Original Article

Identifying the Most Effective Hydatid Cyst Fluid Fraction for Anticancer Vaccination of 4T1 Breast Tumor-Bearing Mice

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Abstract

Background: The hydatid cyst fluid antigens have high homology with cancer cell antigens and also exhibit considerable immunogenicity. Therefore, their utilization for cancer immunization can cause an effective antitumor immune response. However, the main challenge is identifying the most effective antigens for this purpose. **Methods:** Hydatid cyst fluid fractions including the glycolipid fraction, glycoprotein fraction, 78 kDa fraction, and antigen B fraction were prepared. Then, the BALB/c mice were immunized against different antigens and, subsequently, 4T1 cells were subcutaneously implanted. The tumors' growth, metastasis, and tumor-bearing mice survival were assessed in different immunized groups. In addition, IL-2, IL-4, IFN-γ, and TNF-α serum levels were estimated to evaluate the immune system response. **Results:** BALB/c mice immunization against the complete hydatid cyst fluid antigens exhibited more significant inhibition of the tumors' growth and metastasis and increase of tumor-bearing mice survival in comparison with its derived fractions. However, the 78 kDa fraction exhibited the best results according to the same factors in comparison with all the prepared fractions. **Conclusions:** The 78 kDa fraction of the hydatid cyst fluid was the most effective fraction of hydatid cyst fluid for immunization against 4T1 breast tumors.

Keywords: *Breast neoplasm, cyst fluid, immunization, immunotherapy, neoplasm metastasis*

Introduction

Employing the host's immune system to inhibit tumor growth and metastasis has received lots of attention at cancer therapy.[1] Cancers arise from a combination of genetic and epigenetic changes that cause immortality, uncontrolled cancer cells growth, proliferation, and metastasis.[2] Simultaneously, these changes can create new antigens in the cancer cells. These antigens cause the neoplastic cells detection by the immune system.[3-5]

The ultimate goal of cancer immune therapy is to induce a specific immune response against malignant cells. However, malignant cells can manage to escape the immune system recognition and subsequent elimination. Tumors reach this goal by developing multiple resistance mechanisms including local immune system suppression and tolerance induction.[6] However, effective introduction of tumor antigens to the immune system can cause efficient cancer cells recognition and subsequent immune response against tumor.[7-10]

Tumor-associated antigens that are expressed by tumor cells have been used for cancer immunotherapy. However, due to poor immunogenicity, these antigens often fail to induce an efficient immune response.[11] So, immunization with other foreign antigens that have shared homolog epitopes with the cancer antigens may raise a strong immune response. Immune response cross-reaction between the cancer cells and parasite antigens has been reported in many studies. Parasites have high antigens homology with cancer cells and their foreign antigens can significantly activate the immune system.[12-16]

Hydatid cyst is the larval stage of the tapeworm *Echinococcus granulosus* that is located in human and livestock viscera.[17] The cyst is outwardly covered with a carbohydrate-rich material termed laminated layer and then a thinner cellular layer named germinal layer, which is fulfilled with hydatid cyst fluid. The hydatid cyst fluid is a biological mixture of glycoproteins, glycolipids, and carbohydrates that are able to modulate cellular and humoral immune responses.[18] Increase of

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interleukin-2 (IL-2), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-4, IL-5, IL-6, and IL-12 serum levels has been detected in the primary and secondary hydatid cyst infection. Previous studies have exhibited that some antigens in hydatid cyst have high homology with the cancer-associated antigens. So, hydatid cyst may have an appropriate potential for stimulation of the strong immune responses against cancer cells.[19-22]

Identifying the best antigens for arising an effective immune response against tumor is the main challenge for antitumor immunization. Parasite antigens with high homology with cancer antigens and significant immunogenicity can be appropriate candidates. To the best of our knowledge, this is the first time to use different fractions of the hydatid cyst fluid immunization for inhibition of 4T1 breast tumors' growth and metastasis and increase of tumor-bearing mice survival. Antitumor effect of the hydatid cyst fluid has been shown in different previously published works.[23-29] In an investigation effect of two hydatid cyst antigens on the growth of melanoma cancer in C57/black mice has been demonstrated.[23] In another work done by Barrel *et al*., antitumor activity of human hydatid cyst fluid in a murine model of colon cancer has been shown.[24] In another study, the therapeutic effects of hydatid cyst antigens on melanoma cancer in animal model has been shown.[25] It was also reported that Mucin-like peptides from *E. granulosus* increase the number of activated natural killer (NK) cells in the spleens of immunized mice.[26] In addition, in another study, in experimental animals with induced breast cancer the size of breast cancer decreased 20 days following immunization with hydatid cyst fluid.[27] However, it is not clear which specific antigen of the hydatid cyst fluid is responsible for this anticancer activities. So, in this work, the effects of different antigens of hydatid cyst fluid including antigen B, glycolipid, glycoprotein, and 78 kDa fractions on the growth of breast cancer in mouse model have been investigated.

