Vaccine 29 (2011) 9361–9367

Contents lists available at SciVerse ScienceDirect

Vaccine

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

Increasing vaccine potency through exosome antigen targeting☆

Zachary C. Hartman[a,1], Junping Wei[a,1], Oliver K. Glass[a], Hongtao Guo[a], Gangjun Lei[a], Xiao-Yi Yang[a], Takuya Osada[a], Amy Hobeika[a], Alain Delcayre[d], Jean-Bernard Le Pecq[d], Michael A. Morse[b], Timothy M. Clay[a,c], Herbert K. Lyerly[a,*]

[a] Duke Comprehensive Cancer Center, Department of Surgery, Duke University Medical Center, Durham, NC 27710, USA
[b] Department of Medicine, Division of Medical Oncology, Duke University Medical Center, Durham, NC 27710, USA
[c] Department of Immunology, Duke University Medical Center, Durham, NC 27710, USA
[d] ExoThera L.L.C, Menlo Park, CA, USA

ARTICLE INFO

Article history:
Received 4 May 2011
Received in revised form
22 September 2011
Accepted 30 September 2011
Available online 12 October 2011

Keywords:
Cancer vaccines
Exosomes
C1C2 domain
Tumor antigens
Adenovirus vectors
CEA
HER2

ABSTRACT

While many tumor associated antigens (TAAs) have been identified in human cancers, efforts to develop efficient TAA "cancer vaccines" using classical vaccine approaches have been largely ineffective. Recently, a process to specifically target proteins to exosomes has been established [1] which takes advantage of the ability of the factor V like C1C2 domain of lactadherin to specifically address proteins to exosomes. Using this approach, we hypothesized that TAAs could be targeted to exosomes to potentially increase their immunogenicity, as exosomes have been demonstrated to traffic to antigen presenting cells (APC) [2]. To investigate this possibility, we created adenoviral vectors expressing the extracellular domain (ECD) of two non-mutated TAAs often found in tumors of cancer patients, carcinoembryonic antigen (CEA) and HER2, and coupled them to the C1C2 domain of lactadherin. We found that these C1C2 fusion proteins had enhanced expression in exosomes *in vitro*. We saw significant improvement in antigen specific immune responses to each of these antigens in naïve and tolerant transgenic animal models and could further demonstrate significantly enhanced therapeutic anti-tumor effects in a human HER2+ transgenic animal model. These findings demonstrate that the mode of secretion and trafficking can influence the immunogenicity of different human TAAs, and may explain the lack of immunogenicity of non-mutated TAAs found in cancer patients. They suggest that exosomal targeting could enhance future anti-tumor vaccination protocols. This targeting exosome process could also be adapted for the development of more potent vaccines in some viral and parasitic diseases where the classical vaccine approach has demonstrated limitations.

1. Introduction

In most infectious diseases, soluble or particle antigens are circulating in the blood and can easily be captured by the professional antigen presenting cells (APC). Vaccines delivering these antigens in a native or inactivated form associated with proper adjuvant typically elicit a very potent immune response. This classical vaccine approach has been used widely and successfully applied in human and animal populations for preventing deadly diseases.

Unfortunately, this classical approach shows very little efficacy in diseases where antigens remain mainly localized inside cells, such as in some viral and parasitic diseases. The identification of tumor associated antigens (TAA) in human cancers [3,4] triggered an enormous effort in the medical and scientific community to develop "cancer vaccines". Except in the case where viral antigens could be identified [5], the delivery of TAA in various forms by various vectors in association with a variety of adjuvants has led, up to now, to rather disappointing results [6,7]. These studies have revealed several specific difficulties with this type of approach. A primary difficulty is that most TAA are cell-associated and probably not delivered efficiently to professional APCs. While TAA can include proteins [3,4] that are coded by the host and overexpressed in cancer cells (HER2, wild type p53), some are expressed in fetal development (CEA) and/or select tissues, but not widely expressed in adult life such as the cancer–testis antigen family. Others are expressed with somatic mutations (RAS, p53) or translational modifications (MUC1). However, in general, they are poorly antigenic or expressed in an immunosuppressive environment. "Cancer vaccines" are supposed to be used mainly

Abbreviations: TAA, tumor associated antigens; APC, antigen presenting cells; ECD, extracellular domain; CEA, carcinoembryonic antigen; HER2, human epidermal growth factor receptor 2; MUC1, mucin 1; CDC, complement-dependent cytotoxicity; bmDC, bone marrow derived dendritic cells; MVB, multivesicular bodies.

