International Immunopharmacology 25 (2015) 416–424

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Anticancer effects of adenovirus-mediated calreticulin and melanoma-associated antigen 3 expression on non-small cell lung cancer cells

Xinli Liu [a,b], Nan Sun [a], Yinan Dong [a], Jijia Li [a], Yang Liu [b], Yi Ren [a], Chengliang Yang [a], Liang Zhang [a], Yang Zhou [a], Zhuang Tong [a], Jianqiao Ding [a], Xiang Zhao [a], Yu Liu [a], Rong He [a], Ke Xu [a], Yongkai Wu [a], Yanfeng Huang [a], Hui Wang [b], Xiujuan Tao [b,**], Yongyu Liu [a,*]

[a] Department of Thoracic Surgery, Liaoning Provincial Tumor Hospital, Shenyang 110042, China
[b] Department of Medical Oncology, Liaoning Provincial Tumor Hospital, Shenyang 110042, China

ARTICLE INFO

Article history:
Received 5 November 2014
Received in revised form 9 February 2015
Accepted 10 February 2015
Available online 19 February 2015

Keywords:
Calreticulin
Melanoma antigen gene-A3
Non-small cell lung cancer
Dendritic cells
CD8 + T cells

ABSTRACT

Non-small cell lung cancer (NSCLC) is highly prevalent and needs novel therapies. Melanoma-associated antigen 3 (MAGE-A3) is a lung cancer antigen and calreticulin (CALR) can modulate immune responses. Our previous study has shown that up-regulated MAGE-A3 and CALR expression inhibits the proliferation and invasion of glioma cells. In this study, we examined the effect of adenovirus (Ad)-mediated MAGE-A3 and/or CALR expression on the proliferation, invasion, and apoptosis of human NSCLC cells and on the vascular tube formation of human endothelial cells as well as on dendritic cell (DC) activation and induced $CD8^+$ cytotoxic T lymphocyte (CTL) activity in vitro. We found that low levels of CALR and MAGE-A3 were expressed by A549 cells, but only very low CALR was expressed by DC. Up-regulated CALR and MAGE-A3 expression by infection with Ad-CALR/MAGE-A3 significantly inhibited the proliferation and invasion, but promoted the apoptosis of A549 cells. Up-regulated CALR and MAGE-A3 expression significantly inhibited cyclin D1 expression and the AKT, ERK1/2 and NF-κB expression and phosphorylation in A549 cells. Up-regulated CALR expression inhibited the tube formation in human endothelial cells. Up-regulated CALR and MAGE-A3 expression synergistically enhanced classical DC activation by enhancing IL-12, but reducing IL-10 secretion. Furthermore, CTLs induced by up-regulated CALR and MAGE-A3 expressing DCs synergistically triggered A549 cell apoptosis, which was abrogated by treatment with anti-HLA I, but not anti-HLA II antibodies. Moreover, CTLs induced by CALR and MAGE-A3-expressing DCs had a higher frequency of A549-specific IFN-γ-secreting T cells. Our data indicated that up-regulated CALR and MAGE-A3 expression inhibited the carcinogenesis of NSCLC by modulating the AKT, ERK MAPK and NF-κB signaling and enhanced classical DC activation and MAGE-A3-specific CTL cytotoxicity. Therefore, our findings may provide new insights in understanding the role of CALR in modulating antigen-specific T cell immunity and may aid in the design of new therapies for NSCLC.

1. Introduction

Lung cancer is one of the common malignant tumors with the highest morbidity and mortality in the world [1]. Lung cancer, particularly for non-small cell lung cancers (NSCLC), has highly genetic and cellular heterogeneity. Currently, there are several targeted therapies available for compelling lung cancer; however, the therapeutic efficacy is limited. New therapeutic strategies are needed and immunotherapy and gene therapy may be valuable for the control of lung cancer [2]. The major challenge of developing a successful therapy is to deliver agents to the tumor in sufficient quantities for a sufficient period to suppress tumorigenesis and induce tumor-specific immune response. Adenovirus (Ad)-mediated gene therapies and immunotherapies have been tested in clinical trials and are attractive strategies to inhibit carcinogenesis and to attack tumor cells [3].

