Biomaterials 156 (2018) 194–203

Contents lists available at ScienceDirect

Biomaterials

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

Co-delivery of human cancer-testis antigens with adjuvant in protein nanoparticles induces higher cell-mediated immune responses

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ARTICLE INFO

Article history:
Received 14 September 2017
Received in revised form
7 November 2017
Accepted 19 November 2017
Available online 20 November 2017

Keywords:
Nanoparticle
Cancer-testis antigen (CT)
NY-ESO-1
MAGE-A3
HLA-A2
Cancer vaccine

ABSTRACT

Nanoparticles have attracted considerable interest as cancer vaccine delivery vehicles for inducing sufficient $CD8^+$ T cell-mediated immune responses to overcome the low immunogenicity of the tumor microenvironment. Our studies described here are the first to examine the effects of clinically-tested human cancer-testis (CT) peptide epitopes within a synthetic nanoparticle. Specifically, we focused on two significant clinical CT targets, the HLA-A2 restricted epitopes of NY-ESO-1 and MAGE-A3, using a viral-mimetic packaging strategy. Our data shows that simultaneous delivery of a NY-ESO-1 epitope (SLLMWITQV) and CpG using the E2 subunit assembly of pyruvate dehydrogenase (E2 nanoparticle), resulted in a 25-fold increase in specific IFN-γ secretion in HLA-A2 transgenic mice. This translated to a 15-fold increase in lytic activity toward target cancer cells expressing the antigen. Immunization with a MAGE-A3 epitope (FLWGPRALV) delivered with CpG in E2 nanoparticles yielded an increase in specific IFN-γ secretion and cell lysis by 6-fold and 9-fold, respectively. Furthermore, combined delivery of NY-ESO-1 and MAGE-A3 antigens in E2 nanoparticles yielded an additive effect that increased lytic activity towards cells bearing NY-ESO-1^+ and MAGE-$A3^+$. Our investigations demonstrate that formulation of CT antigens within a nanoparticle can significantly enhance antigen-specific cell-mediated responses, and the combination of the two antigens in a vaccine can preserve the increased individual responses that are observed for each antigen alone.

1. Introduction

Boosting a patient's immune system by immunotherapy represents a promising approach in cancer treatment [1], and cancer vaccines in particular enable the recognition of tumor-associated antigens for targeted destruction [2]. Although these cancer vaccines have been shown to elicit CD8 T cell immune responses, the typical response levels generated are usually insufficient to overcome the low immunogenicity and immunosuppressive microenvironment of tumors [3,4]. For example, in a review of clinical studies, an overall objective response rate (50% tumor size reduction) of only 2.6% was observed with peptide vaccines derived from gp100, MART-1, TRP-2, cancer testis NY-ESO-1, MAGE-A12, or HER2 antigens (alone or in combination with adjuvant); these results suggest the need for development of more effective strategies and therapies [1].

The application of nanotechnology has shown exceptional promise towards improvement of cancer diagnosis and treatment in recent years [5–7]. Antigen uptake by dendritic cells (DCs) depends on the antigen properties such as geometry [8], surface charge [9], and importantly, size [10,11]. Some nanoparticles have the advantage of being in the optimal size for DCs uptake and passive transport to the lymphatic system, with prior research demonstrating that particles between approximately 20-45 nm are taken up more effectively by the DCs residing in the lymph-nodes [8,12,13]. Therefore, delivery of vaccine components with these nanoparticles may facilitate and increase DC interaction, resulting

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https://doi.org/10.1016/j.biomaterials.2017.11.022
0142-9612/

in stronger immune responses [14,15].

One nanoparticle with favorable DC uptake properties is the E2 nanoparticle [15–17]. E2 is a 25-nm non-viral protein nanoparticle derived from the pyruvate dehydrogenase complex of *Bacillus stearothermophilus* [18]. It is composed of 60 identical monomer subunits that self assemble into a highly thermostable dodecahedral caged structure with a hollow 12-nm cavity [19], and it can be engineered at the internal, external, and inter-subunit interfaces to change the properties and functionality of the nanoparticle [20–23]. Our research group has also previously demonstrated that the viral-mimetic, simultaneous delivery of adjuvant (internally packaged) and MHC-I restricted antigens (bound on the surface) via E2 nanoparticles resulted in an increase in DCs activation and antigen cross-presentation [16]. This was associated with a significant increase in antigen-specific IFN-γ secretion, tumor cell lysis, delayed B16-F10 tumor growth, and increased survival time in C57BL/6 mice [15]. Cell uptake and biodistribution of E2 have also been reported [17].

The target epitopes in this current study are HLA-A2 restricted peptide sequences from New York esophageal squamous cell carcinoma-1 (NY-ESO-1) and melanoma antigen family A, 3 (MAGE-A3) [24]. While other tumor-associated antigens (TAAs) are often expressed at low levels in healthy tissue, expression of CTs is restricted only to cancer cells and the immune-privileged cells in the testis. Furthermore, CT antigens exist in a high proportion of different human tumors such as melanoma, bladder, lung, prostate, and breast cancers [25,26]. In particular, NY-ESO-1 is expressed in 82% of neuroblastomas and 46% melanomas [27] while MAGE-A3 is also expressed in 76% of melanoma cancers [28]. A phase II clinical trial of NY-ESO-1/ISCOMATRIX vaccine which was recently completed in June 2017 (NCT00518206, ClinicalTrials.gov) resulted in 4% partial response (based on a standard of 30% reduction in tumor size), 48% stable disease, and 48% progressive disease; this result highlights the generation of response to NY-ESO-1, but also the need and potential for development of alternative strategies that will yield more effective therapies.

