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Synergistic tumoricidal effect of combined hPD-L1 vaccine and HER2 gene vaccine

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ABSTRACT

Immunotherapy is gathering momentum as a kind of important therapy for cancer patients. However, monotherapies have limited efficacy in improving outcomes and benefit only in a small subset of patients. Combination therapies targeting multiple pathways often can augment an immune response to improve survival further. Here, the tumoricidal effects of the dual hPD-L1(human programmed cell death ligand 1) vaccination/HER2(human epidermal growth factor receptor 2) gene vaccination immunotherapy against the established HER2-expressed cancers were observed. Animals treated with combination therapy using hPD-L1 vaccine and HER2 gene vaccine had significantly improved survival in a mammary carcinoma model. We observed an increase in tumor growth inhibition following treatment. The percentage of the tumor-free mice (%) was much higher in the combined PD-L1/HER2 group. Furthermore, under the tumor-burden condition, hPD-L1 vaccine enhanced humoral immunity of HER2 gene vaccine. And the combination treatment increased the IFN-γ-producing effector T cells. Additionally, splenocytes from the combined PD-L1/HER2 group immunized mice possessed higher CTL activity. Notably, vaccination with combination therapy induced a significant decrease in the percentage of $CD4^+CD25^+$ Treg cells. Collectively, these data demonstrate that PD-L1/HER2 gene vaccine combination therapy synergistically generates marked tumoricidal effects against established HER2-expressing cancers.

1. Introduction

The human epidermal growth factor receptor-2 (HER2/ErbB-2), is a tumor-associated antigen which is involved in the pathogenesis and progression of many types of human malignancies [1,2]. Many researches have shown that increased HER2 expression not only contributes to the development and progression of human cancers, but also is involved in resistance to chemotherapy, hormone therapy, and radiotherapy. So, molecular strategies that aim to suppress HER2 gene expression or block its function are highly attractive for treating human cancers [3,4]. In 1998, the HER2 membrane receptor-blocking antibody, trastuzumab (Herceptin; Genentech, Inc.), was finally approved by FDA. Since HER2 is selectively overexpressed in cancer cells, and known to be effectively suppressed by antibodies targeting extracellular and intracellular domains, HER2 is also a good candidate for cancer vaccines [5]. Different gene, peptide and/or protein vaccine approaches have been investigated in animal studies and clinical trials in an attempt to develop a potent HER2 vaccine [6–10]. However, in the process of practical study of tumor vaccine, it suffered many kinds of setbacks that the immune systems do not cause tumor regression.

As we know, there are many kinds of mechanism for tumor immune escape [11–14]. For example, the lack of MHC I molecular on the tumor cell surface led to the activation barrier of tumor-specific CTL. Similarly, the lack expression of co-stimulatory molecules B7 family, some cytokines (TGF β) secreted by tumor cells or soluble cytokine receptor analogs all inhibited an effective anti-tumor immune response. In recent years, more and more studies aimed at the effects of the negative immune checkpoints on tumor immune escape. The negative immune checkpoints included CTLA-4, PD-1, the B7 family molecule (PD-L1, PD-L2), and

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$CD4^+CD25^+$Tregs. Their physiological function is to regulate the strength and breadth of the immune response, thus avoiding the damage of normal tissue. Tumor cells are capable of high expressing or inducing the production of immune system "negative regulation point" to escape the attack of immune system. There is growing evidence that blocking one or more "negative regulation point" can produce a lasting and effective anti-tumor immune response. For example, the antibody against CTLA-4, ipilimumab, has been approved by the US Food and Drug Administration (FDA) to treat metastatic melanoma. The antibody against programmed death-1(PD-1), nivolumab and pembrolizumab have been approved by FDA to treat non-small-cell lung cancer.

PD-L1, the homolog of B7.1/2 (CD80/86), shows the ability to co-inhibitory molecules and regulates the immune system [15,16]. The PD-L1 protein is a cell surface glycoprotein which is only expressed on macrophage lineage of cells in normal tissues. Under physiological conditions, the PD-1/PD-L1 signaling pathway inhibits transmission of signals from the activated T cell receptor, plays a key role in self-tolerance [17,18] and prevents T cells from over-activation and tissue damage during infection [19]. The expression of PD-L1 is elevated in many types of cancer and is often correlated with poor patient prognosis. Ligation of PD-L1 on cancer cells with PD-1 on tumor-specific T cells has been demonstrated to suppress T-cell activation and proliferation, and to induce T-cell apoptosis. Tumor cells exploit this regulatory interaction as a mechanism of immune evasion. More recently, the antibody against PD-L1 (MPDL3280A) has been approved by FDA for the treatment of PD-L1-positive NSCLC [20]. Our previous study also showed that the TT-rhPD-L1IgV vaccine induced high-titer anti-PD-L1 antibody, which could inhibit the growth of $PD\text{-}L1^+$SP20 metastatic tumor [21]. We thought that PD-L1 vaccine could not only kill the $PD\text{-}L1^+$tumor cells like other common tumor-associated antigen vaccines, it could block tumor immune escape and increase the body's anti-tumor immune response by blocking PD-1/PD-L1 pathway whether the tumors expressed PD-LI or not.

