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Active immunotherapy for mouse breast cancer with irradiated whole-cell vaccine expressing VEGFR2

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Abstract. As tumor-associated antigens are not well characterized for the majority of human tumors, polyvalent vaccines prepared with whole-tumor antigens are an attractive approach for tumor vaccination. Vascular endothelial growth factor receptor-2 (VEGFR2), as a model antigen with which to explore the feasibility of immunotherapy, has shown great promise as a tumor vaccine. However, the efficacy of immunotherapy is often not ideal when used alone. In this study, we explored the therapeutic efficacy of an irradiated AdVEGFR2-infected cell vaccine-based immunotherapy in the weakly immunogenic and highly metastatic 4T1 murine mammary cancer model. An adenovirus encoding the VEGFR2 gene (AdVEGFR2) was constructed. Lethally irradiated, virus-infected 4T1 cells were used as vaccines. Vaccination with lethally irradiated AdVEGFR2-infected 4T1 cells inhibited subsequent tumor growth and pulmonary metastasis compared with challenge inoculations. Angiogenesis was inhibited, and the number of $CD8^+$ T lymphocytes was increased within the tumors. Antitumor activity was also caused by the adoptive transfer of isolated spleen lymphocytes. *In vitro*, the expression of HMGB1 and HSP70 in the AdVEGFR2-infected 4T1 cells was increased, and was involved in the activation of tumor antigen-specific T-cell immunity. Our results indicate that the immunotherapy based on irradiated AdVEGFR2-infected whole-cancer cell vaccines may be a potentially effective strategy for 4T1 cancer treatment.

Introduction

Whole-cell vaccines are a method for providing target antigens. In this approach, the whole-tumor cell is the source of immunogens with which to induce an antitumor immune response. The advantage of using whole-tumor cell vaccine is that a broad array of tumor-associated antigens (TAAs) is represented, thereby minimizing immune escape. As whole proteins are present, there are no HLA restrictions on who can receive the vaccine. However, it is not ideal to use live pathogens as vaccines due to safety concerns. Killed but maintained immunogenicity via γ-irradiation has been reported to have the benefit of vaccines to induce an appropriate immune response without the issue of pathogen replication in the host (1-5). Meanwhile, the weak immunogenicity of many tumors also represents a barrier to the effective induction of antitumor immunity. Reportedly, cancer cells or other bystander cells included in the vaccine are transfected with vectors containing genes that express potent immunostimulating proteins or cytokines, including B7.1 (CD80), GM-CSF and CCL21 (6-11).

Vascular endothelial growth factor receptor-2 (VEGFR2) is an important receptor responsible for the angiogenic activity of VEGF (12,13). Overexpression of VEGFR2 is found on activated endothelial cells of newly formed vessels and is strongly associated with invasion and metastasis in many types of cancer (14-16). In addition, it has been reported that the inhibition of tumor growth and metastasis in many animal models has been achieved by various techniques that disrupt or neutralize the functions of either VEGF or VEGFR-2 (17-19). In our previous research, a xenogeneic homologous VEGFR2 protein vaccine (qVEGFR) effectively inhibited the tumor growth in LL/2 Lewis lung carcinoma, CT26 colon carcinoma, and Meth A fibrosarcoma models (20). It is, thus, clear that the breaking of the immune tolerance against VEGFR2 of autologous angiogenic endothelial cells is an effective pathway for cancer therapy with active immunity.

In the present study, we explored the therapeutic efficacy of an irradiated AdVEGFR2-infected autologous whole-cell tumor vaccination in the weakly immunogenic and highly metastatic 4T1 murine mammary cancer model. Moreover, we also further discussed its possible mechanism.

Materials and methods

Cell lines and mice. The 293A (human embryonic kidney) and 4T1 cell lines were obtained from the American Type Culture

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Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 10 μg/ml gentamicin sulfate, maintained in a 37˚C incubator with a humidified 5% CO_2 atmosphere. Six- to 8-week-old female BALB/c mice were obtained from the Laboratory Animal Center of Sichuan University and maintained in pathogen-free conditions. All procedures were approved by the institute's Animal Care and Use Committee.

