Immunological consequences of immunization with tumor lysate vaccine and propranolol as an adjuvant: A study on cytokine profiles in breast tumor microenvironment

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ABSTRACT

In this study, we sought out to study the anti-cancer effects of propranolol (Pro) in combination with tumor lysate vaccine on lymphocyte proliferation activities as well as on IL-2, IL-4, IL-10, IL-12, IL-17 and IFN-γ cytokine concentration in the tumor microenvironment (TME). A tumor model was established in inbred Balb/C mice using transplantation of tumor to the flank of native mice. Tumor-bearing mice were immunized with lysate tumor cells (vaccine), a combination of Pro/Vaccine (Vac). Control groups consisted of tumor-bearing mice receiving only propranolol or PBS three times with one week interval via subcutaneous (s.c) injection. One week after the last immunization, tumor was removed, homogenate was prepared and the levels of IL-12, IL-17, 1L-2, IL-10, IL-4, IFN-γ cytokine concentrations were evaluated by commercial ELISA kits. In addition, spleen cell suspension was used for the lymphocyte proliferation assay using the BrdU method. Results from this study indicated that Pro/Vac had the ability to significantly increase lymphocyte proliferation, and to suppress tumor growth. Administration of breast tumor lysates with propranolol increased the concentration of IL-12, IL-17, 1L-2 and IFN-γ cytokines in tumor microenvironment. This study has proved the efficiency of propranolol as an adjuvant in combination with the tumor vaccine model on tumor suppression via cytokine pattern modulation in tumor microenvironment.

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1. Introduction

Breast cancer is one of the leading causes of death worldwide. An estimated 232,670 new cases of invasive breast cancer was diagnosed in 2014 in American women, accounting for 62,570 new cases of non-invasive breast cancer and 40,000 deaths [1]. In Iran, the age of breast cancer onset is 5–10 years lower than the global average [2]. Common treatments for breast cancer include surgery [3], radiation [4], chemotherapy [5], hormonal therapy [6] and biological therapy [7]. Immunotherapy is currently one of the most treatment strategies for patients with breast cancer [8]. Tumor microenvironments contain various molecules that lead to immune surveillance dysfunction and tumor escape from immune system responses [9–11]. Among various compounds of the immune system, cytokines are considered as a potential target for cancer immunotherapy [12]. Interestingly, cytokines can help tumor progression through compromised antitumor immunity in TMEs [13,14]. In tumor microenvironments, secretion of tumor-derived soluble factors, such as IL-10 [15], transforming growth factor-β (TGF-β) [16], vascular endothelial growth factor (VEGF) [17] etc, results in the organized network of local immunosuppressive milieu, as well as promoted immunological ignorance, tumor progression and tumor tolerance [18]. Cytokines both play a critical role in the formation of tumor microenvironment and are responsible for induction of immunogenic or tolerogenic milieu in tumors switched either by tumor cells or by immunotherapeutic agents [19].

During the past three decades, immunotherapy has been used as clinical reality for cancer treatment [20–22]. Immunotherapy, as a

novel approach, reinforces the immune system against tumor cells [23]. Among the various immunotherapy methods, cancer vaccines, as a specific active immunotherapy, is still a desirable and effective treatment for cancer diseases [24].

Tumor cells lead to the production of other extra cytokines in TMEs through absorption, secretion and alteration of local cytokines [25–27]. This process results in the production of Th lymphocytes, such as Th1-secreted cytokines (IFN-γ, IL-12 and IL-2), Th2-secreted cytokines (IL-4 and IL-10) and Th17-secreted cytokines (IL-17) that play an important role in the development or inhibition of tumor growth [28–30]. Propranolol is a non-selective beta adrenergic blocker which binds competitively to beta- adrenoreceptors (β-ARs) and inhibits the functions of norepinephrine (NE) and epinephrine (EPI) [31,32]. Propranolol is widely being used for the treatment of high blood pressure [33]. It is well-demonstrated that propranolol has *in vitro* anti-tumor, anti-proliferative and anti-angiogenesis proprieties against malignancies [34,35]. Preclinical studies on breast and ovarian cancer models demonstrated that propranolol harnesses tumor-related stress and metastasis, specifically through anti-angiogenic and immunostimulatory mechanisms [36,37]. Propranolol leads to cell arrests at the G0/G1 phase of cell cycles through CDK-dependent pathways and inhibition of angiogenic factors [38]. Clinical evidence demonstrated that administration of β-AR antagonists prior to diagnosis or concurrently with chemotherapy enhances the overall survival and decreases tumor recurrence and metastasis in breast cancer patients [39–41]. Different molecular mechanisms have been suggested to describe the efficacy of β-AR antagonists in breast cancer patients [42,43].

