Vaccine 26 (2008) 6433–6440

Contents lists available at ScienceDirect

Vaccine

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

Modification of CEA with both CRT and TAT PTD induces potent anti-tumor immune responses in RNA-pulsed DC vaccination

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ARTICLE INFO

Article history:
Received 2 April 2008
Received in revised form 20 August 2008
Accepted 25 August 2008
Available online 21 September 2008

Keywords:
Calreticulin
HIV TAT
Dendritic cells
Carcinoembryonic antigen
Anti-tumor immunity

ABSTRACT

Carcinoembryonic antigen (CEA) is expressed on human colon carcinomas, is well characterized, and continues to be a promising target for cancer immunotherapy in humans. To enhance the immunogenecity of CEA, we developed a fusion gene (CRT-TAT-ΔCEA) of the TAT protein transduction domain (PTD) and calreticulin (CRT) with human CEA devoid of its signal sequences (ΔCEA) and evaluated anti-tumor immunity using RNA-pulsed dendritic cell (DC) vaccination. Mice vaccinated with DC by electroporation with mRNA encoding TAT-ΔCEA (DC/TAT-ΔCEA) and CRT-ΔCEA (DC/CRT-ΔCEA) had enhanced induction of tumor-specific cytotoxic T lymphocyte (CTL) and increased numbers of IFN-γ-secreting T cells by ELISPOT, as compared to mice vaccinated with DC/ΔCEA. DC/CRT-ΔCEA and DC/TAT-ΔCEA vaccines preferentially stimulated $CD4^+$ and $CD8^+$ T cells, respectively. The DC vaccine by electroporation with mRNA encoding CRT-TAT-ΔCEA (DC/CRT-TAT-ΔCEA) enhanced both $CD4^+$ and $CD8^+$ T cells. DC/CRT-TAT-ΔCEA had the additional effects of CRT and TAT PTD and enhanced the anti-tumor effect against CEA-expressing tumors compared to DC/CRT-ΔCEA or DC/TAT-ΔCEA. These findings suggest that modification of CEA with both CRT and TAT PTD induces potent anti-tumor immune responses in RNA-pulsed DC vaccination and may be a useful approach for DC-based immunotherapy.

1. Introduction

Carcinoembryonic antigen (CEA) is a 180 kDa oncofetal glycoprotein and a well-known soluble tumor marker. It is extensively expressed in colorectal, gastric, and pancreatic carcinomas, and in approximately 50% of breast cancers and 70% of non-small cell lung cancers [1]. Presumably, proteins such as CEA, which are also present in normal tissues, are recognized as self-antigens by the immune system, and thus most individuals, including cancer patients, are immunologically tolerant to these tumor-associated antigens (TAA). CEA is expressed in more than 95% of colon carcinomas, and thus constitutes a good potential target for immunotherapy.

Vaccines using recombinant CEA (rCEA) produced in vaccinia or baculovirus vectors have been used in several studies [2–4]. We previously reported that dendritic cells (DC) transduced with recombinant adenoviruses could induce antigen-specific cytotoxic T lymphocyte (CTL) responses *in vitro* [5] and anti-tumor immunity to challenge with murine colon carcinoma cells expressing human CEA *in vivo* [6]. Moreover, patients with colon cancer immunized with rCEA mount CTL against tumor cell-pulsed CEA peptides [7]. It has been shown that DC pulsed with peptides of TAA, whole cell lysates or mRNA trigger tumor-specific responses [8–10]. mRNA-based vaccines give several conceptual advantages relative to other forms of antigens. RNA transfection has been shown to have a similar or superior efficiency compared with recombinant virus vector transduction without the problems associated with viral vectors [11,12]. We and others have shown that DC transfected with RNA via electroporation effectively receive and process mRNA, then efficiently induce antigen-specific CTL responses [13–15].

