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Systemically administered gp100 encoding DNA vaccine for melanoma using water-in-oil-in-water multiple emulsion delivery systems

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ABSTRACT

The purpose of this study was to develop a water-in-oil-in-water (W/O/W) multiple emulsions-based vaccine delivery system for plasmid DNA encoding the gp100 peptide antigen for melanoma immunotherapy. The gp100 encoding plasmid DNA was encapsulated in the inner-most aqueous phase of squalane oil containing W/O/W multiple emulsions using a two-step emulsification method. *In vitro* transfection ability of the encapsulated plasmid DNA was investigated in murine dendritic cells by transgene expression analysis using fluorescence microscopy and ELISA methods. Prophylactic immunization using the W/O/W multiple emulsions encapsulated the gp100 encoding plasmid DNA vaccine significantly reduced tumor volume in C57BL/6 mice during subsequent B16-F10 tumor challenge. In addition, serum Th1 cytokine levels and immuno-histochemistry of excised tumor tissues indicated activation of cytotoxic T-lymphocytes mediated anti-tumor immunity causing tumor growth suppression. The W/O/W multiple emulsions-based vaccine delivery system efficiently delivers the gp100 plasmid DNA to induce cell-mediated anti-tumor immunity.

1. Introduction

DNA vaccines are plasmid constructs intended to express an encoded protein antigen following *in vivo* administration and subsequent transfection of cells and tissues of interest (Liu, 2011). A gene-based vaccine offers molecular precision with flexibility to induce immune responses against multiple target antigens. Moreover, additional genes encoding for immuno-stimulatory molecule(s) included in a plasmid DNA construct can modulate magnitude and longevity of antigen-specific immunity (Thomas and Steven, 2010). DNA vaccination is a promising cancer treatment and prevention strategy devised on the premise that it would sustain expression of the encoded tumor antigens. This may further enhance *in vivo* sensitization and activation of the T-cells that are capable of recognizing the tumor antigens on malignant cell surface. DNA vaccines offer distinct advantages over traditional vaccines (killed or attenuated pathogens) as well as more recently developed subunit vaccines. Unlike most subunit vaccines, DNA vaccines induce both humoral and cellular immune responses (Allison and Byars, 1991). However, despite its ability to induce potent immune responses, DNA vaccination requires large doses of genetic material in non-human primates and humans (Calarota et al., 1998; Wang et al., 1998). Additionally, concerns on biosafety of the viral vectors based gene therapy have motivated attempts to develop alternative, non-viral means of gene delivery to somatic cells *in vivo*.

To elicit potent immune response a DNA vaccine must be delivered and transported across cell membrane of the antigen presenting cells (APCs) in sufficient concentration while protected from nuclease mediated destruction (Allison and Byars, 1991). Polar and anionic nature of a plasmid DNA molecule does not readily allow transfer across the biological membranes (Kaufman, 2012). Therefore, one of the major challenges in DNA vaccination is optimal gene delivery to target immune cells. The W/O/W multiple emulsions are particulate delivery systems wherein the dispersed oil-droplets containing an internal aqueous-phase are internalized by immune cells (Attarwala and Amiji, 2012; Dupuis et al., 1998) thereby potentially transfect them with encapsulated plasmid DNA. In addition, emulsions-based vaccines have potential to induce protective immunity against protein antigen(s) encoded by encapsulated plasmid DNA (Barut et al., 2005; Guo and Wang, 2011; Yoo et al., 2006; Yu and Vajdy, 2011). The advantages offered by the W/O/W multiple emulsions-based plasmid DNA delivery systems include: (1) physical protection of the labile payload from enzymatic degradation, (2) gene delivery to immune cells by non-viral means, (3) alter or modify transgene expression levels

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over time by multiple gene delivery regimen, (4) modification of oil-droplets surface chemistry for immune-cell specific delivery, and (5) simultaneous delivery of multiple candidate therapeutic agents. Squalene oil-based emulsions demonstrated potent and safe vaccine adjuvant effects in several studies (Kahn et al., 1994; Langenberg et al., 1995; Nitayaphan et al., 2000; Pass et al., 1999) due to particulate-adjuvant effect of the oil-droplets and immune-adjuvant effect of squalane oil. Squalane is a fully saturated analog that is prepared by hydrogenation of squalene (Rosenthal, 2002) derived from shark liver. The W/O/W multiple emulsions were prepared using squalane oil because it offers increased stability against auto-oxidation.

The gp100 glycoprotein is a melanoma differentiation antigen expressed in most melanoma cells (de Vries et al., 1997) and recognized by immune cells including melanoma-derived tumor infiltrating lymphocytes (Bakker et al., 1994). Therefore, gp100 protein is considered as a candidate tumor associated-antigen (TAA) to induce an effective immune response in melanoma. Immunization of C57BL/6 mice with plasmid DNA encoding gp100 melanoma antigen induced T-cell dependent immune responses and provided protection against subsequent tumor challenge (Hawkins et al., 2000; Rakhmilevich et al., 2001; Wagner et al., 2000; Yang et al., 2011). In this study, we have investigated *in vitro* transfection ability of the W/O/W multiple emulsions encapsulated EGFP encoding plasmid DNA in murine dendritic cells. Additionally, *in vivo* effectiveness of the W/O/W multiple emulsions encapsulated the gp100 encoding plasmid DNA vaccine was tested in murine B16 melanoma model. Tumor growth suppression was evaluated in immunized C57BL/6 mice and compared with that in control, placebo, and empty vector treated groups to determine extent of tumor suppression due to vaccination. Moreover, involvement of cell-mediated anti-tumor immunity was confirmed by analyzing serum Th1 cytokine levels and local infiltration of CD4+ and CD8+ cytotoxic T-lymphocytes (CTLs) at tumor site.