Construction of recombinant adenoviral vectors. A recombinant adenovirus carrying the VEGFR2 gene (AdVEGFR2) was constructed by use of the AdEasy system from Qbiogene, Inc., according to the procedure provided by the manufacturer. The recombinant adenovirus without a foreign gene (Adnull) served as a control. All virus particles were amplified in HEK 293A cells and titrated as PFU/ml and stored at -80˚C.

Preparation of whole-cell vaccines. To vaccinate mice, 4T1 cells were infected by AdVEGFR2 or Adnull (control) at a MOI of 100 in serum-free RPMI-1640. Cells were incubated at 37˚C for 48 h. Infected cells and uninfected 4T1 cells were irradiated with 100 Gy and subsequently injected s.c. into the flanks of the mice.

Breast tumor model and immunotherapy. 4T1 breast cancer models were established in BALB/c mice. Six mice in each group were challenged with $1x10^6$ 4T1 cells s.c. in the right flank. For vaccination, 4T1 cells were infected with AdVEGFR2 or Adnull or were uninfected, and then irradiated with 100 Gy of X-rays. Irradiated cells were washed extensively with PBS and vaccination was carried out by s.c. injection of $1x10^6$ cells, 3 times, on Days 7, 21 and 28 in the left flank of mice after tumor cell inoculation. Additional control animals were injected with 0.9% NaCl solution. Thus, mice were divided into 4 groups: the irradiated AdVEGFR2-infected 4T1 cell-treated group (4T1-AdVEGFR2 group), the irradiated Adnull-infected 4T1 cell-treated group (4T1-Adnull group), the irradiated 4T1 cell-treated group (4T1 group) and the saline-treated group (NS group). Tumor dimensions were measured with calipers every 3 days, and the tumor volume (V) was calculated according to the following formula: $V = 0.52 \times \text{length} \times \text{width}^2$.

Adoptive transfer in vivo. Ten mice in each group were immunized with $1x10^6$ irradiated AdVEGFR2-infected 4T1 cells, Adnull-infected 4T1 cells, 4T1 cells or NS s.c. in the right flank 3 times on Days 1, 14 and 28. Sera derived from the mice on Day 7 after the third immunization were adoptively and intraperitoneally transferred 1 day (100 μl serum/mouse) before mice were challenged with $1x10^6$ 4T1 cells s.c. in the right flank and then were treated once per day for 10 days. In addition, isolated spleen lymphocytes from the immunized mice were adoptively and intravenously transferred ($1x10^6$ cells/100 μl/mouse) and were then treated twice per week for 2 weeks. Tumor dimensions were measured with calipers every 4 days for 29 days, and tumor volume (V) was calculated according to the following formula: $V = 0.52 \times \text{length} \times \text{width}^2$.

Histological analysis. Tumors from each group were embedded in paraffin, and sections (3-5 μm) were immunohistochemically stained to determine the infiltration of lymphocytes and quantify the microvessel density in the tumor tissue using rat anti-mouse CD4 antibody, rat anti-mouse CD8 antibody and rabbit anti-mouse CD31 antibody (Abcam, Inc.). Vascular density was quantified by counting the number of microvessels per high power field. Images were acquired using an Olympus BX60 microscope.

Quantitative assessment of apoptosis. Tumor species were prepared as previously described (21). The presence of apoptotic cells within the tumor sections was determined using the In Situ Cell Death Detection kit (DeadEnd™ Fluorometric TUNEL System, Promega, Madison, WI, USA), according to the manufacturer's protocol. In tissue sections, five high power fields were randomly chosen and analyzed. The apoptotic index (AI) was defined as follows: AI (%) = 100 x apoptotic cells/total tumor cells. Images were acquired using a LEICA DM2500 microscope.

Western blot analysis. The 293A cells infected with AdVEGFR2 or Adnull for 48 h were lysed to analysis the expression of VEGFR2 using rabbit anti-mouse VEGFR2 antibody (Abcam). 4T1 cells infected with AdVEGFR2 or Adnull for 48 h and uninfected 4T1 cells were irratiated with 100 Gy, and their lysates were subjected to western blot analysis with rabbit anti-mouse HMGB1 and HSP70 antibodies.

FCAS. For FACS analysis, we prepared single-cell suspensions of tumors from 4T1-AdVEGFR2-treated or 4T1-Adnull-treated mice. Briefly, tumors were minced using a razor blade and digested with collagenase I for 30 min at 37˚C. For extracellular staining of immune markers, $5x10^5$ of freshly prepared cells were stained with PE CD4 and FITC CD8. Fluorescence data were collected on FACScalibur and analyzed using cell quest software (BD Biosciences).