* Correspondence to: Y. Liu, Department of Thoracic Surgery, Liaoning Provincial Tumor Hospital, Shenyang 110042, China.
** Correspondence to: X. Tao, Department of Medical Oncology, Liaoning Provincial Tumor Hospital, Shenyang 110042, China.
E-mail addresses: cinlylau@hotmail.com (X. Liu), nanan1997s@hotmail.com (N. Sun), dongyinan_2012@163.com (Y. Dong), lijijia1982@163.com (J. Li), lyylegend@hotmail.com (Y. Liu), renyi1620@sina.com (Y. Ren), yangliang_0927@163.com (C. Yang), zl750523@sina.com (L. Zhang), janezy1987@163.com (Y. Zhou), 910397562@qq.com (Z. Tong), djq1988@126.com (J. Ding), zhuningzhu13@126.com (X. Zhao), liuyu1984@126.com (Y. Liu), 63788321@qq.com (R. He), 1776119@qq.com (K. Xu), doc.wuyongkai@gmail.com (Y. Wu), huangyanfeng227@163.com (Y. Huang), 550971948@qq.com (H. Wang), liuyang2020@hotmail.com (X. Tao), lyymot@hotmail.com (Y. Liu).

http://dx.doi.org/10.1016/j.intimp.2015.02.017
1567-5769/

Effective delivery of a high immunogenicity of antigen and a potential immunomodulator is crucial for the successful development of new anti-tumor immunotherapies [4,5]. Melanoma-associated antigen 3 (MAGE-A3) is a cancer-testis antigen and highly expressed in different types of tumor tissues [6–8]. MAGE-A3 is expressed in about 35–40% of NSCLC and MAGE-A3-based vaccines have been tested in the clinical trials for the prevention of post-surgical recurrence of MAGE-A3 + NSCLC and other cancers [9–11]. However, the biological function of MAGE-A3, particularly in the pathogenesis of NSCLC, has not been fully understood. Calreticulin (CALR) is an endoplasmic-reticulum (ER) chaperon and can be present on the surface membrane of different types of cells. CALR can bind to calcium and regulate the nuclear receptor-related gene transcription and many cellular functions [12–14]. Its mutation is associated with the development of myeloproliferative neoplasm [15]. CALR can inhibit the growth of prostate cancer [16,17], but promote the growth of pancreatic cancer [18], suggesting that the function of CALR may be tumor type-dependent. CALR can modulate the PI3K/AKT and NF-κB activation in fibrocytes [19]. More interestingly, CALR can modulate antigen-specific immunity [20,21]. Our previous study has shown that up-regulated expression of CALR and MAGE-A3 inhibits the proliferation and invasion of glioma cells [22]. However, the impact of up-regulated CALR and MAGE-A3 expression on the proliferation and invasion of NSCLC has not been clarified. Furthermore, the regulatory role of CALR on the human DC maturation and activation is still unclear. Given that CALR can modulate antigen-specific T cell immunity, we hypothesize that up-regulated CALR and MAGE-A3 expression may inhibit the proliferation and invasion of NSCLC by modulating the AKT, ERK and NF-κB pathways, modulate dendritic cell (DC) activation and induce cytotoxic T cell (CTL) activity.

In the present study, we examined the effects of Ad-mediated CALR and/or MAGE-A3 expression on the proliferation, invasion and apoptosis and the levels of cyclin D1, AKT, ERK and NF-κB expression and phosphorylation in human NSCLC A549 cells. Furthermore, we characterized the impact of these up-regulated molecules on the vascular tube formation in human endothelial cells. Finally, we investigated the effects of up-regulated CALR and MAGE-A3 expression on the activation of human DC and the antigen-specific activity of DC-induced CTLs in vitro. Our data indicated that up-regulated CALR and MAGE-A3 expression inhibited the carcinogenesis of NSCLC by modulating the AKT, ERK MAPK and NF-κB signaling and enhanced classical DC activation and MAGE-A3-specific CTL cytotoxicity.

2. Materials and methods

2.1. Cell lines and cell culture

NSCLC A549 and human umbilical vein endothelial cells (HUVECs) were purchased from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). A549 and HUVECs were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS) and in Kaighn's modification of Ham's F-12 medium (F-12 K, Lifetechologies, Grand Island, USA) supplemented with 0.1 mg/mL heparin, 0.03-0.05 mg/mL endothelial cell growth supplement, and 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2, respectively.