2. Materials and methods

2.1. Human gp100 encoding plasmid DNA construct

The human gp100 (hgp100) protein encoding plasmid DNA was constructed by ligating hgp100 cDNA (SILV) fragment into the multiple cloning site of the OmicsLink™ Expression Clone (Genecopoeia Inc., Rockville, MD). The enhanced green fluorescent protein (EGFP) encoding plasmid DNA and the empty vector contained the expression clone inserted with EGFP expressing cDNA or scrambled sequence fragment respectively into the multiple cloning sites. The plasmid DNA transformed *Escherichia coli* bacterial cultures were purchased from GeneCopoeia, Inc. (Rockville, MD). The plasmid DNA amplification, purification, and isolation from bacterial stock cultures was performed using QIAGEN Plasmid Mega kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions.

2.2. Characterization of human gp100 encoding plasmid DNA

The hgp100 encoding plasmid DNA was characterized for cDNA and expression vector backbone size by restriction enzyme digestion followed by agarose gel-electrophoresis. The hgp100 encoding cDNA was cut from expression clone using combination of XmnI/XhoI and NspV/XhoI restriction enzymes (XmnI and XhoI: New England BioLabs Inc., Ipswich, MA; NspV: Takara Bio Inc., Shiga, Japan) according to manufacturer's instructions. The resulting linear DNA fragments were electrophoresed on an agarose gel (0.8% E-Gel, Invitrogen, Carlsbad, CA) pre-stained with ethidium bromide, and visualized under ultraviolet radiation according to manufacturer's instructions. The 1Kb Plus and a 100 bp DNA Ladders (both from Invitrogen) were used as a molecular ruler.

2.3. Plasmid DNA encapsulation in W/O/W multiple emulsions

The human gp100 plasmid DNA encapsulated W/O/W multiple emulsions vaccine formulation was prepared by a two-step emulsification method previously described by Okochi and Nakano (2000) and Shahiwala and Amiji (2008). The human gp100 plasmid DNA was dissolved in the internal aqueous-phase containing water (3 mg/mL) followed by emulsification with squalane oil (Jedwards International, Quincy, MA) containing 10% (w/v) Span 80™ (Sigma Chemicals, Inc., St. Louis, MO) to a final concentration of 3.3% (w/v) in the W/O/W multiple emulsions. A primary W/O emulsion was prepared using a homogenizer (Model: L4RT-A; Silverson Machines, East Longmeadow, MA) at 10,000 rpm for 5 min. The resulting primary emulsion was further emulsified with the outer aqueous-phase comprising 0.5% w/v Pluronic® F127 (BASF Corporation, Florham Park, NJ) solution using the homogenizer at 10,000 rpm for 10 min to create the W/O/W multiple emulsions. Dispersed oil-droplets size and surface charge was determined using Zetasizer ZS instrument (Malvern Instruments Ltd, Malvern, UK). To determine encapsulation efficiency, 24 h after preparation dispersed oil-droplets were separated from the outer continuous phase by centrifuge filtration and dissolved in iso-propyl alcohol (Acros Organics, Thermo Fisher Scientific Geel, Belgium). The precipitated gp100 plasmid DNA was centrifuged down, the pellet was dissolved in water and concentration was measured using NanoDrop 2000 (Thermo Scientific, Wilmington, DE). For subsequent studies plasmid DNA encapsulated the W/O/W multiple emulsions were prepared freshly, stored at 4 °C and used within 24 h. For *in vitro* transfection study the W/O/W multiple emulsions was prepared using the same method, but the gp100 encoding plasmid DNA was replaced with the EGFP encoding plasmid DNA.

2.4. In vitro evaluations of DNA delivery and transgene expression

2.4.1. Cell line and culture method

In vitro transfection ability of the W/O/W multiple emulsions encapsulated plasmid DNA encoding EGFP reporter gene was investigated in primary murine dendritic cell line (Astarte Biologics LLC, Redmond, WA). The cells were cultured in Dulbecco's Modified Eagle's Medium (GIBCO) supplemented with 10% fetal bovine serum (Gemini Bio-Products) and 5 ng/mL recombinant murine GM-CSF (PeproTech Inc., Rocky Hill, NJ).

2.4.2. In vitro transfection in murine dendritic cells

Approximately 200,000 murine dendritic cells were cultured in each well of a 6-well plate (BD Biosciences, San Jose, CA) for 12 h. After this period serum containing medium was replaced with 40 μL of plasmid DNA containing formulation (20 μg per well) diluted in 960 μL of serum free medium. Incubation was continued at 37 °C for 6 h to allow sufficient uptake of formulation by the cells. The five different treatment groups included: (1) serum free medium (negative control), (2) naked EGFP plasmid, (3) W/O/W squalane-oil multiple emulsion (SME) control, (4) EGFP plasmid:Lipofectin® (Invitrogen) complex (positive control), and (5) EGFP plasmid encapsulated in W/O/W SME. After 6 h formulation containing medium was replaced with DMEM supplemented with 10% fetal bovine serum and 5 ng/mL recombinant murine granulocyte-macrophage colony stimulating factor (GM-CSF). The cell lines were continued to incubate at 37 °C followed by qualitative and quantitative transgene expression analysis at 24 h, 48 h, 72 h, and 96 h.