☆ The work was performed at: Duke University Medical Center, Durham, NC 27710, USA.

* Corresponding author at: 203 Research Drive, Rm 433B Box 2606 Durham, NC 27710, USA. Tel.: +1 919 681 8350.
E-mail address: kim.lyerly@duke.edu (H.K. Lyerly).

[1] Both are co-first authors.

0264-410X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2011.09.133

as therapeutic vaccines when a state of TAA immune-tolerance is established, a situation quite different from that encountered in classical vaccines where xenogeneic antigens are delivered to naïve individuals. Although new immunomodulatory reagents that may reverse tolerance in advanced cancer patients are being developed for cancer immunotherapy, strategies to enhance the potency of cancer vaccines to break established tolerance are essential for vaccines that may be given to a wide range of cancer patients

The lactadherin C1C2 domain is a lipid binding domain, related to the C1C2 domain of factor V. It is responsible for the specific addressing of lactadherin to exosomes as deletion in this domain abolishes exosome addressing [1,8]. It has recently been shown that soluble proteins including intracellular proteins, when fused to the C1C2 domain of lactadherin are no longer found intracellularly but are released in extracellular compartment, almost exclusively associated to exosomes [1,9]. As exosomes transfer intracellular antigens directly to antigen presenting cells (APCs) [2], it was proposed that targeting intracellular antigens to exosomes would increase their trafficking to APCs and therefore stimulate their immunogenicity [1]. These principles were tested in a recent study that compared the tumorigenicities of ovalbumin antigen expressing cells [9]. In the study, the malignant cells expressing the C1C2-lactadherin domain ovalbumin fusion protein released the ovalbumin protein bound to exosomes in contrast to cell expressing unmodified ovalbumin. The authors also found that cells expressing the C1C2-fusion albumin were strikingly less tumorigenic if tested in immune-competent mice but kept their tumorigenity in immune-suppressed mice. Furthermore, some animals treated with cells expressing C1C2-ovabulbin fusion could become immune against the cells expressing the unmodified ovalbumin. These results suggest that despite a tumor cell environment, the C1C2 fusion protein could induce an effective anti-tumor immune response most likely mediated by trafficking to exosomes [9].

In order to test the possibility of using this targeting strategy to improve the potency of vaccines, we generated recombinant adenoviral vectors expressing the extracellular domain (ECD) of carcinoembryonic antigen (CEA) or HER2 linked to the C1C2 domain of lactadherin in addition to native unlinked ECD versions of CEA and HER2. We tested the efficacy of these viruses using mice made transgenic for these antigens to mimic the state of immune-tolerance found in human patients. We found that adenoviral expression of a C1C2 modified CEA/ECD and HER2/ECD resulted in significantly higher protein expression in exosomal fractions compared to non-targeted CEA in both murine cell lines and antigen presenting cells. We also found that secreting the ECD of CEA or HER2 *in vivo* as a vesicle-associated form was superior in inducing antigen specific immune responses in naïve and tolerant animals and enhanced anti-tumor immune responses. Our results thus provide insight into the low immunogenicity of soluble TAAs in cancer patients and suggest new means to improve anti-tumor immune responses for vaccines targeting cancer or potentially other diseases.