ELISA. For ELISA, 96-well plates were coated with 4T1 cells ($1x10^4$ cells/well) in 10% RPMI-1640 overnight at 4˚C. Plates were washed with PBST (0.05% Tween 20 in PBS) and were fixed in 10% formalin for 15 min at room temperature. Then, plates were washed with ddH_2O and blocked for 1 h at 37˚C with 200 μl/well 1% bovine serum albumin (BSA) in PBST. Mouse sera from treated mice diluted serially in PBS were added for 2 h at 37˚C, followed by a dilution of anti-mouse immunoglobulin G (IgG) subclass or anti-IgM or anti-IgA antibody conjugated to alkaline phosphatase. Enzyme activity was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Multiskan MK3).

Statistical analysis. SPSS 16.0 was used for statistical analysis. Data are expressed as means ± SD. The statistical analysis in all the experiments was performed using one-way analysis of variance (ANOVA). P-value <0.05 was considered to indicate a statistically significant result.

Results

Construction of the recombinant VEGFR2-expressing adenovirus. Positive clones were confirmed by restriction enzyme analysis and DNA sequencing. The *Pac*I-digested pAdVEGFR2 plasmid was transfected into 293 cells. At the

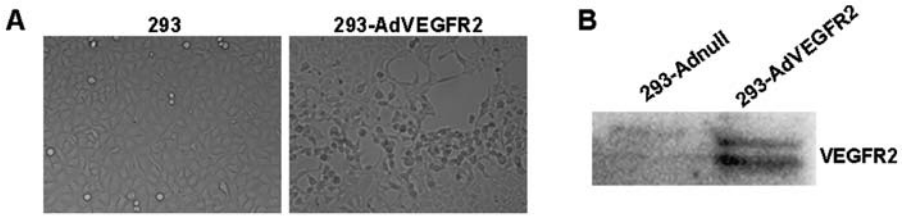

Figure 1. Packaging of AdVEGFR2 and the expression of VEGFR2 in 293 cells. (A) The images show 293 cells transfected with pAdVEGFR2 as they undergo CPE on Days 8-10 post-transfection. (B) Expression of VEGFR2 in AdVEGFR2-infected and Adnull-infected 293 cells. Data are representative of two independent experiments.

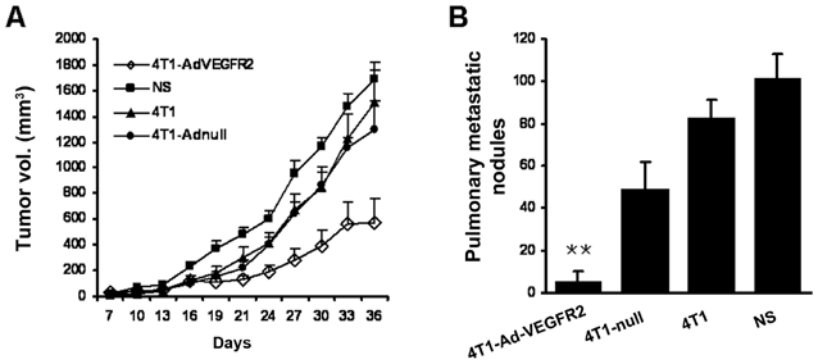

Figure 2. Induction of therapeutic antitumor immunity. Mice (n=6 in each group) were treated s.c. with $1x10^6$ irradiated 4T1 cells infected with AdVEGFR2 or Adnull or were uninfected and treated with 0.9% NaCl solution after challenged with $1x10^6$ 4T1 cells s.c. on Days 7, 21 and 28 for the induction of the therapeutic antitumor immunity. (A) Tumor size of each mouse was monitored every 3 days. The differences between the 4T1-AdVEGFR2-treated group and the control groups were significant (P<0.05) starting on Day 24. (B) Pulmonary metastatic nodules were counted. The differences between the 4T1-AdVEGFR2-treated group and the control groups were significant (P<0.001). Data are representative of two independent experiments. **P<0.001.

early stage, cells producing the adenovirus first appear as patches of rounding, dying cells. As the infection proceeded, cells containing the viral particles lysed and infected neighboring cells. A plaque began to form. On Days 8-10 post-transfection, the infected neighboring cells lysed, forming a plaque that was clearly visible (Fig. 1A). The expression of VEGFR2 in the AdVEGFR2-infected 293 cells was detected using western blotting (Fig. 1B).

