

Prophylactic Autologous Cancer Cells Immunization Inhibits Triple Negative Breast Tumors' Growth and Metastasis in 4T1 Tumor-Bearing BALB/c Mice

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ABSTRACT

Triple negative breast cancer (TNBC), a type of breast cancer, is still incurable and 4T1 tumor is an equal experimental model of TNBC which is highly metastatic and invasive. In the present study, we assessed the prophylactic cancer vaccine effects on survival and metastasis in 4T1 tumor bearing mice. Female BALB/c mice were divided in three groups and subcutaneously (s.c) injected with PBS incomplete Freund's adjuvant (IFA) and a mix of the homogenized 4T1 cancer cells emulsified with IFA, respectively. Also, in the third group, the last immunization was contained just the homogenized

cancer cells. Tumor induction was performed with 4T1 cells injection at the 4th mammary fat pad of each mouse. Tumor diameter and the number of macroscopic metastatic nodules on the lungs' surface were investigated two weeks after the last immunization. We found that the immunization of mice with homogenized 4T1 cancer cells and IFA significantly inhibited 4T1 tumors' growth. Also, metastatic colonies in the lungs significantly decreased in mice which immunized with 4T1 cancer cells and IFA. Overall, the effective induction of immune response by the vaccine used in our study seems to have prevented cancer cells migration and metastasis to other organs. However, precise immunological studies are required to determine the mechanism of such a vaccine.

Key words: Breast cancer; Cancer vaccination; Immunization; 4T1

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INTRODUCTION

Breast cancer is the most frequent cancer among women in the world and after lung cancer is the second cause of death^[1]. Different therapeutic methods including chemotherapy, radiotherapy, surgery, and immunotherapy are applied in the treatment of females with breast cancer^[2]. Despite the many successes in diagnose and therapy of breast cancer, the mortality rate for this cancer is still relatively high^[3-6]. Also, in metastatic patients response to therapies such as chemotherapy is weak and accompanied by consequential side effects such as hypersensitivity, neuropathic pain, and cardiotoxicity^[7,8]. Different immunotherapy methods are used in cancer treatment such as monoclonal antibodies and immune cell therapies which induce immune responses^[9]. Additionally, due to multiple genetic changes which develop during malignancy, tumor cells are included

foreign antigens^[10,11]. Immunizing of cancer patients with their own tumor antigens (cancer vaccination) would induce specific immune responses. Accordingly, studies have shown tumor antigens are recognized by cytotoxic T cells and prime these cells into effector T cells^[12]. Therefore tumor cells are an attractive source of tumor antigens for cancer vaccination. Also, the use of the immune modulatory agents (IMA) such as adjuvants with cancer antigens enhances immune responses and modulates immunosuppressive mechanisms^[13].

A highly metastatic and deadly type of breast cancer is triple negative breast cancer (TNBC) which remains incurable due to the lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression^[14]. The 4T1 tumor cell line is an equal experimental model of TNBC which is highly metastatic and invasive^[15,16].

Because of poor response to common immunotherapies such as monoclonal antibody therapy in TNBC, other immunotherapy approaches like cancer vaccines may be effective.

Accordingly, in the present study, the efficacy of autologous cancer cells antigens' immunization for inhibition of breast tumors growth and metastasis in a mice model of TNBC was evaluated.

METHODS AND MATERIALS

Cell culture

4T1 murine mammary carcinoma cell line was purchased from Pasteur Institute of Tehran, Iran. The cells were cultured in RPMI 1640 medium (Sigma-Aldrich, Germany) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany). Antibiotics mixture (1%) containing penicillin (Sigma-Aldrich, Germany) and streptomycin (Sigma-Aldrich, Germany) was added to the final solution. The cells were incubated in a humidified incubator at 37°C in a 5% CO₂ atmosphere.

