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Original Articles

P5 HER2/neu-derived peptide conjugated to liposomes containing MPL adjuvant as an effective prophylactic vaccine formulation for breast cancer

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ABSTRACT

Vaccines containing synthetic peptides derived from tumor-associated antigens (TAA) can elicit potent cytotoxic T lymphocyte (CTL) response if they are formulated in an optimal vaccine delivery system. The aim of this study was to develop a simple and effective lipid-based vaccine delivery system using P5 HER2/neu-derived peptide conjugated to Maleimide-PEG2000-DSPE. The conjugated lipid was then incorporated into liposomes composed of DMPC:DMPG:Chol:DOPE containing Monophosphoryl lipid A (MPL) (Lip-DOPE-P5-MPL). Different liposome formulations were prepared and characterized for their physicochemical properties. To evaluate anti-tumoral efficacy, BALB/c mice were immunized subcutaneously 3 times in two-week intervals and the generated immune response was studied. The results demonstrated that Lip-DOPE-P5-MPL induced a significantly higher IFN-γ production by CD8+ T cells intracellularly which represents higher CTL response in comparison with other control formulations. CTL response induced by this formulation caused the lowest tumor size and the longest survival time in a mice model of TUBO tumor. The encouraging results achieved by Lip-DOPE-P5-MPL formulation could make it a promising candidate in developing effective vaccines against Her2 positive breast cancers.

Introduction

Despite many decades of research on the cancer treatment, cancer is still a major cause of death. Chemotherapy, radiotherapy and surgery are current treatments for cancers, however chemotherapy destroys cells indiscriminately and radiotherapy and surgery are not able to prevent metastases. Due to the disadvantages of current treatments for cancers, tumor immunotherapy has been paid attention during two past decades [1]. Since humoral immunity has a low potential to eliminate solid tumors individually, induction of an effective cell-mediated immunity based on the activation of cytotoxic T lymphocytes (CTLs), namely $CD8^+$ T cells, is aimed in cancer immunotherapy [2,3].

Vaccines containing synthetic peptides derived from tumor-associated antigens (TAA) can elicit potent CTL response if they are formulated optimally. Ag-presenting cells (APCs) mainly dendritic cells (DCs) present peptide antigens to T cells ($CD4^+$ and $CD8^+$) via MHC molecules and initiate immune responses to infectious diseases and tumors [4,5]. Exogenous peptide antigens which are taken up by DCs, pass the endocytic pathway and they are generally presented to $CD4^+$ T cells on MHC class II molecules whereas endogenous antigens enter into the cytosol, load onto MHC class I molecules in the endoplasmic reticulum and are finally presented to $CD8^+$ CTLs [6] .Therefore, efficient delivery of TAAs to DCs, endosomal escape of antigens to the cytosol and activation of CTLs via MHC class I presentation are crucial to induce an effective immune response leading to tumor regression.

Nanoparticle delivery systems carrying antigens have the potential for achieving all the above mentioned goals. Liposomes can offer several advantages over other particulate systems. Basically, liposomes are safe and well-tolerated carriers. They are also completely biodegradable and versatile to be formulated with different lipid constituents, all types of peptide antigens and adjuvants to induce a robust cell-mediated immunity [7,8].

Adjuvants in liposomal vaccine formulations can enhance and prolong immune responses [9]. Among different adjuvants, Monophosphoryl lipid A (MPL) has been used frequently as an efficient adjuvant in liposomal vaccines. MPL has shown adjuvant activity in both cellular and humoral immunity [10]. MPL is a non-toxic derivative from LPS or endotoxin that drives immunity

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responses via TLR4 stimulation [11,12]. FDA approved MPL as a safe adjuvant for human vaccines [13].

Through developing tumor-specific peptide vaccines, various TAAs have been targeted for cancer immunotherapy. As a TAA, HER2/neu protein has provided an opportunity to develop breast cancer vaccines. HER2/neu is a 185 kDa transmembrane glycoprotein and member of the epidermal growth factor receptor family overexpressed in 20–40% of primary breast cancers [14,15].

In our previous study, four peptides containing MHC class I restricted multi-epitope from rat HER2/neu protein were designed by *in silico* analysis and the effectiveness of these peptides was evaluated by administration to BALB/c mice. As results showed that two of these peptides (p5 and p435) were effective in inducing CTL responses, it was hypothesized that encapsulating P5 or P435 in lipid carriers may enhance CTL immune responses more than peptides alone. Encapsulating peptides in LPD (liposome-polycation-DNA) nanoparticles included DOTAP as a cationic lipid and CpG ODN as an immune-stimulatory adjuvant confirmed the hypothesis [16]. However, LPD is a complex carrier and PS-type CpG ODN at high dose may elicit systemic toxicity [17].

