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Vaccination with FasL-/TCL plus MHSP65 induces improved anti-lung cancer immunity in mice

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ABSTRACT

In a previous study, we constructed a MHSP65-TCL anti-lung cancer vaccine with Lewis lung carcinoma TCL plus MHSP65, and illustrated its anti-lung cancer effect through specific and nonspecific anti-tumor immunity. However, TCL contains some immunoinhibit components such as FasL. If this component can be eliminated from TCL, the anti-tumor immunity of MHSP65-TCL constructed with TCL should be improved. In the present study, we knocked down FasL from Lewis lung carcinoma cells and prepared MHSP65-(FasL-/TCL) with this cell line's TCL. After further investigation, MHSP65-(FasL-/TCL) exhibited a better ability to reduce splenocytes apoptosis, promote its activation and secretion of secretingTNF-β, IL-2 compared with MHSP65-(FasL + /TCL). Accordingly, specific and nonspecific antitumor immunity induced by MHSP65-(FasL-/TCL) is stronger than that of MHSP65-(FasL + /TCL). In vivo, MHSP65-(FasL-/TCL) immunization can prolong survival of Lewis lung carcinoma bearing mice. Thus, we report that the anti-lung cancer effect of MHSP65-TCL can be improved by removal of FasL from the TCL. It provides a new route to construct MHSP65-TCL and other antitumor vaccines based on TCL.

1. Introduction

Tumor cell lysate (TCL) contains all antigens and proteins present in tumor cells. Therefore, it can activate immune cells and induce anti-tumor immunity [1]. However, it has been shown in our previous studies that TCL may contain certain components that can suppress immune cells. While the cell lysate prepared from Lewis lung carcinoma can activate the lymphocytes in a mouse spleen, it can also induce the early stage apoptosis of lymphocytes. The onset of such apoptosis may be related to the presence of apoptosis-inducing proteins, such as Fas-L, in TCL [2]. Fas-L is an important molecule of the Fas/FasL apoptotic signal transduction pathway. By binding to the Fas receptor on the cell membrane, Fas-L can trigger the intracellular apoptotic cascade and programmed cell death of the target cells [3].

A number of different cells, such as lymphocytes, thymocytes, kidney cells and cardiomyocytes, can induce apoptosis through the Fas/FasL pathway [4–7]. Particularly, the apoptosis of lymphocytes is closely related to the Fas/FasL pathway. In the Fas/FasL pathway, Fas participates in the removing of peripheral self-reactive T-cell clones and the clearance of activated T cells by activation-induced cell death (AICD). With the induction of TCR, T cells are activated while at the same time Fas and FasL were induced to express. The Fas and FasL on adjacent activated T lymphocytes can interact and kill each other, whereas the interaction between the Fas/FasL on the membrane of activated T cells can also lead to the direct apoptosis of T cells.

In addition, FasL can also detach from the cell membrane and form a soluble ligand molecule, then it act on its host cells as well as adjacent activated T cells by autocrine and paracrine signaling, leading to autocrine and paracrine killing of these T cells respectively [8]. Similarly, after the activating of B cells by antigens, the expression level of Fas on their cell surface has also significantly increased, which can result in the killing of these activated B cells by the binding of FasL on the surface of CTL cells [9]. In lpr and gld mice, accumulation of activated B cells in the body can be triggered due to a blocked Fas/FasL mechanism, thus generating a large amount of immunoglobulins that also include self-reactive antibodies that act as a main reason for the onset of autoimmune diseases in these mice [10].

The Fas/FasL signaling system plays an important role in the anti-apoptosis and immune escape of tumor cells. On the one hand, the expression of Fas in tumor cells is lower than normal cells, which abnormally increases the proliferation capability of tumor cells and prevents apoptosis [11]. On the other hand, FasL is expressed in the cells of

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multiple cancers such as lung cancer, liver cancer, colon cancer and esophageal cancer. These tumor cells can attach to T and B lymphocytes or other immune cells by synthesizing and secreting FasL, and further inducing the apoptosis of these immune cells through the Fas/FasL apoptosis pathways. Immune cells apoptosis cannot mount an attack against tumor cells, thus allowing them to escape [12]. Therefore, regulating the expression of Fas or FasL in cancer cells is an effective strategy for treating cancer.

The TCL prepared in this study was a mixture of intracellular proteins from the tumor cells. Therefore, such TCL must contain the FasL synthesized by tumor cells. After being in contact with T and B lymphocytes or other immune cells such as macrophages and dendritic cells, FasL can bind to the Fas expressing on the membrane of these cells and lead to their apoptosis. On the contrary, if we can remove FasL from TCL, the ability of such processed TCL to elicit the apoptosis of immune cells will decrease, whereas its ability to activate immune cells will be enhanced accordingly. Anti-tumor vaccines based on such TCL may have a higher therapeutic efficacy.

In order to validate the above hypothesis, we used FasL knocked down Lewis lung carcinoma cells to prepare TCL and construct a FasL-/TCL-MHSP65 anti-tumor vaccine. Through in vivo and in vitro experiments, the efficacy of this vaccine against lung cancer was compared with a homologous FasL + /TCL-MHSP65 vaccine.

2. Materials and methods

2.1. Animals and cell lines

Female C57BL/6 mice were purchased from Anhui medical university Laboratory Animal Center (Hefei, China) and maintained in a microisolator cage under pathogen-free conditions. All mice were 6–8 weeks of age. Experimental manipulation of the mice was undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Science and Technology of Anhui Province. The mouse Lewis lung cancer cell line was purchased from the Wuhan Boster Biotechnology Co. (Wuhan, China) and cultured in high-glucose Dulbecco's modified Eagle's medium (Hyclone Biotechnology Co., Tianjin, China) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). This study was approved by the Ethics Committee of Wannan Medical College (Wuhu, China).