Mice immunization method and schedule

Female BALB/c mice (23 ± 2 g, 6 to 7 weeks old) were purchased from Pasteur Institute of Tehran, Iran. All animal experiments and procedures were approved by the Ethics Committee on Animal Experiments of the Isfahan University of Medical Sciences. The mice were randomly divided into three groups (*n* = 15). Mice of group 1 were subcutaneously (s.c) injected with 100 µL PBS as the no-treatment group. Mice of group 2 were s.c injected with 100 µL incomplete Freund's adjuvant (IFA) which was formulated with PBS in 50% V/V. The 3rd group was immunized by a mix of the homogenized 4T1 cancer cells (10⁷ cells in 50 µL of PBS) emulsified with 50 µL IFA 50% V/V. The last immunization for this group contained just the homogenized cancer cells. The homogenized cancer cells mixture was prepared with homogenizing of 4T1 cancer cells in a final concentration of 10⁷ cells/ 50 µL PBS. All the injections in all the groups were subcutaneous with one-week intervals (Table 1). The immunized mice were injected with the cancer cells and interred the tumor challenge two weeks after the last immunization time.

Breast tumors' implantation in BALB/c mice

4T1 cancer cells were harvested from culture flasks by trypsin (Sigma, USA) and washed three times with PBS. 10⁷ cells which were suspended in 50 µL PBS (Sigma, USA) was subcutaneously (s.c) injected at the 4th mammary fat pad of each mouse, two weeks after the last immunization, tumor diameters were measured with a digital caliper each 3 days. The tumor volume was calculated by the below formula:

$$\text{Tumor volume} = \text{Length} \times \text{Width}^2 \times 0.5$$

The mice were sacrificed and their lungs were harvested after 35 days from 4T1 cancer cells implantation. The number of macroscopic metastatic nodules on the lungs' surface were investigated.

Statistical analysis

The JMP 11.0 software was employed for performing statistical analyses and graphics. All data were compared between different groups by One-way ANOVA. Statistical significance was set at *p* < 0.05.

RESULTS

Immunization with homogenized 4T1 cancer cells inhibited 4T1 tumors' growth

Information about immunization schedule of mice has indicated

Table 1 Vaccination schedule at different groups.

Group	1st injection	2nd injection	3rd injection	4th injection
1 (n=15)	PBS	PBS	PBS	PBS
2 (n=15)	IFA	IFA	IFA	IFA
3 (n=15)	IFA + CCA	IFA + CCA	IFA + CCA	CCA

IFA: Incomplete Freund's adjuvant, PBS: Phosphate buffer solution, CCA: Homogenized 4T1 cancer cells antigens, n: Number of mice per group.

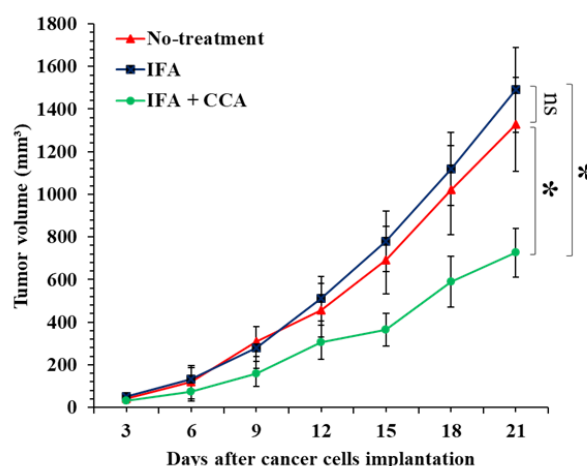


Figure 1 The tumor' volumes progression at different treatment groups. (*:*p* < 0.05; ns: not significant; *n* = 15).

