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Combination immunotherapy with extract of heated 4T1 and naloxone in mouse model of breast cancer

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Background/aim: This study was designed to investigate the efficacy of a new vaccine against breast cancer, which was made by mixing the extract of heated 4T1 cells and naloxone, as an adjuvant.

Materials and methods: Female BALB/c mice of 6–8 weeks old were challenged subcutaneously in the right flanks with 4T1 cells. When all animals developed a palpable tumor, immunotherapy was initiated. Mice in the experimental groups received, twice with a 1-week interval, either the extract of heated 4T1 alone or in combination with naloxone, and mice in the negative control group received phosphate-buffered saline. One week after the last immunotherapy, half of the mice were euthanized in order to determine the immune response profile. The remaining animals were kept until the time when death occurred spontaneously.

Results: The combined-treated mice with mammary tumors showed a more favorable survival curve and slower rate of tumor development compared to the mice with tumors that received only heated 4T1 and/or negative control mice. Moreover, the combined immunization significantly amplified the respiratory burst potential and the secretion of IFN-γ, and, conversely, diminished the secretion of IL-4, IL-10, and TGF-β in the splenocyte population compared to splenocytes from other groups.

Conclusion: The combined naloxone and heated 4T1 cells promote beneficial outcomes in the mouse model of breast cancer.

Key words: 4T1 cell line, breast cancer, tumor vaccine, naloxone

1. Introduction

It appears that the immune system has the ability to recognize and eliminate some tumor cells, at least in the early stages of their development (1). Nonetheless, many tumors escape from the pressure of the immune system and become clinically significant. Therefore, improvement of immunity responses against tumor cells may be a useful strategy to control malignancy (1–3). Therapeutic cancer vaccines are one of the best approaches to elicit an antitumor immune response (2). On the other hand, the polarization of immunity towards the cellular arm plays an essential role in defense against tumor cells (4). Adjuvants are useful materials to induce an appropriate response in clinical vaccines (5,6). Unfortunately, alum, the only adjuvant approved worldwide for human use, is able to enhance only the Th2-specific response (7). Meanwhile, strong Th1 adjuvants, like complete Freund's adjuvant, cannot be tolerated by humans (8). Thus, researching new and safe adjuvants for induction of robust cellular immunity would be a logical decision.

Some evidence has suggested that naloxone (NLX), an opioid receptor antagonist, acts as an appropriate adjuvant in enhancing vaccine-induced cellular immunity and Th1 immune responses against viruses and intracellular bacteria or parasites (9–13).

It is worth indicating that breast cancer is the most commonly diagnosed form of cancer and the second leading cause of death among Western women (14). The 4T1 mammary carcinoma is an easily transplantable, highly tumorigenic, and invasive tumor cell line that can be used as an experimental animal model for human mammary cancer (15,16). The 4T1 cell lines, unlike most tumor models, can spontaneously metastasize from the primary tumor in the mammary gland to multiple distant sites like lymph nodes, blood, liver, lung, brain, and bone, in a very similar manner to human mammary cancer (15,17).

This study was carried out to investigate the efficacy of a new vaccine against breast cancer, made by mixing the extract of heated 4T1 cells and NLX, as an adjuvant.

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2. Materials and methods

2.1. Materials

Fetal calf serum, Dulbecco's modified Eagle's medium (DMEM), and RPMI 1640 medium were purchased from GIBCO/Life Technologies Inc. (Gaithersburg, MD, USA). In addition, naloxone, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), nitro tetrazolium blue chloride (NBT), dimethyl sulfoxide (DMSO), phytohemagglutinin (PHA), dioxane, and phosphate-buffered saline (PBS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits were procured from QIAGEN (Hilden, Germany).

2.2. Cells and culture conditions

4T1 cells were supplied from the Pasteur Institute of Iran. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 and maintained in monolayer cultures in DMEM supplemented with 10% FBS.