Given the wide range of tumors that express CT antigens, their relatively high level in cancer, their restricted expression, and their potential for vaccine improvement, the CT class of antigens is an important and significant clinical target. In this study, we examined the feasibility of using the E2 nanoparticle to induce cell-mediated immune responses against NY-ESO-1 and MAGE-A3 in a mouse model that is transgenic for the human major histocompatibility complex, HLA-A2. While our prior work examined gp100, an antigen that has mouse and human analogues, this current study focuses on antigens that are specifically expressed in humans.

We also investigate the extent of cell-mediated and cytolytic responses by simultaneous delivery of NY-ESO-1- and MAGE-A3-containing nanoparticles. Tumor escape after single-epitope vaccination is common since cancers often lose expression of the targeted antigen to evade the immune system [29]. Immunization with combined antigens can possibly decrease the risk of tumor escape resulting from antigen loss [30,31]. Furthermore, increasing the number of different antigen targets in a vaccine can induce a broader range of T cell responses simultaneously, which could be effective in a higher number of patients. Because there is a lack of immune-competent murine tumor models expressing these CT antigens to examine *in vivo* anti-tumor efficacy in the most physiologically relevant way possible, we examined lytic ability *ex vivo* using human cancer cell lines expressing both NY-ESO-1 and MAGE-A3. To our knowledge, our study is the first to test the efficacy of cell-mediated responses to clinically-relevant CT peptide epitopes formulated as a nanoparticle, and it examines these human epitopes in nanoparticles both individually and in combination.

2. Methods

2.1. Materials

Reagents were purchased from Fisher Scientific unless otherwise noted. Complete RPMI used in this study for splenocytes was compromised of RPMI 1640 (Mediatech) with 10% heat-inactivated FBS (Hyclone), 1 mM sodium pyruvate (Hyclone), 100 μg/ml of streptomycin (Hyclone), 0.1 mM nonessential amino acids (Lonza), 2 mM L-glutamine (Lonza), and 100 units/ml penicillin. Cancer cell lines used in this study were cultured in DMEM media (Sigma) supplemented with 10% heat-inactivated FBS (Hyclone).

2.2. Peptides and CpG

CpG 1826, a bacterial DNA ligand for TLR9, was purchased from Invivogen, and 5′ benzaldehyde-modified CpG 1826 with a phosphorothioated backbone was synthesized by Trilink. The NY-ESO-1 and MAGE-A3 peptide epitopes were synthesized by Genscript or Genemed Synthesis (Table 1). Peptides were synthesized both with and without an N-terminal cysteine; the thiol on the cysteine-modified peptides was used for conjugation to E2, whereas peptides with no cysteine were used as controls. In this study, the abbreviation (*e.g.,* NYESO, MAGE) refers to the peptide, while the names NY-ESO-1 and MAGE-A3 refer to the whole protein.

2.3. E2 purification and characterization

In this study, we used the D381C mutant of the E2 nanoparticle, which has an aspartic acid-to-cysteine mutation at position 381 in the internal hollow cavity of the nanoparticle. The cysteine of D381C can be used for site-directed conjugation, and this nanoparticle is abbreviated as “E2” in this study. Expression, purification, and characterization of E2 (D381C mutant) were performed as previously described [19]. In summary, *E. coli* strain BL21(DE3) containing the E2 gene was cultured in Luria-Bertani medium containing 100 μg/ml ampicillin. Expression was induced by adding 1 mM of IPTG when the culture reached the optical density of 0.6–0.9 measured at 600 nm. Cells were harvested and stored at −80 °C. Cells were lysed using a French pressure cell (Thermo Scientific), and the insoluble fraction was removed by centrifugation. The soluble fraction was heated at 70 °C for 20 min. Denatured native *E. coli* protein aggregates were removed by centrifugation. The recovered supernatant was loaded to a HiPrep Q Sepharose anion exchange column followed by a Superose 6 size exclusion column [19]. Purity and the molecular weight of purified E2 were confirmed with SDS-PAGE and electrospray ionization mass spectrometry. Dynamic light scattering and transmission electron microscopy were used to check the size, assembly, and monodispersity of the particles. As previously described, lipopolysaccharide was removed using Triton X-114 (Sigma) extraction, and endotoxin levels were evaluated using an LAL ToxinSensor kit (Genscript) [16].

2.4. CpG and peptides conjugation

CpG 1826 modified with a 5′-benzaldehyde was attached to the TCEP-reduced cysteines in the internal cavity of E2 nanoparticles using a N-β-maleimidopropionic acid hydrazide (BMPH) linker. The average number of CpG molecules conjugated to the internal cavity of E2 nanoparticle was estimated with intensity analysis in ImageJ software, using standardized concentrations [16]. Peptides with N-terminal cysteines were conjugated to the native lysines on the surface of the E2 nanoparticle by mixing the nanoparticle with a sulfo-SMCC linker in the presence of a 10-fold excess of TCEP-