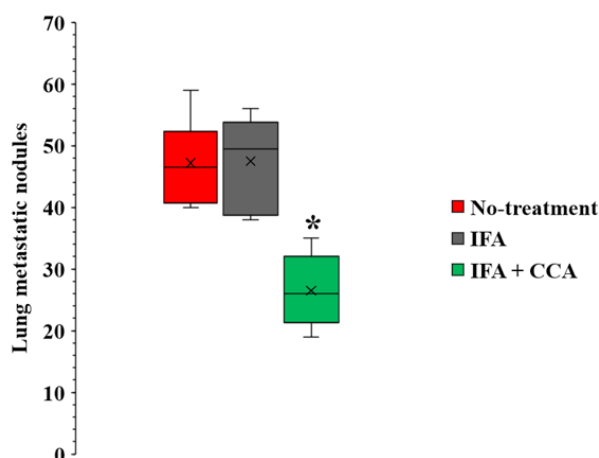


Figure 2 Metastatic nodules on the surface of lungs at different treatment groups, 35 days after the breast cancer cells implantation.

in table1. We found that the utilized adjuvant was approximately ineffective for inhibition of breast tumors growth. But, the immunization at the third groups of treatment, with homogenized cancer cells as the mixture of the breast cancer cells antigens and IFA significantly decreased tumors growth. In addition, lower tumors' volume was observed at the last day of measuring tumors diameters in this group in comparison with two other groups (Figure 1).

Immunization with homogenized 4T1 cancer cells inhibited 4T1 tumors' metastasis

Metastasis is the main cause of breast cancer patients' mortality and lung is the main vital organ for breast cancer metastasis. Introduction of the cancer cells' antigens to the mice immune system through homogenized cancer cells and IFA immunization significantly decreased metastatic colonies (Figure 2) and significantly ($p < 0.05$) lower number of metastatic nodules were observed at the tumor-bearing mice lungs.

DISCUSSION

Triple negative breast cancer (TNBC) as a metastatic cancer poorly responds to common treatments. Also, cancer vaccination with tumor associated antigens (TAAs) as an anticancer therapeutic approach targets tumor antigens. 4T1 tumor cells are a suitable model of triple negative breast cancer. Therefore we immunized mice with the homogenized 4T1 cells and then induced tumor model with these cells in immunized mice. We found that immunization of mice with homogenized 4T1 cancer cells and IFA significantly inhibited 4T1 tumors' growth. However, mice immunized with IFA alone failed to inhibit breast tumors growth. These results are expected because the adjuvants usually increase the humoral and cellular immune response and when administered with antigens, help the immune system to boost immunogenicity, accelerate the immune responses, and improve efficient immune responses^[17,18]. Most of cancer vaccine trials have been done in people who have been diagnosed with cancer and usually are immunosuppressed patients. Therefore preventive breast cancer vaccines seem to be effective in women who are high-risk patients^[18]. Recently prevention experimental studies have performed which used highly expressed cancer antigens^[19-21]. For example Xia *et al.* have used a fibroblast activation protein a (FAPa) vaccine using recombinant adenovirus (rAd) vector and they found a prime-boost strategy in the process of vaccination significantly increased survival time of 4T1 tumor-bearing mice^[19]. Our findings are consistent with these reports, although we used total cancer cells homogenate as a source of antigens. In another study Ravindranathan *et al.* immunized mice with two murine breast carcinomas, 4T1 and EMT6, as a prophylactic vaccine and they found EMT6 vaccination was accompanied by significantly higher protective immunity compared to 4T1 vaccination^[22]. The lower protective effect of 4T1 vaccination in their study may be due to the use of primary and booster vaccine without adjuvant administration.

Also, we found that introduction of the cancer cells' antigens to the mice immune system through homogenized cancer cells and IFA immunization significantly decreased metastatic colonies in the lungs. Like to human breast cancer, the 4T1 tumor develops metastasis to other tissues and quantitation of metastatic cells could be performed even in distant organs^[23].

The effective induction of immune response by the vaccine used in our study seems to have prevented cancer cells migration and metastasis to other organs. However, precise immunological studies are needed to determine the mechanism of such a vaccine.

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