2.3. Animals and tumor challenge

Female BALB/c mice of 6–8 weeks old were brought from the Pasteur Institute of Iran. The mice were maintained under constant temperature (22–24 °C) and a 12-h light/dark cycle, and they received food and water ad libitum. Animal welfare was observed in compliance with the regulations of the Ministry of Health and Medical Education of Iran, approved by the medical ethics committee of the university for animal studies. Animals were allowed to rest for 1 week before any treatment and were then challenged subcutaneously in the right flanks with 1×10^5 viable tumor cells in 50 µL of PBS. Tumor growth was monitored every 5 days by a caliper. Tumor volume in mm^3 was estimated by using the formula of an ellipsoid (length × width × height × 0.5236).

2.4. Vaccination of the mice

Vaccination was initiated when all animals developed a palpable tumor. At this time, the mice were randomly divided into 3 groups. Each group had 10 animals with tumors and all vaccines were administrated by subcutaneous injection to the left flank. In the first group, with the control tumor mice, the animals received 100 µL of PBS twice at a 1-week interval. In the second group, the heated 4T1-treated group, the mice were immunized twice at a 1-week interval with the frozen and thawed extract of 10^3 heated 4T1 cells in 100 µL of volume. The heated 4T1 cells were provided by the exposure of 4T1 cell lines to nonlethal heat shock (43 °C, 30 min). Finally, in the last group, with the combined NLX and heated 4T1-treated mice, the animals were vaccinated twice at a 1-week interval with the frozen and thawed extract of 10^3 heated 4T1 cells and NLX (6 mg/kg) in 100 µL of volume. One week after the last immunotherapy, half of the mice were euthanized in order to evaluate their immune responses.

2.5. Splenocyte proliferation

The proliferation potential of lymphocytes in the splenocyte population was evaluated by MTT assay. In brief, spleen cells were aseptically isolated from mice at the time of bleeding. The single-cell suspensions of splenocytes were prepared in RPMI 1640 medium, supplemented with 10% fetal calf serum, and red blood cells (RBCs) were removed by RBC lysis buffer (18). The splenocytes were plated in 96-well flat-bottomed plates in RPMI 1640 medium, supplemented with 10% fetal calf serum (1×10^5 cells 100 μL^{-1} $well^{-1}$) and stimulated with 50 µL of PHA solution (1 mg/mL) or medium alone. After 72 h of incubation, the cultures were pulsed with 20 µL of the MTT solution (5 mg/mL) for 4 h at 37 °C. Then 150 mL of DMSO was added and shaken vigorously to dissolve the formazan crystals. The optical density (OD) at 550 nm was measured using a microplate reader (Dynatech, Denkendorf, Germany). The experiments were done in triplicate sets. The results were expressed as the proliferation index according to the ratio of the OD_{550} of stimulated cells with PHA to the OD_{550} of nonstimulated cells (18).

2.6. Cytokine production

The splenocytes (2×10^6 cells/mL) were incubated in 24-well plates and pulsed with 50 µL of PHA solution (1 mg/mL). The culture supernatants were collected after 72 h. IFN-γ, IL-4, IL-10, and TGF-β production was assumed by ELISA according to the manufacturer's instructions.

2.7. Respiratory burst in the splenocyte population

Respiratory burst of phagocytic cells in the splenocyte population was checked by using NBT dye reduction as described previously (19). In brief, 100 µL of the suspension of splenocytes with 0.1 mL of *S. aureus* suspension (10^8 cell/mL) and 0.1 mL of 0.1% NBT in PBS (pH 7.4) were mixed. The mixture was incubated at room temperature for 15 min and subsequently kept at 37 °C for an additional 15 min. The reduced dye was extracted in dioxane and quantitated at 520 nm.

2.8. Statistical analysis

The statistical analysis was performed with the Kruskal–Wallis test, followed by pair-wise comparisons using the Mann–Whitney U test with Bonferroni adjustment. The results were shown as mean ± SD. $P < 0.05$ was considered statistically significant.