Induction of therapeutic antitumor immunity. We tested the therapeutic efficacy of lethally irradiated AdVEGFR2-infected 4T1 cells used as vaccines in established tumors. We treated the mice on Day 7 after 4T1 cell inoculation, when the tumors were visible and palpable. Following treatment with the vaccine 3 times on Days 7, 21 and 28, the size of the tumor nodes in the 4T1-AdVEGFR2-treated group was significantly smaller in comparison with those in the control groups starting on Day 24 (P<0.05) (Fig. 2A). Furthermore, lung metastatic nodules of mice sacrificed at the termination of the experiment were counted under a dissecting microscope. Lung metastatic nodules in the 4T1-AdVEGFR2-treated group were nearly absent compared with the control groups (P<0.05) (Fig. 2B). We monitored the mice treated with the vaccines every 3 days throughout the entire experiment. No severe toxic effects were observed in terms of gross measures, such as weight loss, ruffling of fur and feeding. Thus, the therapy with the irradiated AdVEGFR2-infected cell vaccine not only inhibited the growth of the implanted tumors, but also restrained tumor metastasis.

Induction of tumor apoptosis and inhibition of tumor angiogenesis. To explore the role of the irradiated AdVEGFR2-infected 4T1 cell vaccine on the apoptosis of tumor cells, TUNEL assay of tumor sections was performed. As shown in Fig. 3A, within a similar high-power field, more apoptotic cells were noted in the tumor tissues from the 4T1-AdVEGFR2-treated mice, and the differences were significant compared with those of the control groups (P<0.001) (Fig. 3B). As VEGFR2 is closely related to tumor angiogenic blood vessels, we hypothesized that the therapy with the irradiated AdVEGFR2-infected 4T1 cell vaccine would act partly via an antiangiogenic mechanism to promote tumor regression. Thus, we investigated the microvessel density in the tumor sections by immunohistochemistry using an antibody specific for CD31. Results showed that the 4T1 tumor regression after irradiated AdVEGFR2-infected 4T1 cell vaccine treatment was accompanied by a corresponding decrease in microvessel density compared with the controls (P<0.001) (Fig. 3C).

Cellular and humoral immune response in irradiated AdVEGFR2-infected 4T1 cell vaccine-induced antitumor activity. To explore the possible mechanism through which the antitumor activity was induced by the irradiated AdVEGFR2-infected 4T1 cell vaccine, anti-CD4 and anti-CD8 monoclonal antibodies were used in immunohistochemical staining and FACS. As shown in Fig. 4A, the infiltration of $CD4^+$ and $CD8^+$ T lymphocytes was apparently increased in the 4T1-AdVEGFR2-treated group. Results from FACS indicated the number of $CD8^+$ lymphocytes was increased by 63.8% and