2.2. Adenovirus and infection

Ad-vector, Ad-CALR, Ad-MAGE-A3 and Ad-CALR/MAGE-A3 vectors were preserved in our laboratory. A549 cells (2×10^6/well) were infected in triplicate with null (buffer only), Ad-vector, Ad-CALR, Ad-MAGE-A3 and Ad-CALR/MAGE-A3 at 100 multiplicity of infection (MOI) for 48 h.

2.3. Cell proliferation assay

The impact of CALR or/and MAGE-A3 expression on A549 cell proliferation was determined by methyl-thiazolyl-tetrazolium (MTT) assay. Briefly, A549 cells (1×10^4 cells/well) were cultured in 96-well plates overnight and infected in triplicate with null (control), Ad-vector, Ad-CALR, Ad-MAGE-A3 or Ad-CALR/MAGE-A3 (MOI = 100) for 24–96 h. During the last 4 h incubation, the cells were exposed to MTT (20 μl/well, 5 mg/mL, Sigma, St Louis, USA) and the resulting formazan was solubilized in 150 μL/well of dimethyl sulfoxide (Sigma), followed by detecting the absorbance at 490 nm in a microplate reader (Bio-Tek ELX800, USA). The inhibition rate was calculated.

2.4. Invasion assay

The impact of CALR or/and MAGE-A3 expression on A549 cell invasion was determined by a transwell invasion assay. Briefly, the infected A549 cells (2×10^4 cells/well) were cultured in triplicate in FBS-free medium for 12 h in the top chamber of 24-well transwell plates (8 μm pore, Corning, USA) that had been coated with Matrigel (BD Biosciences, San Jose, USA). The bottom chambers were added with 10% FBS RPMI-1640 and the cells were cultured for 48 h. The cells invaded to the bottom surface of the top chambers were stained with crystal violet. The numbers of invaded cells in five fields (magnification x 100) selected randomly were counted under a light microscope in a blinded manner.

2.5. Tube formation assay

HUVEC cells (2×10^5/well) were cultured in triplicate in the conditional medium from the different groups of infected A549 cells for 48 h in 96-well plates that had been coated with Matrigel. The tube formation in individual wells was quantified by counting the numbers of connected cells in randomly selected fields (magnification × 100).

2.6. Preparation of dendritic cells

Peripheral venous blood samples were collected from two healthy donors from the Liaoning Cancer Hospital and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-paque™ PLUS (Haoyang, Tianjin, China). PBMCs (1×10^7 cells/flask) were cultured in 10% FBS RPMI-1640 medium at 37 °C 5% CO_2 for 2 h and the non-adherent cells were removed. The adherent cells were cultured in RPMI-1640 medium supplemented with recombinant granulocyte-macrophage colony stimulating factor (GM-CSF, 1000 IU/ml) and recombinant interleukin-4 (IL-4, 500 IU/ml; R&D, USA) for 7 days and exposed to fresh medium every 2–3 days. The DCs were characterized longitudinally by flow cytometry and the generated mature DCs were used for subsequent experiments.

2.7. Infection of DCs with adenovirus

The immature DCs were collected on day 5 post stimulation and DCs (1×10^6 cells/well) were infected with null, Ad-vector, Ad-CALR, Ad-MAGE-A3, or Ad-CALR/MAGE-A3 at a MOI of 100 in FBS-free medium for 2 h, followed by culturing in 10% FBS RPMI 1640 medium containing recombinant GM-CSF (1000 IU/ml) and IL-4 (500 IU/ml) for 48 h.

2.8. Western blot analysis

The infected A549 cells were lysed in radioimmunoprecipitation (RIPA) buffer, heated and centrifuged. After the quantification of protein concentrations by a bicinchoninic acid (BCA) assay (Beyotime, China), the cell lysates (20 μg/lane) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were washed, blocked with 5% fat-free dry milk, and incubated with rabbit polyclonal antibodies against CALR (1:1000), MAGE-A3 (1:200), NF-κB (1:400), Cyclin D1 (1:10000), phosphorylated (p)-Akt (1:500), Akt (1:1000), Erk1/2 (1:1000), p-Erk1/2 (1:100), β-actin (1:1000, Abcam, Cambridge, USA) and NF-κBp65 (1:1000, Cell Signaling, USA) overnight at 4 °C. After being washed, the bound