3. Results

Following tumor induction, mice were monitored every 5 days for the first signs of palpable tumors. All immunization protocols were started on day 12 after tumor induction, when individual mice developed a palpable tumor, and continued to day 59, when all mice in the groups died. As illustrated in Figure 1, mice that received the combined heated 4T1 and NLX showed a more favorable survival curve compared to mice in other groups. At least 20% of

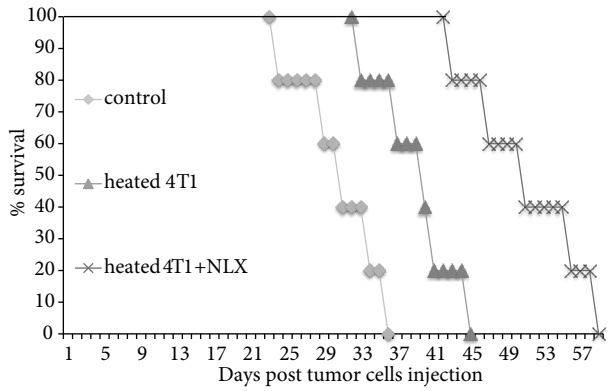

Figure 1. Survival of BALB/c mice challenged with 4T1 cells after immunotherapy. Immunotherapy was started when all animals had developed a palpable tumor. Combined extract of heated 4T1 and naloxone (NLX) led to more favorable outcome compared to other groups.

mice in the combined immunization group were alive until day 58 after tumor induction, while all mice in the group treated with heated 4T1 alone were dead by day 45 after tumor induction. The survival rate of the control tumor mice was very poor compared to other groups as all mice in this group were dead by day 36 after tumor induction.

Moreover, tumors developed at a significantly slower rate in the mice that received the combined immunotherapy in comparison with other groups (Figure 2). Mice in this group showed a significant reduction in tumor growth rate from day 27 after tumor induction compared to other groups. The tumor volume changes were not statistically different between mice that received the extract of heated 4T1 and control tumor mice (Figure 2).

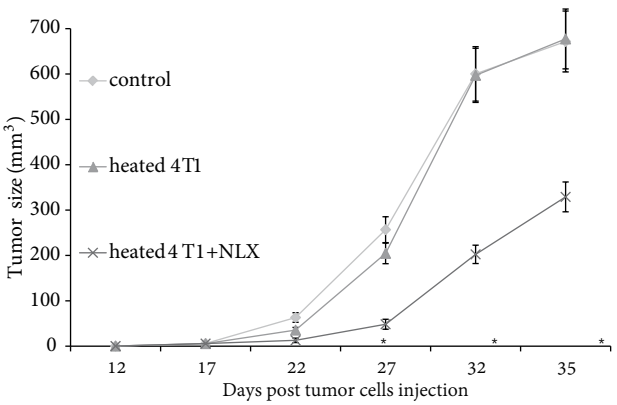

Figure 2. Evaluation of mammary tumor size after immunotherapy. Tumor volume was estimated as detailed in Section 2. Combined extract of heated 4T1 and NLX decreased tumor growth rate more profoundly than in other groups (*P < 0.05 versus control tumor mice and/or mice with tumors that received only the extract of heated 4T1).

The ex vivo cytokine assay demonstrated that combined immunization significantly upregulated the secretion of IFN-γ and conversely downregulated the secretion of IL-4, IL-10, and TGF-β in the splenocyte population compared to the splenocytes from other groups (Figure 3). Although a similar pattern of change in the secretion of these cytokines was observed in heated 4T1 immunized mice compared to the control group, these changes, except for IL-10, were not statistically significant. Moreover, a significant increase in splenocyte proliferation was observed in mice with tumors that received the combined heated 4T1 and NLX compared to other animals (Figure 4A).