In the present study, the TME of experimental breast cancer-bearing mice immunized with tumor lysate vaccine and propranolol as an adjuvant was evaluated for cytokine patterns. We hypostatized that anti-tumor effects of propranolol may be accompanied with the change of TMEs, especially cytokine patterns in the tumor milieu.

2. Materials and methods

2.1. Cell culture and propagation

The cell line 4T1 was purchased from National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran. Cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM L-glutamine, 1 mM sodium pyruvate and 50 μm 2-mercaptoethanol (2-ME) and kept at 37 °C in humidified incubator with 5% CO2. When cells reached full confluency, the cells were harvested with Trypsin/ethylenediaminetetraacetate (EDTA) (GIBCO, Germany), washed three times with cold phosphate-buffered saline (PBS) and resuspended in cold PBS. The 4T1 cell suspension was used for tumor induction and preparation of tumor cell lysate as a vaccine model.

2.2. Tumor antigen preparation

Cell suspension (5×10^6 cells/ml) in cold PBS containing 1 mM of Phenyl Methane Sulfonyl Fluoride (PMSF) was prepared and disrupted by 5 cycles of freezing-thawing using liquid nitrogen and water bath at 37 °C, followed by sonication (60 HZ, 0.5 amplitude, 10 cycles). Afterwards, fragmented cells were centrifuged at 10000 RPM/10 min and supernatant was harvested. The harvested part containing cell lysates was dialyzed versus PBS in the cold room (4 °C). The sample was passed through a 0.2 μm filter to eliminate the possible microbial contamination; then, the protein concentration in the sample was measured by the Bradford method. The

resulting sample was used as the vaccine model throughout the study, as well as the antigen for in vitro experiments.

2.3. Mice

Six-to-8 week-old inbred female Balb/C mice were purchased from Pasteur Institute of Iran (Karaj, Iran) and kept in animal house (temperature, 20–22 °C, 12/12 light/dark) with ad libitum access to food and water. One week after for mouse adaptation, the experiments began. All experiments on mice were carried out in agreement with the Animal Care using the protocol of Pasteur Institute of Iran.

2.4. Tumor stock preparation

To establish a tumor stock, 1×10^6 4T1 cells were injected to the flank of inbred Balb/c mice. After the tumor developed and growth reached 20 mm, the samples were used as a stock for tumor induction in naïve Balb/c mice via the surgery method.

2.5. Tumor transplantation

In order to establish a breast cancer model of Balb/c mice, we used the surgery method for tumor modeling in our study cohorts, according to our previous experiences. This method gives uniform population of tumor sizes with lower variance in tumor sizes of experimental mice in the beginning of study. For this purpose, Balb/C mice bearing mammary adenocarcinoma, as a stock, were dislocated and the tumor was harvested and placed in cold PBS containing 100 IU/ml penicillin and 100 μ/ml streptomycin under laminar hood class 2. Then, the tumor was dissected into 3 mm length pieces and used for transplantation. The right flanks of experimental mice were shaved and, 2 days later, the mice were anesthized with intraperitoneal (I.P) injection of ketamine/xylazine. Afterwards, tumor pieces were subcutaneously placed in mice and the skin was stitched with glue stitches after sterilization with alcohol. Three days after transplantation, the experimental mice were used for the study.

2.6. Experimental groups and immunization

After establishment of tumor model mice, when tumor length reached 3–4 mm, mice were randomly divided into four groups as below:

Group 1: mice immunized subcutaneously with 100 μg of tumor lysate vaccine in combination with 3 mg/kg propranolol (Sigma, USA) and continuous injection of propranolol for 5 days each 12 h.

Group 2: mice immunized subcutaneously with 100 μg of tumor lysate vaccine in combination with PBS and continuous injection of PBS for 5 days each 12 h.

Group 3: mice immunized with 3 mg/kg propranolol and continuous injection for 5 days each 12 h.

Group 4: mice immunized with PBS and continuous injection for 5 days each 12 h.

Experimental groups of mice were immunized three times with 7-day intervals and immunoassay was carried out one week after the last immunization.

2.7. Preparation of spleen cell suspension

Mice were dislocated and treated with 70% ethanol and their spleens were removed and dissected mechanically in 2% cold PBS/FBS under class II laminar hood. Then, red blood cells were lysed via RBC lysis buffer and cell suspension was centrifuged at $300 \times g$ for 10 min at 4 °C. The cell pellet was resuspended in RPMI-1640 supplemented with 10% FBS, 4 mM L.glutamin, 1 mM with