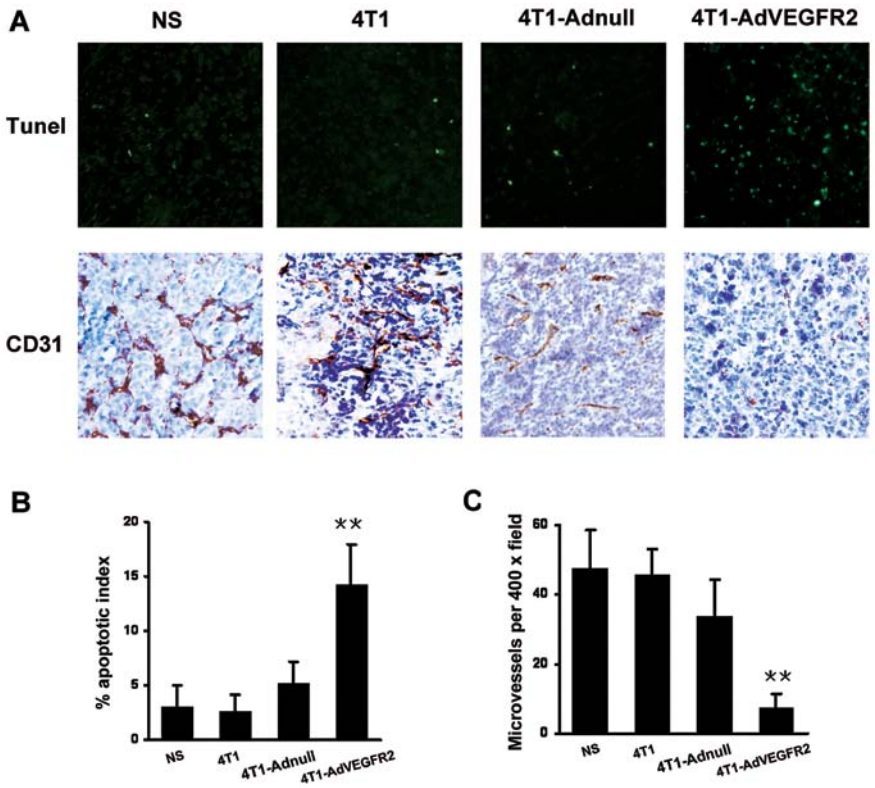

Figure 3. Histological and histomorphometric analysis. (A) Top panel: TUNEL assay revealed that 4T1-AdVEGFR2 induced a significant enhancement in the number of apoptotic cells versus the controls (magnification, x200). Bottom panel: Vascularization within tumors was detected by an antibody to CD31 (magnification, x100). Shown are representative sections from each group. (B) The percentage of apoptosis in each group. The number of apoptotic tumor cells in the tumor sections from the 4T1-AdVEGFR2-treated group was increased compared with the control groups (P<0.001, respectively). (C) CD31-positive microvessels in each group. Vascular density was quantified by counting the number of microvessels per high power field. There was a decrease in the density of CD31-positive microvessels in the 4T1-AdVEGFR2-treated group (P<0.001). Values are expressed as the means ± SD. **P<0.001.

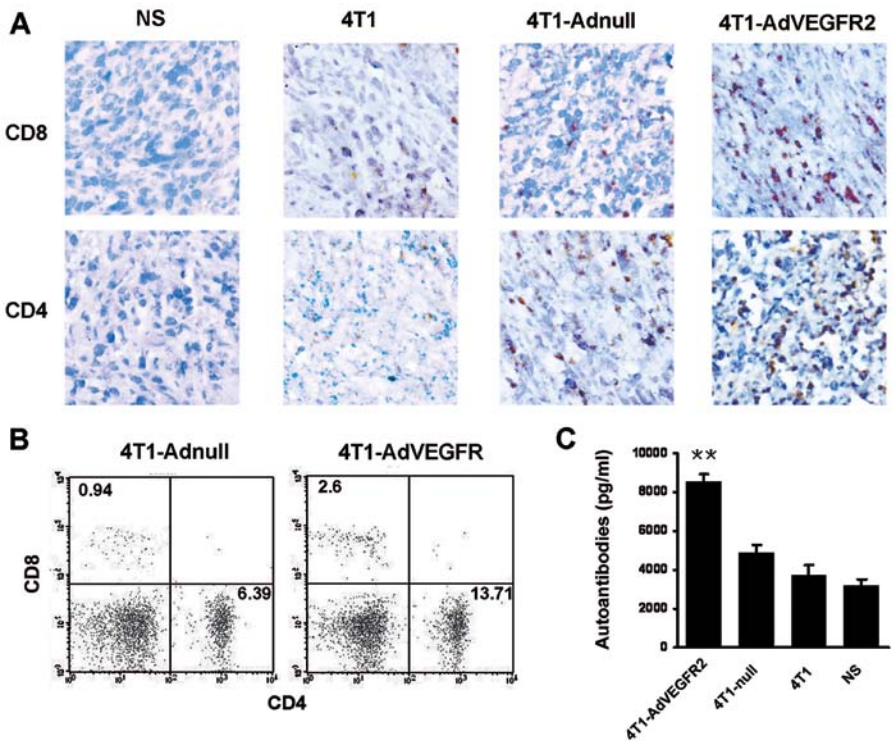

Figure 4. Analysis of the cellular and humoral immune response. (A) Immunohistochemical staining of CD4 and CD8 was used to detect lymphocyte infiltration into tumors *in situ*. Representative staining at x200 is shown. (B) The number of $CD8^+$ and $CD4^+$ cells were found by FACS to be increased in the 4T1-AdVEGFR2-treated group compared with the 4T1-Adnull-treated group. Numerals indicate the percentage of $CD8^+$ or $CD4^+$ cells within the total cell gate. A representative flow cytometric analysis is shown. (C) Autoantibodies against 4T1 cells were detected by ELISA. The differences between the 4T1-AdVEGFR2-treated group and the control groups were significant (P<0.001). Data are representative of two independent experiments. **P<0.001.