Materials and Methods

Preparation and extraction of the hydatid cyst fluid antigens

Sheep hydatid cysts were collected from lungs and livers of slaughtered animals. The hydatid cyst fluid was aspirated from the cysts and, with the observation of the protoscolices, the fluid was collected in 50 mL test tubes. The tubes containing fluids were then centrifuged at 2000 g for 2 min and the supernatant was lyophilized and stored at −20°C as crude hydatid fluid antigen. The antigen B (Ag B) fraction was prepared according to the previously described method. Briefly, 50 mL tubes containing hydatid cyst fluid were centrifuged at 1500 g for 15 min. The supernatant was then dialyzed against 0.005 M acetate buffer (pH = 5) overnight at 4°C and centrifuged at 5000 g for 30 min. Then, the precipitate was dissolved in 10 mL of 2 M phosphate buffer (pH = 8). The preparation was saturated with 5 mL ammonium sulfate 40% and mixed for 1 h and centrifuged at 5000 g for 15 min. The supernatant was incubated in the water bath for 15 min and centrifuged at 5000 g for 30 min. Finally, the supernatant containing the Ag B was collected and stored at −70°C until further use. The 78 kDa fraction of hydatid cyst fluid was prepared using gel filtration chromatography and loaded to Hi-Load 16/600 Superdex 75 prep grade column. The column was then washed with 150 mL PBS buffer, and the fractions were collected with the flow rate of 1 mL/min at room temperature.

The purified fractions were then analyzed on 12% SDS-PAGE and then stained with silver nitrate as we published earlier.[30,31] The glycoprotein and glycolipid of hydatid cyst fluid were purified by chloroform–methanol extraction. The distilled water, methanol, and chloroform were added to the crude hydatid cyst fluid with 3:4:1:1 ratio, respectively, and centrifuged for about 5 min and at 5000 g. Then, the pellet was resuspended in 4 mL methanol and centrifuged for 20 min. The lower section of the tube was obtained as the glycoprotein fraction and the supernatant as the glycolipid fraction.[32-34]

Cell culture

4T1 mouse 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 (Sigma-Aldrich, Germany), and 1% antibiotics mixture 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% CO_2 atmosphere.[35]

4T1 breast cancer animal model

Six- to eight-weeks-old BALB/c female mice were purchased from the laboratory animal center of Pasteur Institute of Tehran, Iran. All procedures were verified according to the guidelines of the Institutional Animal Care and Ethics Committee of Isfahan University of Medical Sciences. The mice were housed in an appropriate animal research facility and kept in groups of five per cage, with complete access to the clean food and water. For animal experiments, enough number of mice were purchased (n = 8 for each group) and seven groups were designed for the study. Groups 1–5 immunized with the complete hydatid cyst mixture, the glycolipid fraction, the glycoprotein fraction, the 78 kDa fraction, and the Ag B fraction, respectively. All antigens were mixed with the Alum adjuvant before injection for more provocation of the immune system. Two control groups were injected with Alum alone or PBS. The boosters were given to mice every 5 days up to five injections. At each injection, 50 μg of each antigen was

injected. Moreover, 5 days after the last injection, all of the mice were injected with the 4T1 breast cancer cells (1×10^6 per mice) at the mammary fat pad. The size of the developed tumors was measured every 3 days. For this purpose, two dimensions of every tumor were measured using a digital clipper. Then, the tumor volumes were calculated according to below formula. Each group contained eight mice ($n = 8$).

Tumor volume = Length × Width^2 × 0.52

Histopathological analysis

Fifty days after the cancer cells implantation, mice were sacrificed and their lungs and livers were harvested and preserved in 10% formalin for histopathological investigations and to evaluate the metastasis nodules. Paraffin-embedded tissues were cut and stained with hematoxylin and eosin (H and E), and the metastatic colonies were examined using a bright-field microscope.