CEA contains signal peptides that target proteins through the endoplasmic reticulum (ER) and the cell membrane. It has been hypothesized that the intracellular retention of truncated CEA (ΔCEA), devoid of its signal peptides, enhances MHC class I presentation of CEA peptides, thus favoring cellular immune responses. It has recently been reported that the ΔCEA protein accumulates better in aggregates with a perinuclear or cytoplasmic

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doi:10.1016/j.vaccine.2008.08.072

distribution than full-length CEA protein containing signal peptides [16].

Many investigators have begun to explore the utility of HIV TAT protein transduction domain (TAT-PTD) as a primary DC-based tumor vaccine. It has been demonstrated that HIV TAT-PTD-containing whole protein antigen-transduced DC stimulate antigen-specific $CD8^+$ and $CD4^+$ T cells [17,18]. In addition, calreticulin (CRT) stimulates the immune system to induce both protective immune responses and therapeutic tumor rejection [19,20]. CRT are resident ER proteins that bind transiently to many newly synthesized glycoproteins as they pass through the ER [21,22]. Previous studies have shown that pulsing of DC with soluble, exogenous CRT yields presentation of CRT-derived peptides in association with DC class I molecules and lysis by an MHC-restricted CTL line [23,24].

In this study, we investigated whether the modification of CEA with CRT and TAT PTD could enhance CEA-specific immune responses, as well as anti-tumor immunity by RNA-electroporated DC vaccine. Our results showed that the combination of CRT-TAT-ΔCEA exhibited a dramatic increase in CEA-specific CTL responses and an impressive anti-tumor effect against CEA-expressing tumors compared with CRT-ΔCEA or TAT-ΔCEA.

2. Materials and methods

2.1. Plasmid DNA constructs and preparation

For the generation of pcDNA3-CEA, CEA was amplified with a reverse transcriptase-polymerase chain reaction (RT-PCR) using LoVo cell cDNA as the template and a set of primers (5′-CGAAGAATTCATGGAGTCTCCCTCGGCCCC-3′ and 5′′-GCGCCAATTCC TATATCAGAGCAACCCCAACC-3′). The amplified products were then cloned into the EcoRI site of the pcDNA3 vector (Invitrogen Corp., Carlsbad, CA, USA). For the generation of pcDNA3-ΔCEA and pcDNA3-TAT-ΔCEA, the ΔCEA and TAT-ΔCEA were amplified with PCR using pcDNA3-CEA and pcDNA3-TAT-CEA as the template and a set of primers (5′-CCGGGGATCCAAGCTCACTATTGAATCCACG-3′ and 5′-TGCGCGGCCGCCT ATATCAGAGCAACCCCAACC-3′). The amplified products were cloned into the BamHI/NotI site of the pcDNA3 vector (Invitrogen Corp.). ΔCEA is the designation for a signal peptide-deleted form of the full CEA gene. For the generation of pcDNA3-CRT-ΔCEA and pcDNA3-CRT-TAT-ΔCEA, the CRT was first amplified with PCR using murine DC cDNA as the template and a set of primers (5′-ACGAAGCTTACATGCTCCTTTCGGTGCC-3′ and 5′-TGCGGATCCCTACAGCTCATCCTTG GCTTGG-3′). The amplified product was then cloned into the HindIII/XbaI sites of pcDNA3-ΔCEA and pcDNA3-TAT-ΔCEA vectors. The accuracy of the DNA construct was confirmed by DNA sequencing.

2.2. Mice and cell lines

Female, 6–8-week-old C57BL/6 mice were purchased from Orient Bio (Kapyung, Kyounggi, Korea). We performed all experiments in accordance with the guidelines of the local Council for Animal Care. During experimentation, the mice were maintained in a pathogen-free animal facility with controlled humidity and temperature under a 12-h light/dark cycle. All animals were acclimated to the environment for at least 1 week before the experiments were conducted.