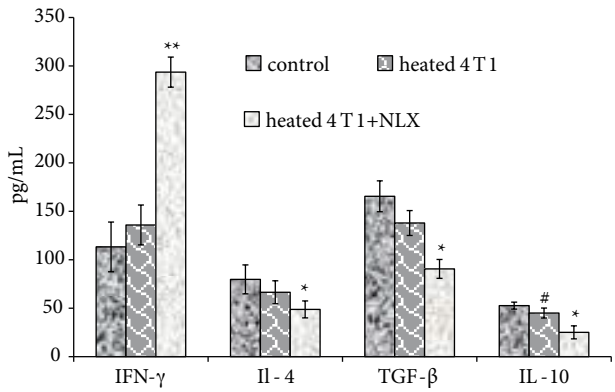

Figure 3. Effects of immunotherapy on cytokine production of splenocyte population. One half of mice in each group were euthanized 1 week after last immunotherapy and splenocytes were isolated and cultured for 72 h as described in Section 2 (*P < 0.01; **P < 0.001 versus control tumor mice and/or mice with tumors that received only the extract of heated 4T1; #P < 0.05 versus control tumor mice).

As shown in Figure 4B, the respiratory burst was significantly increased in the splenocytes from the combined immunized mice with mammary tumors and the mice with mammary tumors that received only heated 4T1, as compared to control tumor mice. This increase was significantly more profound in mice with tumors that received combination immunotherapy as compared to mice with tumors that received only the extract of heated 4T1.

4. Discussion

The malignant tumors induced by 4T1 cells are able to frequently grow and evade destruction by the immune responses because of their low immunogenicity (20,21). In this regard, enhancement of the immunogenicity of 4T1 cells was considered as the first goal of the current study.

Heat shock proteins (HSPs) are highly conserved housekeeping proteins, found in all organisms, that

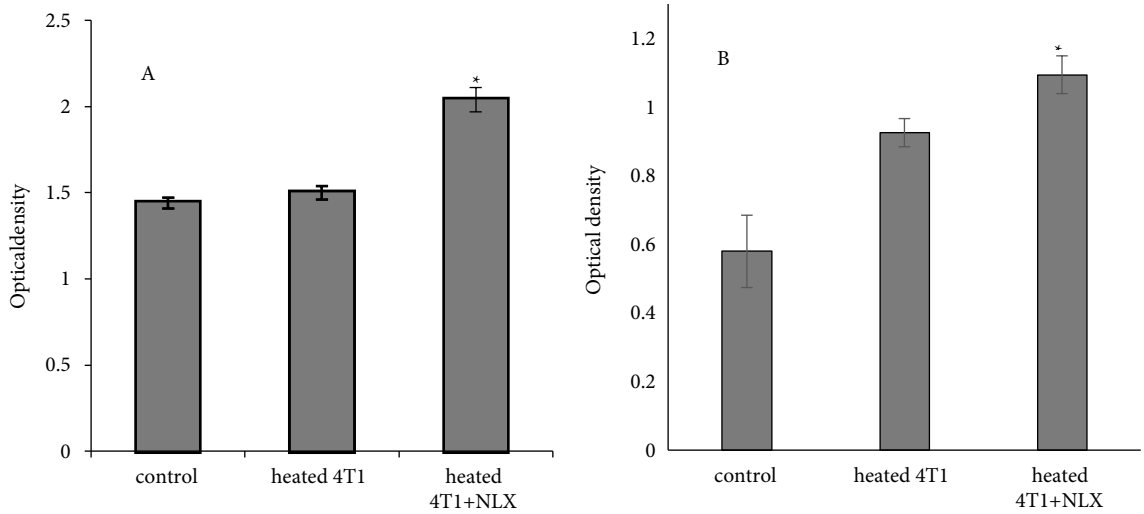

Figure 4. Modulation of splenocyte proliferation (A) and respiratory burst (B) after immunotherapy. Lymphocyte proliferation in splenocytes was determined by MTT test as described in Section 2 (A). The respiratory burst of phagocyte cells in splenocytes was assumed by NBT reduction assay as detailed in Section 2 (B) (*P < 0.001 versus control tumor mice and/or mice with tumors that received only the extract of heated 4T1).