Enzyme-linked immunosorbent assay (ELISA)

Three days after the last immunization injection of the mice, their blood was collected and the sera were extracted. ELISA kits (eBioscience, USA) were used to measure IL-2, IL-4, TNF-α, and IFN-γ. The method was performed according to the kit's manufacturer. Each test was performed three times.

Statistical analysis

Statistical analyses were performed using JMP 11.0. All data were analyzed by One Way ANOVA. Statistical significance was set at $P < 0.05$. All experiments were performed in triplicate with results expressed as mean ± SD.

Results

Production of hydatid cyst fluid fraction

Hydatid cyst fluid was subjected to chromatography, and the main products of this method were analyzed in SDS-PAGE. Following silver nitrate staining, results revealed that a band with a molecular weight of about 78 kDa is the main constituent of hydatid cyst fluid [Figure 1].

Assessment of the hydatid cyst fluid fractions immunization effect on the 4T1 tumors' growth

Five groups of BALB/c female mice were immunized with the hydatid cyst fluid, the glycoprotein fraction, the glycolipid fraction, the Ag B fraction, and the 78 kDa antigen fraction, respectively. Two groups of mice were also immunized with Alum alone or PBS as controls. All antigens were dissolved in the Alum as adjuvant, and each mouse was injected five times. After 5 days following the last vaccination, 4T1 cancer cells were implanted subcutaneously. As Figure 2a illustrates, the breast tumor growth was inhibited especially in mice injected with hydatid cyst fluid or the 78 kDa fraction.

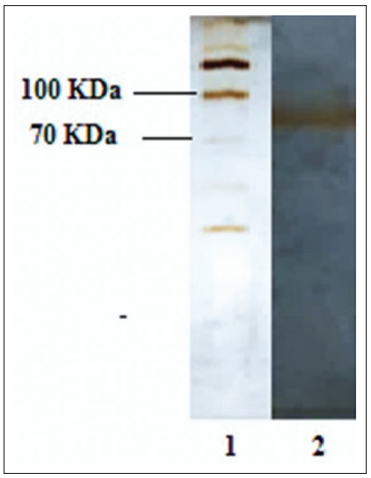

Figure 1: SDS-PAGE of products of chromatography of hydatid cyst fluid following staining with silver nitrate (lane 2) in comparison with molecular weight marker (lane 1)

Assessment of the hydatid cyst fluid fractions immunization effect on the tumors' metastasis

Cancer cell metastasis is the main cause of cancer patients' death. Therefore, inhibition of the metastasis is one of the main concerns of cancer treatment. In this study, 4T1 breast tumor metastasis to the lung was evaluated in the five groups of BALB/c female mice immunized with the hydatid cyst fluid, the glycoprotein fraction, the glycolipid fraction, the Ag B fraction, and the 78 kDa antigen fraction in comparison with metastasis in control animals (injected with Alum alone or PBS). Complete crude hydatid cyst fluid and the 78 kDa fraction-immunized mice exhibited the lowest rate of lung metastasis [Figure 2b].

Assessment of the hydatid cyst fluid fractions immunization effect on the breast tumor-bearing mice survival time

The introduced antigens immunization effect on breast tumor-bearing mice survival was evaluated. Although the 78 kDa fraction antigens and less effectively other fractions immunization increased the mice survival time, the complete hydatid cyst fluid antigens immunization had the most efficacy for the enhancement of the tumor-bearing mice survival [Figure 2c and Table 1].

Assessment of the cytokines level in the immunized mice with different hydatid cyst fluid fractions

The serum level of IL-2, IFN-γ, IL-4, and TNF-α were estimated in different immunized groups to evaluate the immune system response. The results revealed that the IL-2, IFN-γ, and TNF-α serum level in the crude hydatid cyst fluid and 78 kDa fraction antigens immunized mice were significantly ($P < 0.05$) higher than the PBS, Alum, and the other groups [Figure 3].

