions of dendritic cells/cytokine-induced killer (DC/CIK) cells; and PBS group as controls. After therapy, the cellular immunity of mice in GM-CSF group and DC–CIK group was obviously increased, especially for GM-CSF group ($P < 0.05$), tumor regression was obviously observed in GM-CSF group. The survival rate of mice in GM-CSF group was significantly higher compared to DC–CIK group and PBS group. These results indicated that tumor immunotherapy manifested strong killing activity against TNBC. The therapeutic effect of GM-CSF combined with antigens and CpG was better than DC–CIK cells.

1. Introduction

The triple negative breast cancer (TNBC) which is negative for estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 over-expression, is the most clinically aggressive breast tumor and account for about 10–17% of all breast cancer cases. Due to a high histological grade, patients with TNBC are often unresponsive to endocrine agents and less responsive to standard adjuvant therapy, so relapses quickly in response to clinical treatment [1–3]. Recent evidence has suggested that TNBC is composed of heterogeneous cell types and that tumor initiation and growth are driven by a subset of cells termed tumor-initiating cells or cancer stem cells (CSCs) [4]. This subpopulation of cells seem resistant to conventional chemotherapies and lead to tumor metastasis. Cancer immunotherapy is considered to be the fourth cancer therapy after the three major cancer therapies of surgery, chemotherapy and radiotherapy. It is thought that complete eradication of cancer stem cells is essential for the cure of cancer and that immunotherapy is capable of killing non-dividing, quiescent cancer stem cells. Therefore, ideal and future immunotherapy to improve clinical outcome among TNBC patients should be approached as soon as possible.

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a well known hematopoietic cytokine and also an important regulator of inflammation associated infection and autoimmunity. It is reported that GM-CSF can induce the transcription of genes important for T cell activation, chemotaxis, antigen processing, innate immunity, and immunosuppression [5–7]. Cytosine–phosphorothioate–guanine oligodeoxynucleotides (CpG-ODNs) are unmethylated DNA sequences containing characteristic CpG dinucleotides and can initiate a potent immune response in mice, primates, and humans. A recent study in patients with melanoma had shown that s.c. injection of CpG-ODN was associated with tumor regression in 2 of 20 patients [8]. CpG-ODNs have been reported to have greater adjuvant activity when combined with GM-CSF [9] and has been shown for tumor immunity in murine lymphoma and neuroblastoma models [10]. Also in a phase I study of CpG-ODN, GM-CSF, and peptide vaccination, administration of CpG-ODN and GM-CSF induced marked alteration of lymphocyte trafficking and resulted in accumulation of activated PDC, T cells, and B cells at the injection site, with local and systemic IFN-a release and ultimately tumor regression [11].

Ex vivo expansion of monocyte-derived dendritic cells (mDCs) and subsequent coculture with autologous cytokine-induced killer (CIK) cells is an established system to create specific and non-specific anti-tumoral immunity in vitro and in vivo [12–14]. CIK cells are a $CD3^{+}CD56^{+}$ rich cytotoxic effector T cell population generated and expanded in vitro from peripheral blood cells. CIK cells have been shown to eradicate established tumors in a nude mouse

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xenograft model [15] and have already achieved promising results in clinical studies [16–18].

The two immunotherapies of GM-CSF/CpG and DC/CIK cells have been used alone in some kinds of cancers, including Wilms' Tumor, multiple myeloma, lung cancer, bladder cancer and hepatocellular carcinoma [11,19–23], but not in TNBC. The characteristics of breast cancer in TA2 mice approximated the basal-like breast cancers [24] and about 70% of TNBCs presented with basal-like molecular characteristics according to gene-expression profiling [1]. In this study, we use spontaneous breast cancer TA2 mice as TNBC model to investigate if the therapy of GM-CSF combined cancer stem cells associated antigens and CpG, or infusions of DC–CIK cells expanded ex vivo with antigens loaded could improve the outcome of TNBC.

2. Materials and methods

2.1. Animals

This study was approved by the Institutional Animal Ethical Committee, Qingdao and Ethics Committee of the Affiliated Hospital of Medical College, Qingdao University. Spontaneous breast cancer TA2 mice with breast cancer lump appearance (about 0.5 cm^3), average age 330 days (156 ± 460 days), were purchased from Laboratory Animal Center of Medical College, Tianjin University. All mice were housed in a certified SPF animal facility, fed on regular rat chow and maintained under optimal ambience of temperature (22–23 °C), light (12 h dark/light cycles), oxygen, humidity (60%) and ventilation till sacrificed.

2.2. Experimental plan

Sixty mice with spontaneous breast cancer lump appeared (mean tumor volume 0.5 cm^3) were randomly divided into three groups ($n = 20$): GM-CSF group, DC–CIK group and PBS group. In GM-CSF group, GM-CSF was injected subcutaneously into mice, 10 μg/200 μl/d, persistently for 7 days (day 1–7). At day 8, CpG and tumor antigens (a total volume of 200 μl) were injected subcutaneously. In DC–CIK group, peripheral blood derived DC–CIK cells were cultured. The DC–CIK cells (200 μl) were injected intravenously through caudal vein at day 8. In PBS group, mice were injected intravenously through caudal vein with phosphate buffered solution (PBS, 200 μl, day 8).