upregulate under a wide variety of physiological and pathological stimuli such as heat shock (22,23). The extracellular and membrane-bound HSPs, especially Hsp70 families, participate in binding and presenting tumor antigens to the antigen-presenting cells as a mechanism for host-priming of T-cell-mediated antitumor immunity (24–26). A prior study revealed that the exposure of 4T1 cell lines to nonlethal heat shock induced the surface expression of Hsp72, a member of the Hsp70 family, and concurrently reduced the growth and metastatic potential of these cells in vivo (27). Accordingly, the 4T1 cells used in the current study were sublethally heated before undergoing the freeze/thaw procedure. Based on the results, vaccination with the extract of heated 4T1 cells could reduce the tumor growth rate and increase the survivability of the mice with breast tumors; however, these changes were not significant. Nevertheless, the vaccine made with the extract of heated 4T1 cells and NLX as an adjuvant significantly reduced the tumor growth rate, and consequently the survivability of these mice significantly increased.

The previous available data showed that the interaction between immune and nervous systems plays a substantial role in the fate of immune responses (28,29). Naloxone is routinely used to rapidly and safely reverse opioid-induced respiratory depression (30). It is now clear that opioid receptors, particularly κ- and δ-opioid receptors, are expressed in immune cells. Opioid receptors can modulate both innate and acquired immune responses (31–33). Earlier evidence suggests that naloxone may induce a proinflammatory milieu by increasing the release of local proinflammatory neuropeptides, such as substance P, from the nerve fibers or via a direct effect on innate immune cells, including monocytes, macrophages, and dendritic cells (9,34,35). Antigens in this proinflammatory milieu can promote specific immunity toward cell-mediated immunity and a Th1 pattern response (9,36). On the other hand, neuropeptides may promote the maturation and migration of local antigen-presenting cells to the draining lymph nodes and skew immune responses toward a Th1 pattern (10).

The combined heated 4T1 cells and NLX significantly increased splenocytes' proliferative response, more so than that observed after treatment with heated 4T1 cells. In this regard, previous reports showed that the opioid possesses an antiproliferative effect on T-cells, and, in contrast, naloxone accelerates the mitogen-induced splenocyte proliferation (10,37,38).

It is clear that IFN-γ production by the immune system correlates with antitumor responses (3). The obtained results showed that IFN-γ production in the splenocyte population was significantly increased more than that observed in other groups. Importantly, the level of IFN-γ did not show any significant difference among the control tumor group and the mice immunized with heated 4T1.

Tumors are able to evade immunity by secreting some mediators like TGF-β and IL-10. TGF-β and IL-10 tend to inhibit the proliferation and activation of lymphocytes and macrophages and consequently suppress cell-mediated immunity, and they are required to control tumor growth (37,39). Both cytokines can induce the development of regulatory T cells, which have been found in a variety

of tumors, and may suppress T cell responses to tumors. Interestingly, regulatory T cells are the other source of TGF-β and IL-10 (39). Besides TGF-β and IL-10, IL-4 can diminish the essential arms of antitumor immunity including macrophages and Th1 responses. Moreover, it seems that IL-4 directly promotes the tumor cell growth in human breast cancer (40). Our data demonstrated that the combined heated 4T1 and NLX significantly lowered the levels of TGF-β, IL-10, and IL-4 further than in other groups.

Previous works showed that IFN-γ-activated macrophages display various antitumor functions, such as profound production of reactive oxygen species (ROS) (41). Unfortunately, malignant tumors may suppress macrophage activation and promote macrophages to secrete soluble factors such as TGF-β, which confers a local state for tumor growth (42). It has been reported that the injection of IL-4-treated monocytes into the mammary fat pads of mice with 4T1 cell-induced tumors increased the solid tumor growth and lung metastasis of 4T1 cells (42). In the current survey, the NBT reduction assay in splenocytes was used to estimate the level of production of ROS by macrophages in the tumor microenvironment. The findings showed that the production of the ROS was significantly increased in vaccine-treated mice compared with the control tumor mice.

As a result, it was concluded that the combined NLX and heated 4T1 cells promote beneficial outcomes in the mouse model of breast cancer. It is of note that the immunogenicity of 4T1 cells is very low (20,21). Therefore, the achieved findings are beneficial. However, it should be stated that this survey is a preliminary study, and further studies need to be designed and developed.

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