The MC38/CEA2 adenocarcinoma cell line was kindly provided by Dr. J. Schlom (Division of Tumor Immunology and Biology, NIH, Bethesda, MD, USA). The murine colon adenocarcinoma cells expressing human CEA (MC38/CEA2) were generated by retroviral transduction of MC-38 cells with CEA cDNA [25]. The EL-4 and YAC-1 lymphoma cell lines were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). The EL-4 and YAC-1 cells were used as control targets in the CTL assays. The MC38/CEA2 and EL-4 cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM; GibcoBRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. To enhance the expression of MHC class I, MC38/CEA2 cells were treated with 50 U/ml of murine IFN-γ (Endogen Inc., Cambridge, MA, USA) for 48 h at 37 °C before re-stimulation and analysis for cytotoxicity. The YAC-1 cells were cultured in complete RPMI 1640 (GibcoBRL) medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

2.3. Generation of DC from bone marrow

Primary bone marrow DC were obtained from mouse bone marrow precursors as previously described [6]. Briefly, bone marrow was prepared from the femur and tibia by flushing with media. The tissue pieces were chopped through a nylon mesh into a single-cell suspension. Next, erythrocytes were lysed by resuspending the cell pellet in an ammonium chloride potassium carbonate buffer (ACK lysis buffer, 0.15 M NH_4Cl, 10.0 mM $KHCO_3$ and 0.1 mM EDTA) and incubating for 10 min on ice. The cells were washed twice in serum-free RPMI 1640 media and cultured in a six-well plates at 5×10^6 cells/well with RPMI 1640 media containing 20 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D System, Minneapolis, MN, USA) and 20 ng/ml recombinant murine interleukin-4 (IL-4; R&D System). On day 2, non-adherent granulocytes were gently removed, and fresh cytokine media was added. On day 6 of culture, the non-adherent cells were considered to be immature bone marrow-derived DC. They were harvested, washed, and placed in 100 mm Petri dishes at 10^6 cell/ml for 24 h in the presence of fresh GM-CSF, IL-4, and 1 μg/ml LPS (Sigma, St. Louis, MO, USA) to allow the DC to fully mature. The phenotypes of DC were characterized by cell-surface markers using fluorescently labeled monoclonal antibodies and quantified using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). The cells were stained with the following antibodies: CD3 (145-2C11), CD4 (GK1.5), CD8a (53-6.7), CD11b (M1/70), CD11c (HL3), CD14 (rmC5-3), CD19 (1D3), CD40 (HM40-3), CD54 (3E20), CD80 (16-10A1), CD86 (GL1), MHC class I (SF-1.1), MHC class II (2G9), and NK-1.1 (PK136; PharMingen, San Diego, CA, USA).

2.4. RNA electroporation to DC

For *in vitro* transcription, pcDNA3-EGFP, pcDNA3-CEA, pcDNA3-ΔCEA, pcDNA3-TAT-ΔCEA, pcDNA3-CRT-ΔCEA, and pcDNA3-CRT-TAT-ΔCEA plasmids were linearized with Sma I, purified by phenol/chloroform extraction and ethanol precipitation, and used as DNA templates [26]. *In vitro* transcription was performed using T7 RNA polymerase (mMESSAGE mMACHINE kit; Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA quality was verified by agarose gel electrophoresis. RNA concentration was measured spectrophotometrically and was stored at −70 °C.

DC were harvested from the cell factories and washed once with serum-free RPMI 1640 medium at room temperature. The cells were resuspended in OptiMEM without phenol red (Invitrogen Life Technologies, Grand Island, New York, USA) at 5×10^6 cells/ml. 20 μg of RNA was transferred to a 2 mm cuvette. A column of 200 μl of the cell suspension was electroporated with a square-wave pulse (300 V for 500 μs) in a Gene-pulser (BTX, San Diego, CA, USA). Immediately after electroporation, the cells were transferred to autologous medium supplemented with 1 μg/ml of LPS.