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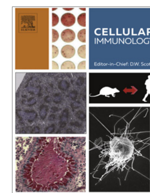

Research paper

Calreticulin acts as an adjuvant to promote dendritic cell maturation and enhances antigen-specific cytotoxic T lymphocyte responses against non-small cell lung cancer cells

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ABSTRACT

Dendritic cell (DC)-based immunotherapy has promising for treatment of non-small cell lung cancer (NSCLC). Melanoma-associated antigen 3 (MAGE-A3) is a tumor-specific antigen and expressed in approximately 35–40% of NSCLC tissues. Calreticulin (CALR) is a protein chaperone and can enhance DC maturation and antigen presentation. In this study, we evaluated the adjuvant activity of CALR in human DC maturation and their capacity to induce MAGE-A3-specific CD8+ cytotoxic T lymphocyte (CTL) responses to NSCLC in vitro. Infection with recombinant Ad-CALR and/or Ad-MAGE-A3, but not with control Ads, induced CALR and/or MAGE-A3 expression in DCs. Infection with Ad-CALR significantly increased the percentages of CD80+, CD83+, CD86+ and HLA-DR+ DCs and IL-12 secretion, but reduced IL-10 production in DCs. Co-culture of autologous lymphocytes with DC-Ad-CALR or DC-Ad-CM significantly increased the numbers of induced CD8+ CTLs. The percentages of IFNγ-secreting CTLs responding to SK-LU-1 and NCI-H522 NSCLC, but not to non-tumor NL-20 cells in Ad-C-CTL, Ad-M-CTL and Ad-CM-CTL were significantly higher than that of DC-CTL and Ad-null-CTL. Ad-C-CTL, Ad-M-CTL and Ad-CM-CTL, but not control DC-CTL and Ad-null-CTL, induced higher frequency of MAGE-A3+HLA-A2+ NCI-H-522 cell apoptosis, but did not affect the survival of MAGE-A3+HLA-A2- SK-LU-1 and non-tumor NL20 cells in vitro. Treatment with anti-HLA-I antibody, but not with anti-HLA-II, dramatically diminished the cytotoxicity of Ad-CM-CTLs against NCI-H522 cells. Our data indicated that CALR acted as an adjuvant to promote DC maturation, which induced CTL development and enhanced MAGE-A3-specific CTL cytotoxicity against NSCLC.

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1. Introduction

Non-small cell lung cancer (NSCLC) is the most prevalent type of lung cancer and the most common cause of cancer-related death [1]. Many patients are usually diagnosed at advanced stage of NSCLC [2]. Although target therapies have significantly prolonged the survival of NSCLC patients the long-term efficacy of current therapies is disappointed. Therefore, development of more effective therapies for NSCLC patients will be of great significance.

Dendritic cell (DC)-based antigen-specific immunotherapies have emerged as promising therapies for NSCLC [3] and have been applied for several types of cancers [4]. Theoretically, cancer-specific antigen is crucial for the efficacy of immunotherapies. Melanoma-associated antigen 3 (MAGE-A3), a cancer/testis antigen, is a member of the melanoma-associated antigen family. MAGE-A3 is highly expressed in 35–55% of NSCLC tissues and is an independent predictor of poor survival in patients with NSCLC [5,6]. Furthermore, MAGE-A3 has high immunogenicity and has been tested as a vaccine with adjuvants of liposomal, CpG, monophosphoryl lipid or QS-21 for immunotherapy in patients with MAGE-A3+ NSCLC [7–10]. However, the clinical efficiency of MAGE-A3-based immunotherapies is unsatisfactory in the clinic. Given that adjuvant is critical for inducing antigen-specific T cell immunity new adjuvant may need to improve the effectiveness of MAGE-A3-based immunotherapies.

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Calreticulin (CALR) is a protein chaperone with glycoprotein- and polypeptide-specific binding sites [11,12]. CALR has been shown to enhance DC maturation and MHC I-based antigen presentation, particularly to CD8+ cytotoxic T lymphocyte (CTL) [13]. A previous study has shown that administration of recombinant CALR enhances tumor immune recognition [14]. Accordingly, we hypothesize that induction of MAGE-A3 and CALR over-expression in DCs can promote DC maturation and antigen-presenting, and induce potent CTL responses against MAGE-A3+ NSCLC.

In this study, we employed recombinant adenoviruses to induce MAGE-A3 and/or CALR expression in DCs and evaluated the effect of adenovirus-mediated MAGE-A3 and/or CALR expression on DC maturation and cytokine production. Furthermore, we tested the efficacy of MAGE-A3 and/or CALR expressing DCs in inducing MAGE-A3-specific CTL and their cytotoxicity against NSCLC in vitro.

2. Materials and methods

2.1. Study participants

Informed consent was obtained from individual participants and the experimental protocol was approved by the Medical Ethics Committee of Liaoning Cancer Hospital and Institute, Shenyang, China. Three healthy female volunteers (24–33 years old, HLA-A*0201) donated their peripheral blood samples.

