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Microencapsulation of tumor lysates and live cell engineering with MIP-3α as an effective vaccine

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The combination of several potential strategies so as to develop new tumor vaccines is an attractive field of translational medicine. Pulsing tumor lysates with dendritic cells (DCs), *in-vivo* attraction of DCs by macrophage inflammatory protein 3α (MIP-3α), and reversion of the tumor suppressive microenvironment have been tested as strategies to develop tumor vaccines. In this study, we generated an alginate microsphere (named PaLtTcAdMIP3α) that encapsulated tumor lysates, live tumor cells engineering with a recombinant MIP-3α adenovirus and BCG. We used PaLtTcAdMIP3α as a model vaccine to test its antitumor activities. Our results showed that PaLtTcAdMIP3α expressed and excreted MIP-3α, which effectively attracted DCs *ex vivo* and *in vivo*. Injection of PaLtTcAdMIP3α into tumor-bearing mice effectively induced both therapeutic and prophylactic antitumor immunities in CT26, Meth A, B16-F10 and H22 models, but without any ensuing increase in adverse effects. Both tumor-specific cellular and humoral immune responses, especially the $CD8^+$ T cell-dependent cytotoxic T immunity, were found in the mice injected with PaLtTcAdMIP3α. The anti-tumor activity was abrogated completely by depletion of $CD8^+$ and partially by $CD4^+$ T lymphocytes. In addition, the number of IFN-γ-producing $CD8^+$ T cells in spleen and tumor tissues was significantly increased; but the number of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) in tumor tissues was decreased. These data strongly suggest that a combination of multi-current-using strategies such as the novel approach of using our PaLtTcAdMIP3α microspheres could be an effective tumor model vaccine.

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1. Introduction

Dendritic cells (DCs) are the most effective antigen-presenting cells which can act as major activators of quiescent T cells in launching an immune response [1–5]. Thus, recent strategies for developing therapeutic tumor vaccines have focused on targeted delivery of tumor antigens to DCs [4–6]. However, the effects of tumor vaccines are still unsatisfactory in the clinic. The major reason for this is likely the difficulty in inducing an effective anti-tumor response in patients with advanced disease; this is due to already established immune tolerance mechanisms that actively disable effector T cells and/or turn off immune recognition in the tumor microenvironment. At present, it is well acknowledgment that whole tumor lysates can offer a universal source of all potential tumor antigens, and that multiple tumor antigens can thereby be targeted at once. Thus, several clinical trials have used whole tumor lysates as a source of antigens for loading DCs, and these showed significant immune responses [3,7–11].

It is well known that suppressive microenvironments created by growing tumor cells become a major barrier for inducing effective antitumor immunity and successful tumor immunotherapy [12–14]. Tumor cells can express immune inhibitory molecules (such as PD-L1 and FasL), secrete suppressive factors (such as TGF-β, IL-10 and IDO), directly induce T-cell apoptosis and proliferation, or even inhibit tumor-specific T-cell expansion [13–16]. In addition, tumor cells are capable of utilizing various strategies to recruit and expand multiple types of suppressive tumor-infiltrating lymphocytes (TILs), including regulatory T cells (Treg), myeloid suppressor cells (MSCs), tumor-derived macrophages and tolerogenic DCs [17–21]. Thus, it is reasonable to believe that if one directly uses

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live tumor cells for vaccine generation, the cells should induce additional immune responses against these various suppressive factors, leading to possible change over of the suppressive microenvironments.

In general, most of the DC vaccine strategies rely on the activation and maturation of DCs *ex vivo*, and their subsequent return to tumor-bearing recipients after being pulsed with tumor lysates or specific antigens [4–6]. These procedures are complex and difficult to standardize. To avoid these disadvantages, an alternative approach is to use the endogenous precursor population through the chemokine-attracted migratory and homing properties to increase the number of endogenous DCs at a tumor site [6,22–24]. Macrophage inflammatory protein-3α (MIP-3α, also called CCL20) is a C–C chemokine and a potent chemoattractant for immature DCs [25,26], Previous studies have used MIP-3α to attract endogenous dendritic cells to tumor tissues, leading to inducing tumor-specific cellular immune response and thereby suppressing the preexisting tumor growth [6,24]. Thus, we hypothesized that by using a combination of tumor lysate, live tumor cells, and *in-vivo* attracting DCs induced by MIP-3α in one approach, the attracting DCs might process both the original tumor and excreting antigens, and therefore induce stronger immunity with consequent suppression of tumor growth. To evaluate this concept, we have made an alginate microsphere (named PaLtTcAdMIP3α) that encapsulated tumor lysates, live tumor cells transfected with a recombinant MIP-3α adenovirus and Bacillus Calmette–Guerin (BCG) in several mouse tumor models. The results demonstrate that this strategy does induce both specific cellular and humoral immunities, and that it reverses the suppressive microenvironment of tumors, resulting in suppression of preexisting tumor growth.