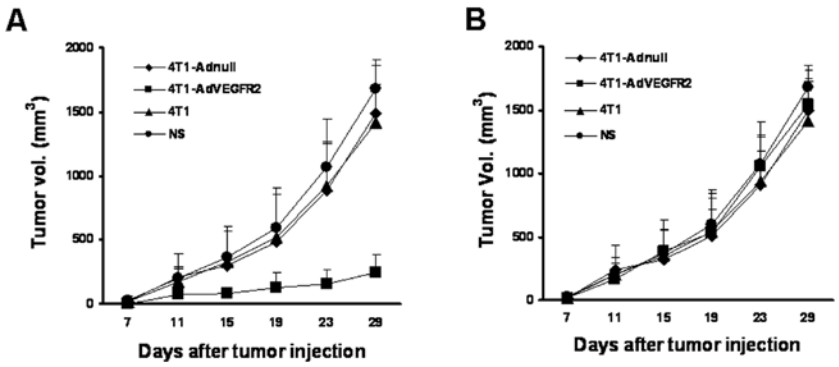

Figure 5. Antitumor effect by the adoptive transfer of lymphocytes *in vivo*. The protective antitumor effect against 4T1 cells was tested with isolated lymphocytes and sera from mice immunized with 4T1-AdVEGFR2, 4T1-Adnull, 4T1 or NS. (A) Treatment with lymphocytes isolated from 4T1-AdVEGFR2 immunized mice showed an apparent protective antitumor effect, compared with the controls (P<0.05). (B) The adoptive transfer of sera from mice immunized with 4T1-AdVEGFR2 did not significantly inhibit tumor growth (P>0.05). Results are expressed as the means ± SD. Data are representative of two independent experiments.

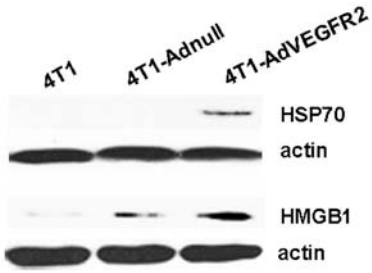

Figure 6. HMGB1 and HSP70 are selectively released by dying tumor cells. HMGB1 and HSP70 were detected by western blot analysis in whole-cell lysates of irradiated 4T1, AdVEGFR2-infected 4T1 and Adnull-infected 4T1 cells. HMGB1 and HSP70 were increased in irradiated AdVEGFR2-infected 4T1 cells.

the number of $CD4^+$ lymphocyte cells was increased 53.4% in the 4T1-AdVEGFR2-treated group compared with the 4T1-Adnull-treated group (Fig. 4B). These results indicate that both $CD4^+$ and $CD8^+$ lymphocytes are important for the therapeutic activity of the 4T1-AdVEGFR2 vaccine against 4T1 breast tumors. To identify the autoantibodies against 4T1 cells within sera from treated mice, we investigated the sera by ELISA. The autoantibodies were increased in the 4T1-AdVEGFR2-treated group (8566.667±351.1885 pg/ml) when compared with the control groups (P<0.001) (Fig. 4C).

Serum and lymphocyte adoptive transfer in vivo. Given that the autoantibodies and T lymphocytes were increased in the 4T1-AdVEGFR2-treated mouse blood and tumor sections, we sought to investigate the protection from tumor growth of serum and lymphocyte adoptive transfer. As expected, treatment with lymphocytes from the spleens of the mice immunized with the irradiated AdVEGFR2-infected 4T1 cell vaccine resulted in apparent inhibition of tumor growth, compared with those from mice immunized with 4T1-Adnull, 4T1 or NS (Fig. 5A). Yet, the adoptive transfer of sera from mice immunized with 4T1-AdVEGFR2 did not effectively inhibit tumor growth (Fig. 5B). These results indicated that the immune responses to the irradiated AdVEGFR2-infected cell vaccine were mainly cellular immune responses.