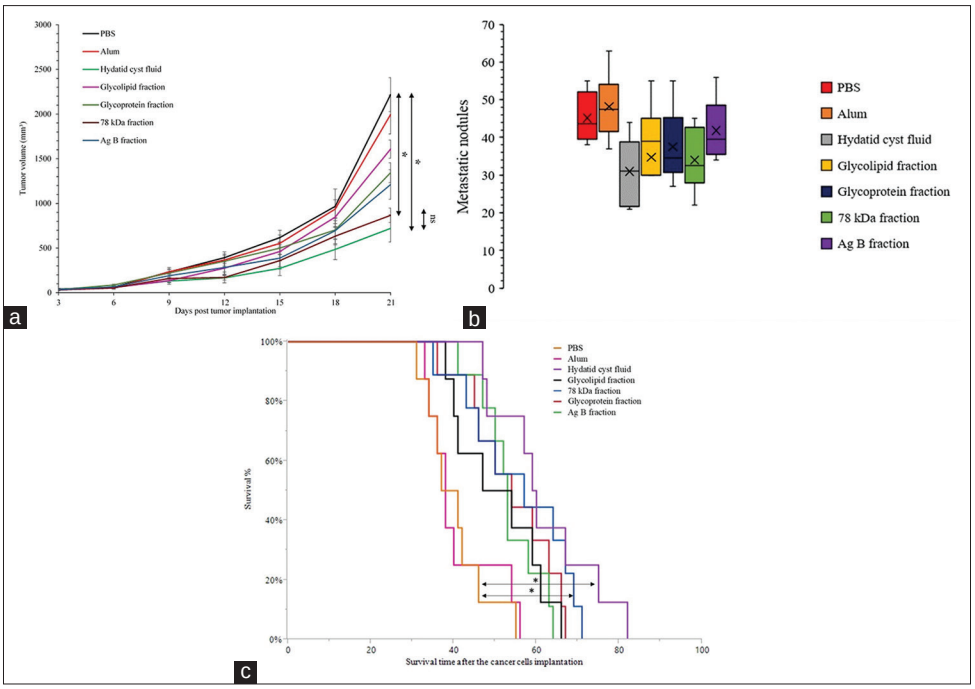

Figure 2: 4T1 breast tumor growth (a), tumor metastasis (b), and Kaplan–Meier survival curve (c) in BALB/c mice immunized with the hydatid cyst fluid, the glycoprotein, the glycolipid, the Ag B, and the 78 kDa fractions that subsequently challenged with 4T1 breast cancer cells in comparison with that of control mice (injected with BPS or Alum alone)

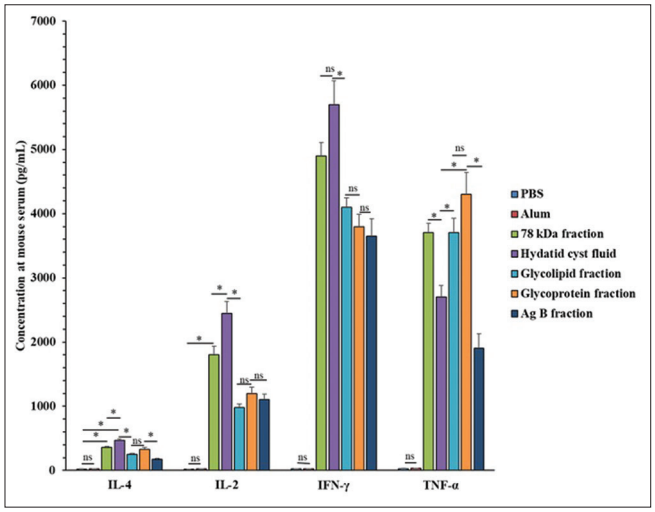

Figure 3: The serum level of IL-2, IFN-γ, IL-4, and TNF-α in immunized mice with the crude hydatid cyst fluid antigens and its derived fractions that subsequently challenged with 4T1 breast cancer cells. It seems that the crude hydatid cyst antigens immunization can significantly increase IL-2, IFN-γ, and TNF-α cytokines serum level. In addition, the 78 kDa fraction of immunized mice had a significant increase at IFN-γ, TNF-α, and IL-2 in comparison with the nonimmunized mice (PBS- or Alum-injected groups)

Table 1: Survival time (days) of BALB/c mice immunized with the hydatid cyst fluid, the glycoprotein, the glycolipid, the AgB, and the 78 kDa antigen fractions and subsequent injection with 4T1 breast cancer cells in comparison with the nonimmunized mice

Groups (immunized with)	Mean survival time (Days)
PBS	40.2±7.6
Alum	41.1±8.8
Hydatid cyst fluid antigens	61.8±12.2
Glycolipid fraction	50.7±10.7
Glycoprotein fraction	52±10
78 kDa fraction	56.7±12.8
Ag B fraction	53.4±7.3