For each group, 20 TA2 mice were divided again into two subgroups ($n = 10$). Mice in subgroup I were observed for tumor growth and survival for 60 days. Mice in subgroup II were sacrificed on day 15 to collect the blood serum and tumor tissue for analysis (showed in Fig. 1).

2.3. Preparation of breast cancer stem cells and tumor antigens

Tumor tissue from TA2 mice were cut into 1-mm^3 pieces and digested with type I collagenase/hyaluronidase (2 mg/ml, Sigma–Aldrich) for 30 min at 37 °C, then filtered through 70 μM mesh to discard clumps. The suspension was centrifuged and washed once in PBS, then remained cells were seeded in a 24-well plate and cultured in serum-free medium (SFM), a stem-cell permissive DMEM/Ham's F12 (1:1) medium without FBS, supplemented with epidermal growth factor (EGF, 20 ng/ml, PeproTech), basic fibroblast growth factor (bFGF; 10 ng/ml, PeproTech, London, UK), 0.4% bovine serum albumin (w/v, Sigma–Aldrich) and insulin (5 μg/ml, Sigma–Aldrich). The formation and morphology of clusters and spheres were evaluated after at least 1 week of culture by microscopic observation and monitored for up to 21 days after initial seeding.

Mammosphere cells from five TA2 mice were mixed to get a mixture of cancer stem cells, in further to get a mixed breast cancer stem cell associated antigens. Mammosphere cells suspension (10^7 approximately) was subjected to three cycles of rapid freeze in liquid nitrogen (−196 °C), thawed (37 °C) and then centrifuged at 400 rpm for 10 min to remove large debris. After that, the supernatants were passed through a 0.2-μm pore filter and the filtrate was collected as tumor antigens. Protein concentrations of tumor cells lysates were determined using BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). The resulting tumor lysates were aliquoted and stored at −40 °C until use.

2.4. Preparation of DC–CIK cells

Peripheral blood from TA2 mice were separated by density-gradient centrifugation and suspended in RPMI1640. Following 4 h of incubation at 37 °C in a humidified atmosphere of 5% CO_2, adherent cells were cultured in RPMI1640 supplemented with 10% FBS, 10 ng/mL IL-4 (1000 units/mL; R&D Systems, Minneapolis, MN) and 20 ng/mL GM-CSF (Schering-Plough, Madison, NJ). After 5 days of culture, DCs were incubated overnight with 4 μg/mL CpG-ODN (5′-TCCATGACGTTCCTGACGTT-3′) and tumor antigens (40 μg/ml).

Non-adherent cells from above suspension were cultured in RPMI1640 supplemented with 10% FBS, IL-1α (Roche Diagnostics; 1000 UI/mL), 50 ng/mL CD3McAb (Cilag GMBH, Sulzbach, Germany) and 300 UI/mL IL-2 (Roche Diagnostics) to acquire CIK cells. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and restimulated every third day with IL-2. CIK cells were harvested on day 6 and co-cultured with mature DC at a stimulator: effector ratio of 1:5. DC–CIK cells were collected at day 8 and resuspended in PBS for injection.

2.5. Assessment of antitumor effects

Tumor size was examined three times weekly and quantitated with spring-loaded calipers (Dwyer Corporation, Michigan City, IN) for two perpendicular dimensions. Tumor volume (mm^3) was calculated as the cross product of $0.52 \times width^2 \times length$. The tumor was monitored until tumor volume exceeded 2500 mm^3 or mice were died. Living time of every mouse was recorded carefully.

2.6. Spleen cell preparation and flow cytometric analysis

Single-cell suspensions were obtained by passing spleen through a 70-μm pore size cell strainer (Falcon, Heidelberg, Germany), followed by lysis of erythrocytes (Ortho-Clinical Diagnostics, Neckargemund, Germany). The cell suspensions were used for flow cytometer analysis.

Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or allophycocyanin (APC)-conjugated monoclonal antibodies (mAbs) against CD4, CD8, CD3, CD25, CD19, CD20 (all from eBioscience, San Diego, CA) were added to splenocytes, incubated for 30 min at 4 °C, washed twice, and analyzed using a FACS Calibur flow cytometer (BD, America).

2.7. Histological examination of tumor tissue

Tissue samples from each mouse were divided into two halves and one half was fixed in 4% formalin solution overnight and embedded in paraffin by the conventional method, then cut into 4-μm thick sections. The sections were stained with hematoxylin–eosin (H&E) for gross histological examination, the areas of necrosis and inflammatory infiltrations were evaluated.

The other half of tumor tissues were embedded in O.C.T. medium (Tissue-Tek; Sakura Finetek), snap-frozen immediately in liquid nitrogen and stored at −80 °C until use. For indirect