2. Materials and methods

2.1. C1C2 cloning and Ad vector construction

Briefly, the extracellular domain of either human CEA (nt 1-2025) or human HER2/neu (nt 1-1953) were inserted into the mouse Lactadherin expression plasmid p6mLC1C2 as described [1] to create exosomal cassettes containing the leader signal and C1C2 domains of mouse lactadherin fused in-frame to the respective constructs. Vectors were created using the pAdEasy system [10] and all stocks titered using AdEasy viral titer kit (Stratagene, Santa Clara, CA).

2.2. In vivo experiments

C57BL/6J and BALB/c mice were obtained from Jackson Labs (Bar Harbor, MA), human CEA-transgenic mice were a kind gift from Jeff Schlom (National Cancer Institute, Bethesda, MD), and HER2 transgenic mice were obtained from Dr. Wei-Zen Wei (Wayne State University, Detroit, MI). Adenoviral vectors were administered via the footpad at indicated times of 4–12-week-old mice. All animal work was performed in accordance with Duke IACUC approved protocols.

2.3. ELISPOT and antibody procedures

Mouse IFN-gamma ELISPOT assay (Mabtech Inc., Cincinnati, OH) was performed according to according to published methods [11]. Briefly, harvested splenocytes were stimulated with CEA peptide mix (2.6 μg/ml: BD Bioscience, San Jose, CA), HER2 overlapping peptide mixtures (1 μg/ml of 15mer peptides overlapping by 11 amino acids for HER2) or irrelevant HIV gag or CMV pp65 antigen controls (2.6 μg/ml: BD Bioscience, San Jose, CA). PMA (50 ng/ml) and Ionomycin (1 μg/ml) were used as positive controls for splenocyte responsiveness. Anti-HER2 IgG antibodies were detected by FACS analysis of BT474 and SKBR3 HER2+ cells using PE-conjugated anti-Mouse IgG (Dako, Cat # R0480) as a secondary detection antibody. CEA ELISA used recombinant CEA (TriChem Resources, Inc., West Chester, PA, 10ug/ml) as the capture antigen and an anti-mouse IgG secondary antibody (Jackson Immunoresearch, West Grove, PA) as the detection antibody.

2.4. In vitro assays

The human BT474 and SKBR3 HER2+ breast cancer lines and the murine JAWSII DC lines were obtained, tested for contamination (cellular and mycoplasma), and maintained according to ATCC recommendations. The human-HER2 expressing 4T1 mouse mammary tumor line (4T1-HER2) was kindly provided by Dr. Michael Kershaw (Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia). Murine bone marrow derived dendritic cells (bmDCs) were prepared and cultured with standard methods using GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) (PeproTech, Rocky Hill, NJ).

For assessment of exosome protein expression, cleared supernatants were harvested and exosomes concentrated using standard ExoQuick (SBI Biosciences, Mountain View, CA) procedures [12]. Briefly, cleared supernatants (centrifuged at $3000 \times g$ for 15 min) were filtered using a .45um PVDF filter and mixed with ExoQuick precipitation solution and incubated overnight at 4 °C. After incubation, precipitated exosomes were centrifuged at $1500 \times g$ for 30 min at 4 °C following ExoQuick protocols. Concentrated exosomal fractions were resuspended in then quantified used a BCA assay (Thermo Fisher, Rockford, IL), and subjected to western blot analysis using anti-CEA (Cell Signaling Technology, Danvers, MA) and tsg101 as an exosome marker loading control (Abcam, Cambridge, MA) antibodies.

For exosome-capture ELISA to measure CEA and HER2 protein levels, supernatants from transduced cells were used in an anti-CD81 cross-capture ELISA [13] with monoclonal anti-CD81 (BioLegend, San Diego, CA) as a capture antibody and a polyclonal anti-CEA rabbit Ab (AbCam, Cambridge, MA) or an anti HER2 (N-terminal) rabbit Ab (Santa Cruz Biotechnology, Santa Cruz, CA), and an anti-rabbit HRP linked antibody (AbCam, Cambridge, MA) as the detection antibody set. In all experiments, uninfected control