Discussion

Current cancer treatments including chemotherapy and radiation therapy have many side effects due to their low specificity for the malignant cells. Immunotherapy is an alternative method for cancer treatment.[36] It has many branches such as T-cell therapy, dendritic cells therapy, and vaccination, which is one of the most popular and interesting techniques of immune therapy. Immunization is a safe, low-cost, and effective therapeutic method.[5] Its goal is to introduce cancer antigens to the immune system and flaring the weakened host immune response against tumors. This will cause a specific immune response against cancer cells and tumor destruction. However, the poor immunogenicity of the tumor-associated antigens often fails to induce an effective immune response. To overcome this problem and activate an effective antitumor immune response, utilizing parasite antigens that have high homologies with cancer-associated antigens can be a good choice. This is because these antigens as foreign antigens are strong immunogenic antigens and can significantly activate the immune system.[5,37-39]

Common antigens between parasites and tumors have been reported in previous investigations. Unusual mucin

antigens such as the TF antigens that are overexpressed by cancer cells have considerable similarities with helminths mucin.[15] *Schistosoma mansoni* and *Echinococcus granulosus* express Tn antigen. Tk antigen, which is expressed at the surface of human colorectal cancer cells, has also been detected in *Taenia crassiceps, Mesocestoides vogae*, and *Taenia hydatigena*. In another investigation, it was shown that sera from *S. mansoni*-infected mice react with antigens of some human carcinomas that demonstrates antibodies cross-reaction due to antigens homology.[14,40]

Therefore, immune response to the parasite antigens that have a high degree of homology with the cancer antigens may stimulate a strong immune response against the malignant cells. This response can significantly inhibit the tumors growth and metastasis and subsequently, increase tumor-bearing mice survival. Cancer-specific antigens are usually poor immunogens, but parasite antigens that are similar to the cancer antigens may retrieve the poor immunogenicity of cancer cells and cause considerable immune response against their homolog cancer-associated antigens.[16,41] Many studies have shown immunization of mice with *Trypanosoma cruzi* and *Toxoplasma gondii* antigens resulted in antitumor effects.[42-44] In the mice that had been infected with the malaria parasite and then challenged with murine Lewis lung cancer, inhibition of the tumor growth was observed.[45]

The immune system has two arms including cellular and humoral immunity. CD4+ cells are the main regulators of immune system and have two subtypes (Th1 and Th2) with different functions.[46] Th1 stimulation can activate the cellular immune response by activating CD8+ T-cell lymphocytes by secretion of IL-2, IL-12, and IFN-γ. Cellular immunity has the main role at eradication of cancer cells.[47] The most important produced cytokine by Th2 is IL-4, which activates the humoral immune response.[48,49] The crude hydatid cyst antigens and the 78 kDa fraction immunization caused the highest serum level of IFN-γ and IL-2, which exhibit the cellular immune arm activation. These results are in agreement with significant inhibition of the tumors growth and metastasis in these groups. Therefore, both cellular and humoral immune responses are involved in the antitumor effects of crude hydatid cyst and 78 kDa antigens immunization. TNF-α has been demonstrated to be part of the cytotoxic effectors of the immune cells such as NKs and CD8+ T lymphocytes, which are the main players in the anticancer immune response. The crude hydatid cyst and 78 kDa fraction-immunized groups had significant ($P < 0.05$) increase of TNF-α serum level in comparison with PBS- and Alum-immunized groups.

In this investigation, mice immunization with the hydatid cyst fluid antigens results in inhibition of 4T1 breast tumors' growth, metastasis, and the increase of the 4T1 tumor-bearing mice survival. The mice immune system exhibited high activation after immunization according to immune system cytokines. Among different antigens, crude hydatid cyst fluid and the 78 kDa fraction exhibited the best efficacy in the inhibition of tumor growth and metastasis. The mice weight was monitored from the first day of vaccination until the cancer cells implantation. No sign of body weight loss, cachexia, and anorexia was observed (data not shown). In addition, no death due to immunization was observed during this time period. Therefore, due to availability, easy purification, low cost, immunization safety, effective immune system activation, and also significant antitumor efficacy, the 78 kDa antigen can be an appropriate candidate for future clinical trials for cancer immunotherapy.

Conclusions

Employing parasites antigens due to high immunogenicity and homology with cancer cells for antitumoral immunization and immune therapy has gained many attentions. The hydatid cyst fluid is a mixture of different antigens with high ability to activate the immune system. BALB/c mice immunization with the total hydatid cyst antigens mixture and the 78 kDa fraction increased the interleukin levels in the mice sera and subsequent inhibition of tumor growth and metastasis and increase of tumor-bearing mice survival. Therefore, these two antigens can be a good choice for breast cancer immunotherapy in human.

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Conflicts of interest

There are no conflicts of interest.

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