2. Materials and methods

2.1. Cell culture and preparation of tumor lysates

Murine colorectal carcinoma cell line CT26, fibrosarcoma cell line Meth A, hepatoma cell line H22 and melanoma cell line B16-F10 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). These tumor cells were cultured in RPMI1640 or DMEM (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 mg/mL streptomycin and 100 IU/mL penicillin at 37 °C in a humidified atmosphere in 5% CO_2 in compressed air. All the experiments related to the use of tumor cells in this study entailed cells in the logarithmic phase. For preparation of tumor lysates, the tumor cells were quickly washed twice with distilled purified water and then dissolved in 1 mL distilled purified water. Thereafter, these tumor cells were dissolved in 2 cycles of freezing-thawing and then lyophilized using a lyophilizer (FreeZone 4.5, Labconco, Kansas City, MO) and stored at 4 °C.

2.2. Construction of the recombinant MIP-3α adenovirus and preparation of the alginate microspheres

The Adeno-X™ Adenoviral System 3 (Clontech, Mountain View, CA) was used to construct the recombinant MIP-3α adenovirus (AdMIP3α), following the recommended protocols in the manual. The murine MIP-3α gene (intronless ORF) from the ATG to the stop codon was amplified from the expression plasmid pORF5-MIP-3α (Invivogen, San Diego, CA). The AdMIP3α and AdNull (no MIP-3α transgene in the expression cassette as the control) adenoviruses were packaged and propagated in Adeno-X 293 cells and purified by CsCl discontinuous density gradient centrifugation. The typical vector titers determined by Adeno-X qPCR Titration Kit (Clontech Laboratories, Palo Alto, CA) were greater than 1.0×10^{10} IFU/mL.

Alginate microspheres encapsulated various ingredients including tumor lysates, live tumor cells (transfected with or without adenovirus) and BCG for this study were performed similarly to previous studies [27–29]. For the preparation of PaLtTcAdMIP3α, 300 μg tumor lysates, 1×10^6 live tumor cells transfected with 10 MOI AdMIP3α in 0.5 mL PBS and 100 μg BCG vaccine (Chendu Institute of Biological Products, Chendu, China) were suspended in 1 mL each of 2% solution of sodium alginate (Sigma, St. Louis). Thereafter, the mixture of the solution was adjusted to a final concentration of 1.5% alginate. This mixture solution was sprayed into a 250 mM calcium chloride solution at 37 °C with constant agitation. In this way, alginate microspheres were formed. The resultant microspheres were passed through a 200-mesh sieve and then cultured for 24 h to eliminate the larger spheres and the possibly unencapsulated adherent tumor cells. The same method was performed to prepare the microspheres of PaLtTcAdnull (tumor cells transfected with AdNull instead of AdMIP3α), PaLtTc (normal tumor cells instead of transfected tumor cells), PaLt (no tumor cells) and Pa (no tumor cells and no tumor lysates). The morphological features of the microspheres stained with or without Wright–Giemsa (Baso, Taiwan) were observed under a stereomicroscope (SMZ800, Nikon, Tokyo) and an inverted microscope (TS100-F, Nikon).

2.3. Northern and Western blot assays

Northern blot assay was used to detect the MIP-3α mRNA expressed in the microspheres and transfected tumor cells. The CT26 and Meth A tumor cells were used to prepare microspheres and infected with AdMIP-3α or the AdNull adenovirus (each at 10 MOI). After 48 h, total RNA was extracted using the Trizol reagent (Gibco-BRL, Carlsbad, CA) as recommended by the manufacturer's instructions. RNA samples were separated by electrophoresis on a 1% agarose gel containing 0.66 M formaldehyde, transferred to Hybond N^+ membranes and then hybridized with full-length cDNA probes for mouse MIP-3α and β-actin in PerfectHyb Plus hybridization buffer (Sigma–Aldrich, St. Louis, MO).