2.2. Transfection of siRNA

A total of 20%–30% confluent Lewis lung cancer cells were transfected with FasL short hairpin RNA (shRNA) LV or control LV according to the manufacturer's protocols (GeneChem, Shanghai, China). Forty-eight hours after the transfection, the cells were purified with 5 μg/ml puromycin overnight. The Lewis lung cancer cell lines with FasL knockdown (KD) and transfected with control LV (NC) were established. Then the transfection efficiency was evaluated by observing green fluorescence of GFP report gene expression.

2.3. TCL, MHSP65 and MHSP65 plus TCL (MHSP65–TCL)

To prepare TCL, cultured KD, NC or wild Lewis cells were lysed using a freeze-thaw cycle in 1 × phosphate buffered saline (1 × PBS) solution between − 70 °C and 37 °C for five times. Then the prepared TCL was stored in a − 70 °C freezer until use. Each of the TCLs were detected under a microscope (Olympus Corporation, Tokyo, Japan) using trypan blue staining (Sigma-Aldrich, St. Louis, MO, USA) after the final cycle. TCL of the KD, NC and wild Lewis lung cancer cell is named as FasL-/TCL, control/TCL or TCL individually. Mycobacterial heat shock protein 65 (MHSP65)–TCL was prepared by directly mixing MHSP65 (Prospecbio, Ness-Ziona, Israel) with different TCL in vitro. FasL-/TCL plus MHSP65 is MHSP65-(FasL-/TCL), FasL + /TCL plus MHSP65 is MHSP65-(FasL + /TCL), control/TCL plus MHSP65 is MHSP65-(control/TCL).

2.4. Q-PCR

Total RNA was extracted from mouse Lewis lung cancer cells using RNA extraction kit (TIANGEN BIOTECH CO. LTD, Beijing, China) and reverse transcribed to cDNA using a reverse transcription kit (TIANGEN BIOTECH CO. LTD, Beijing, China). Then quantitative real-time PCR (Q-PCR) was conducted with the cDNA to evaluate mRNA expression of FasL or GAPDH in mouse Lewis lung carcinoma cells transfected with control LV (NC), FasL knocked down Lewis lung carcinoma cell (KD) or wild Lewis lung cancer cell using Q-PCR kit (TIANGEN BIOTECH CO. LTD, Beijing, China). The Q-PCR data were analyzed with Q-PCR equipment (ABI Step one, CA,USA).

2.5. Western blot analysis

TCL was prepared from Lewis lung carcinoma cells (1×10^6), FasL knocked down Lewis lung cancer cells, Lewis lung cancer cells transfected with control LV or TCL prepared with wild Lewis lung cancer cell was separated using SDS-PAGE. The protein was transferred from the gel onto a PVDF membrane. The membrane was incubated with an anti-FasL mAb (Abcam Co. LTD, MA, USA) and an anti-rabbit polyclonal IgG-horseradish peroxidase antibody successively (LI-COR Co, LTD. NE, USA). Finally, the membrane was scanned and analyzed using an Odyssey fluorescent scanning system (LI-COR Co, LTD. NE, USA) to detect the protein on the membrane.

2.6. Flow cytometric analysis

Mouse spleen cells (1×10^6/ml) from naive mice were co-cultured with either MHSP65-(FasL + /TCL), MHSP65-(FasL-/TCL), MHSP65-(control/TCL) or 1 × PBS for 48 h. TCL is prepared with wild, FasL knocked down or control LV transfected Lewis lung cancer cell accordingly. The ratio of spleen cells: TCL is 2:1. MHSP65 is 10 μg/ml. Then the cells were collected, washed and re-suspended in 1 × PBS supplemented with 1% heat-inactivated fetal bovine serum. Thereafter, the mouse spleen cells were stained with Annexin V (Kaiji Biotechnology Co., Nanjing, China) to analyze apoptosis or stained with fluorescein isothiocyanate labeled anti-CD69 (QED Bioscience; San Diego, CA, USA) to detect the activation of spleen cells. The cells were then stored in a 4 °C refrigerator for 30 min, washed with 1 × PBS and analyzed by flow cytometry (FACS Calibur; Becton-Dickinson, San Jose, CA, USA). For NK cell activation analysis, spleen cells from naive mice were stimulated as above. The spleen cells of different groups were stained with anti-NK1.1 (QED Bioscience; San Diego, CA, USA) and analyzed by flow cytometry. For DC activation analysis, matured mouse DC were cocultured with MHSP65-(FasL + /TCL), MHSP65-(FasL-/TCL), MHSP65-(control/TCL) or 1 × PBS. The different DC was stained with anti-CD86 and used to process flow cytometry.

2.7. Nonspecific cytotoxicity assay

1×10^6/ml spleen cells of naive mice were cultured in a medium added with 1 × PBS, HSP65-(FasL + /TCL), MHSP65-(FasL-/TCL), MHSP65-(control/TCL) for 48 h, spleen cells: TCL at a ratio of 2:1. MHSP65 is 10 μg/ml. Mouse IL-2 (20 U/ml) was added into the culture at the same time. The spleen cells were used as effector cells. The effector cells were cultured with 5×10^3 Lewis lung cancer cells per well in a 96-well plate at effector/target ratios of 25:1, 50:1 and 100:1 at 37 °C. 24 h later, the supernatant in the culture was discarded and remaining adherent cells were collected, stained with trypan blue and counted. The amount of the remaining live cells was calculated