In this study, we chose breast cancer cell line EMT6 and its syngenic host (the BALB/c mouse) as the model, and we wanted to identify whether the rhPD-L1 protein vaccine could enhanced the immune responses and tumor suppress effect of corresponding tumor antigen through blocking the immune escape of general metastatic tumor. In short, our study confirm that rhPD-L1 protein vaccine and HER2 gene vaccine have synergism effects, rhPD-L1 protein vaccine can increase HER2-specific humoral and cellular immunity responses and then increase the effect of HER2 vaccines against EMT6 tumors. The study provides new idea and evidence for designing tumor vaccine which not only can induce strong tumor-specific immune response, but also can block tumor immune escape.

2. Materials and methods

2.1. Animals

Healthy female BALB/c mice (6–8 weeks old; purchased from the Experimental Animal Center of the Fourth Military Medical University, Xi'an, China) were cared for under institutional animal care protocols in the Experimental Animal Center of the Fourth Military Medical University.

2.2. Cell line

EMT6 is a HER2/neu + mouse breast cancer cell lines that don't express PD-L1. It was propagated in RPMI 1640 medium (Sigma, USA). The tumor cells were obtained from biotechnology center of the Fourth Military Medical University.

2.3. Vaccine

2.3.1. HER2 gene vaccine

In order to obtain the fusion expression vector pRC-CMV-signal peptide, the signal peptide sequence of human immunoglobulin was obtained by oligonucleotide synthesis according to the sequences PTF and PER listed in Supplementary Table 1. The sequences was linked with pRC-CMV which was digested with Hind III and BamH I then the ligation reaction was transformed into the competent *Escherichia coli* cells, amplification and extracted pRC-CMV-signal peptide plasmid.

To obtain the sequence of HER2-ECD(extracellular region of HER2/neu antigen), the $HER2^+$ human breast cancer cell line SKBR3 was used as a template and PCR was carried out with corresponding primers P1 and P2 (Supplementary Table 1). The HER2-ECD segment was gel purified, digested with *Bam*H I and *Xba I*, and clone d into the fusion expression vector pRC-CMV-signal peptide. Large scale preparation of plasmid DNA was carried by Giga kit (Omega, USA) according to the manufacturer's instructions.

2.3.2. PD-L1 protein vaccine

The vaccine hPD-L1 was constructed and expressed in *Escherichia coli* and purified as described in our previous study [30]. In brief, the gene fragment encoded for IgV-like domain of hPD-L1 was amplified by reverse transcription-polymerase chain reaction (RT-PCR) and fusion expressed with TT (T-helper epitope) in *E. coli* and purified as recombinant vaccine for later use.

2.3.3. Grouping and immunization

Grouping: Five groups of 8 BALB/c mice were vaccinated with normal saline control group (control), empty plasmid group (CMV), rhPD-L1IgV protein group, HER2 gene group, and combined PD-L1/HER2 group respectively.

Immunization: Plasmid DNA for immunization consisted of three intramuscular (i.m.) injections of 50 μg of plasmid diluted to a final volume of 50 μl per mouse in final concentrations of 0.9% NaCl [33]. Proteins for immunization were emulsified 1:1 (v/v) in IFA (Sigma-Aldrich). Mice were injected s. c. in the neck region with 40 μg of protein in a total volume of 100 μl[32].

2.3.4. Enzyme-linked immunosorbent assay (ELISA)

The 96-well plates (Costar, USA) were coated with 100 ng rhPD-L1/Fc or rhHER2/Fc protein (R&D, USA). Diluted serum samples were added to duplicate wells and were used as primary antibodies. Goat-anti mouse IgG antibody conjugated to HRP (Zhongshan, China) was added as secondary antibody. After washing, *o*-phenylenediamine (OPD) substrate buffer was added, and the plates were read at 490 nm in an ELISA plate reader. The experiments were repeated at least three times.

2.3.5. ELISPOT assay

IFN-γELISPOT kit (Invitrogen, USA) was used. Splenocytes from vaccinated mice were isolated and restimulated with EMT6 cells (splenocytes: tumor cells, 30: 1) and cultured for 24 h. EMT6 cells were killed by 60 Gy irradiation before and immediately used as antigen presenting cell specifically for HER2. The restimulated splenocytes were then added to anti-mouse IFN-γ mAb precoated 96-well plates (2.5×10^5/well) and further incubated at 37 °Cfor 4 h. The spots were counted using a dissecting microscope. The spot numbers were the mean of triplicates in each vaccinated group.

2.3.6. Cytotoxicity assay

Determination of cytotoxic T lymphocyte (CTL) activity was performed using a colorimetric lactate dehydrogenase (LDH) assay kit (Cayman, USA) according to the manufacturer's manual.

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