Western blot assay was performed to evaluate the expression of the MIP-3α protein in the microspheres and transfected tumor cells, and the production of antibodies against tumor cells in the microsphere-treated mice. The CT26 and Meth A tumor cells were used to prepare microspheres and infected with AdMIP-3α or the AdNull adenovirus (each at 10 MOI). Microspheres were first dissolved as lysates using a tissue lyser (QIAgen, Valencia, CA). These lysates from microspheres and tumor cells transfected with or without adenovirus and serum from the injected mouse were separated using 12% SDS-PAGE. Gels were then transfered onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) by a mini trans-blot system (Bio-Rad). Thereafter, the membrane blots were blocked at 4 °C in 5% non-fat dry milk, washed, and probed with antibodies against murine MIP-3α or IgG at a 1:500 dilution. The results were detected using an enhanced chemiluminescence system (Amersham Biosciences, Amersham, UK) as previously reported [30].

2.4. DC chemotaxis assay

Mouse DCs were isolated from bone marrow precursors as described previously [6]. Briefly, erythrocyte-depleted mouse bone marrow cells were harvested and cultured with recombinant mouse GM-CSF (100 U/mL) and IL-4 (20 ng/mL). Non-adherent granulocytes were gently removed on days 2 and 4. On day 6, the loosely adhering proliferating DC aggregates were dislodged and replated. After culture for another 6 days, the non-adherent cells with the typical morphological characteristics of DCs were used for the migration assay. DC chemotaxis assay was performed with pore polycarbonate filters in 24-well Transwell chambers (Corning Costar, Corning, NY) as previously reported [30]. In brief, 5×10^4 DCs in 200 μL of 0.5% BSA medium were added to the top chamber, and the lower parts of the chambers were filled with different percentages of supernatants from various cultured microspheres or tumor cells in a volume of 600 μL. The plates were incubated in a 5% CO_2 atmosphere at 37 °C for 2 h. Thereafter, the filter was washed by HBSS, fixed, stained and assembled on a microscope slide. The chemotactic DCs on the lower chamber was quantified by averaging five high power fields (hpf) under a microscope (80i, Nikon) at 200× magnification.

2.5. Flow cytometric analysis

The expression markers and cytokines on/in DCs or CD4/CD8 T cells were determined by flow cytometry after surface or intracellular stain with antimouse-specific Abs conjugated with either FITC or PE. These Abs were anti-CD4, anti-CD8, anti-CD11c, anti-DEC205, anti-IFN-γ, anti-CD25 and anti-FOXP3, which were purchased from eBioscience (San Diego, CA) or BD Biosciences (Franklin Lakes, NJ). For intracellular staining, the detecting cells were first fixed, permeabilized and stained with corresponding PE- or FITC-conjugated anti-mouse Abs. Flow cytometry was performed with a FACS Canto II (BD Biosciences) and the data were analyzed with BD FCSDiva Software (Franklin Lakes, NJ).

2.6. Tumor models and injection of the microspheres

The animal protocols involved in this study were approved by the Animal Care and Use Committee of Hainan Medical College. Mice at 6–8 weeks of age were subcutaneously injected with 2×10^6 live tumor cells in the right flank to establish tumor models. To evaluate the therapeutic effects, microspheres, including PaLtTcAdMIP3α, PaLtTcAdNull, PaLtTc, PaLt or Pa, were injected into the mouse abdominal cavity of 10 mice in each group on the same day when the tumor models were established. To investigate the prophylactic effect of the microspheres, 10 mice in each group were first injected with PaLtTcAdMIP3α, PaLtTcAdNull, PaLtTc, PaLt or Pa microspheres. Fourteen days later, tumor cells were injected into mice to establish the tumor models. The CT26, Meth A and H22 models were established in BALB/c syngeneic mice; and the B16-F10 models were established in C57BL/6 syngeneic mice. Tumor volumes and survival time were monitored at three-day intervals to evaluate the antitumor effects. Tumor volume was acquired using the formula $V\ (mm^3) = 0.52a \times b^2$, where *a* and *b* represent the largest and the smallest superficial diameters, respectively.

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