2.2. Cell lines and culture

Human bronchial epithelial NL20, non-small lung cancer NCI-H522 (HLA-A*0201), and SK-LU-1 (HAL-Aw2432) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute (RPMI1640) medium supplemented with 10% fetal bovine serum (FBS, complete medium, Gibco/Invitrogen, Grand Island, NY, USA) at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Preparation of DCs

Peripheral blood mononuclear cells (PBMCs) were isolated from individual healthy female volunteers using Ficoll-Paque™ PLUS (Haoyang, Tianjin, China). PBMCs (3×10^6 cells/ml) were incubated in RPMI1640 medium for 2 h at 37 °C. The non-adherent cells were collected for isolation of CD8+ T cells and the adherent cells were cultured in complete medium containing recombinant human granulocyte–macrophage colony stimulating factor (GM-CSF; 1000 IU/ml) and interleukin-4 (IL-4; 500 IU/ml; R&D Systems, Minneapolis, USA) for 7 days. Some immature DCs were harvested at five days post incubation for identification by flow cytometry and virus infection, and mature DCs were harvested at 7 days post incubation for subsequent experiments.

2.4. Infection with adenovirus

Recombinant adenoviruses Ad-null, Ad-CALR, Ad-MAGE-A3, and Ad-CALR/MAGE-A3 (serotype 5) were prepared, according to a previous protocol [15]. Briefly, a DNA fragment for CALR was excised from xxx plasmid (company name, city, country) by EcoRI/KpnI digestion and cloned into the plasmid of pShuttle-CMV-EGFP (company name, city, country) to generate pCMV-EGFP-CALR. Furthermore, the pCMV-EGFP-CALR was digested with I-CeuI and I-SceI and the resulting DNA fragment was cloned into the corresponding sites of pAd to generate plasmid of pAd-EGFP-CALR. A similar procedure was used to generate pAd-EGFP-MAGE-A3 using the BglI/XhoI fragment. In addition, the CALR NheI/PmeI DNA fragment containing another CMV promoter was cloned into pAd-EGFP-MAGE-A3 to generate the plasmid of pAd-EGFP-CALR/MAGE-A3. After sequencing, the control pAd-EGFP, pAd-EGFP-CALR, pAd-EGFP-MAGE-A3 and pAd-EGFP-CALR/MAGE-A3 were transfected into HEK 293LP cells, respectively, and the generated Ad-EGFP, Ad-CALR, Ad-MAGE-A3 and Ad-CALR/MAGE-A3 virions were purified by cesium chloride density gradient centrifugation. The infection efficacy of those viruses was determined by EGFP expression.

After culture for five days, immature DCs (1×10^6/well) were cultured in serum-free RPMI1640 in six-well plates and infected in duplicate with Ad-null, Ad-CALR, Ad-MAGE-A3, and Ad-CALR/MAGE-A3 at a multiplicity of infection (MOI) of 100 for 2 h. The cells were then cultured in complete medium containing recombinant GM-CSF (1000 IU/ml) and IL-4 (500 IU/ml) for 48 h.

2.5. Flow cytometry

DCs were collected at 7 days post stimulation and re-suspended in cold phosphate-buffered saline (PBS) and stained with PE-conjugated mouse anti-human CD80, PE-conjugated mouse anti-human CD83, PE-conjugated mouse anti-human CD86, or PE-conjugated mouse anti-human HLA-DR antibody (Abcam, Cambridge, MA, USA). The corresponding isotype control antibody (Abcam) was used as a negative control. After incubation on ice for 40 min, the DCs were washed three times with PBS, and fluorescence was detected using a FACScan (BD Biosciences, Franklin Lakes, USA). Similarly, immature DCs were collected at five days post incubation and stained with PE-anti-CD49, PE-anti-CD1a, PE-anti-CD11c, and PE-anti-CD123, using the same subclass of fluorescent antibodies as the controls. Subsequently, the stained cells were analyzed by flow cytometry analysis (Supplementary Fig. 1).

2.6. Western blot analysis

NL20, SK-LU-1 and NCI-H522 cells were lyzed in radioimmunoprecipitation (RIPA) buffer, and after being centrifuged, their protein concentrations were determined using bicinchoninic acid (BCA) assay (Beyotime, Beijing, China). The cell lysates (20 μg/lane) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 10% gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% fat-free dry milk in TBST, and incubated sequentially with specific primary antibodies, rabbit polyclonal anti-CALR (1:1000), anti-MAGE-A3 (1:200) and anti-β-actin (Abcam). The bound antibodies were detected using HRP-conjugated goat anti-rabbit secondary antibodies (Abcam, 1:10,000) and visualized using enhanced chemiluminescence reagents.

Different groups of DCs were lysed in RIPA buffer, and the cell lysates were subjected to Western blot analysis using anti-CALR and anti-MAGE-A3.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-12 and IL-10 in the supernatants of cultured different groups of DCs were measured using ELISA kits (Biolegend, San Diego, USA), according to the manufacturers' instructions.

2.8. Preparation of MAGE-A3-specific CD8$^+$ CTLs

The non-adherent autologous peripheral blood lymphocytes from individual subjects (1×10^6/well) were co-cultured with individual groups of DCs (5×10^4/well) in 24-well plates in complete medium containing 50 U/ml of IL-2 for 7 days. The half-volume

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