Expression of HMGB1 and HSP70 in tumor cells infected with AdVEGFR2. Reportedly, HSP70 and the alarmin high-mobility-group box 1 protein (HMGB1) are involved in the activation of tumor antigen-specific T-cell immunity (22-25). Our findings showed that the therapeutic antitumor immunity of the vaccine was mainly cellular immunity. Thus, we investigated the expression of HMGB1 and HSP70 by western blot analysis in whole-cell lysates of irradiated 4T1 tumors infected with AdVEGFR2, Adnull or uninfected. As shown in Fig. 6, the surface expression of HMGB1 and HSP70 in irradiated AdVEGFR2-transfected 4T1 cell tumors was obviously increased, and HSP70 was scarcely expressed in the groups treated with 4T1 cells.

Discussion

In the present study, we demonstrated that the immunotherapy based on the irradiated AdVEGFR2-infected 4T1 cell vaccine had an increased antitumor effect when compared with the irradiated Adnull-infected 4T1 cell or irradiated 4T1 cell vaccines. *In vivo*, irradiated AdVEGFR2-infected 4T1 cell vaccine significantly prevented local tumor growth and pulmonary metastasis. The autoantibodies against 4T1 cells were increased in the vaccine-treated mouse sera, yet the antitumor activity was not caused by the adoptive transfer of sera. Instead, the adoptive transfer of spleen lymphocytes caused an apparent antitumor activity. The number of $CD4^+$ and $CD8^+$ T lymphocytes was increased in the tumors treated with the irradiated AdVEGFR2-infected cell vaccine, and angiogenesis was markedly inhibited. The surface exposures of HMGB1 and HSP70 in the 4T1 cells were apparently increased *in vitro*. The antitumor mechanisms of the vaccine may be due to induction of celluar immunity by targeted inhibition of tumor cells and tumor vessels.

Whole-cell vaccines are characterized by their broad array of tumor-associated antigens (26,27). Vaccination with irradiated tumor cells has been studied in various animal models as early as the 1970s. Yet, tumor cells are not very immunogenic, thus many proteins or cytokines were infected into tumor cells to stimulate immunogenicity. These immunostimulating proteins include B7.1 (CD80), CCL21 and GM-CSF (8-11).

Reportedly, vaccination with irradiated tumor cells engineered to secrete murine GM-CSF stimulated potent, specific, and long-lasting antitumor immunity (28).

Angiogenesis is important not only for normal embryonic development but also for the development of pathologic conditions such as cancer, retinopathies and rheumatoid arthritis (29-32). There is accumulating evidence that the growth and persistence of solid tumors and their metastasis are angiogenesis-dependent (14,33,34). VEGFR-2 is the main receptor responsible for the angiogenic activity of VEGF. Antiangiogenic therapy targeting VEGFR2 represents a good alternative for the treatment of tumors (35,36). Our previous studies demonstrated that a quail homologous VEGFR2 protein vaccine effectively induced protective and therapeutic antitumor immunity in several solid and hematopoietic tumor models in mice (20), which suggested that VEGFR2 gene therapy warrants further research.

In the present study, 4T1 cells were infected with the VEGFR2 gene, which synchronously stimulating the immune response to tumor cells and tumor vessels. The increase in $CD4^+$ and $CD8^+$ T lymphocytes in tumors after treatment with the AdVEGFR2-infected cell vaccine showed that cellular immunity was involved in the antitumor immune response, which was further confirmed by the significant inhibition of tumor growth by spleen lymphocyte adoptive transfer. Reportedly, the activation of tumor antigen-specific T-cell immunity involves secretion or surface exposure of the high-mobility-group box 1 (HMGB1) alarmin protein and HSP70 by dying tumor cells (22-25). Our results demonstrated that HMGB1 and HSP70 were upregulated in the irradiated AdVEGFR2-infected 4T1 cells. Thus, cell immunity played an important role in the irradiated infected VEGFR2 whole-cell vaccine treatment.

Collectively, our data in the present study suggest that immunotherapy with AdVEGFR2 whole-cell vaccine was effective for therapeutic antitumor immunity in a breast tumor model. This antitumor effect may result from eliciting the host CTL response against 4T1 cells and tumor vessels. These findings may be of importance in further exploration of the potential application of this vaccine in the treatment of breast cancer.

Acknowledgements

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