For these reasons, in the present study, we utilized liposomes composed of DMPC:DMPG:Chol:DOPE containing MPL for efficiently introducing P5 peptide to cytosol of APCs and generating a strong CTL response. In our earlier challenging study, we developed an optimized procedure for encapsulating P5 peptide in the inner cavity of liposomes by passive loading [18]. As encapsulation efficiency was low, in this study, P5 peptide (ELAAWCRWGFLLALLPPGIAGGGC) was covalently conjugated to Maleimide-PEG_{2000}-DSPE to improve peptide incorporation into liposomes. The effectiveness of liposomal formulation of P5 peptide in the induction of CTL response was evaluated in BALB/c mice and in TUBO *in vivo* tumor mice model, which overexpresses the HER2/neu oncogene.

Materials and methods

Materials

Peptide P5 (ELAAWCRWGFLLALLPPGIAGGGC, purity > 95%) was synthesized by ChinaPeptides Co. (Shanghai, China). Dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphoglycerol (DMPG), dioleoylphosphatidylethanolamine (DOPE) and distearoylphosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (Maleimide-PEG2000-DSPE) were purchased from Avanti Polar Lipid (Alabaster, USA). Cholesterol and Monophosphoryl lipid A from *Salmonella enterica* (MPL) were purchased from Sigma-Aldrich (Steinheim, Germany). Cytofix/Cytoperm™ Plus, PMA/ionomycin cocktail, anti-CD8a-PE-cy5, anti CD4-PE-cy5, anti-IFN-γ- FITC and anti-IL-4-PE antibodies were purchased from BD Biosciences (San Diego, USA). All other solvents and reagents were used as chemical grade.

Animal and cell lines

Four to six week old female BALB/c mice were purchased from Pasteur Institute (Tehran, Iran). The experimental protocols were approved by the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences in accordance with animal welfare guidelines.

TUBO, a cloned cell line that overexpresses the rHER2/neu protein, was kindly provided by Dr. Pier-Luigi Lollini (Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy) and was cultured in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 20% fetal bovine serum (FBS). A murine colon carcinoma cell line, CT26, was purchased from Pasteur Institute (Tehran, Iran) and cultured in RPMI-1640 medium supplemented with 10% FBS.

Conjugation of P5 peptide to PEG_{2000}-DSPE

P5 peptide was conjugated to Maleimide-PEG_{2000}-DSPE through covalent binding between the thiol group of cysteine residue of peptide and the pyrrole group of maleimide. Peptide was reacted with Maleimide-PEG_{2000}-DSPE in a molar ratio of 1.2:1 (peptide:maleimide) in DMSO:chloroform (1:1) solution at room temperature for 24 h. Thin layer chromatography (TLC) was used to confirm the formation of P5-PEG_{2000}-DSPE. A TLC plate (silica gel 60 F254, Merck, USA) was placed in a TLC chamber containing mobile phase composed of chloroform, methanol, and water at 90:18:2 (v/v). The chamber was saturated with iodine vapor to stain the TLC plate. The conjugation of peptide with PEG_{2000}-DSPE was also ascertained indirectly by determining unconjugated peptide fraction using HPLC. KNAUER smart line HPLC (Berlin, Germany) was equipped with a Nucleosil C18, 5 μm, 150 × 4.6 mm, 100A° column (KENAUER) and an UV detector (KENAUER S2600) set at 220 nm. The mobile phases employed were A (water + 0.1% TFA) and B (acetonitrile + 0.1% TFA). Elution program was a gradient starting with 100% A and increasing to 30% B in 2 min, 60% B in 10 min and 90% B in 2 min. The flow rate was set to 1 ml/min.

Liposome preparation

Liposomes (Lip-DOPE) composed of DMPC:DMPG:Chol:DOPE at a molar ratio of 30:4:6:10 were prepared using lipid film hydration method. Control liposomes (Lip) were also prepared in the same molar ratio as above without using DOPE. Lipids were first dissolved in chloroform and then they were combined in sterile glass tubes. The required amount of MPL and P5-PEG_{2000}-DSPE conjugate was added to the lipid solutions to prepare liposomes containing P5 peptide and MPL (Lip-P5-MPL). The lipid solutions were dried to a thin film by rotary evaporation (Heidolph, Germany) under reduced pressure. Films were freeze-dried (VD-800F, Taitech, Japan) overnight to remove the solvents completely. Lipids were then hydrated in HEPES buffer (10 mM, pH 7.2) containing 5% dextrose, vortexed and bath-sonicated to disperse completely the lipids into the buffer. The resulting multilamellar vesicles (MLVs) were extruded using a mini extruder (Avestin, Canada) to form 100 nm small unilamellar vesicles (SUVs) with a uniform size. The final formulations contained 0.1 mg/ml P5 peptide and 0.25 mg/ml monophosphoryl lipid A in liposome with a lipid concentration of 50 mM.

Liposome characterization

The P5 peptide content in liposomal formulations was determined by the same HPLC method as described in "Conjugation of P5 Peptide to PEG_{2000}-DSPE." Liposome preparations were disrupted with 1.5% (v/v) $C_{12}E_{10}$ detergent and then assayed to determine MPL content by an LAL chromogenic endpoint assay (QCL-1000, Lonza, Walkersville, MD) [19]. The amount of total lipids was determined based on phospholipids by using a phosphorus assay method [20]. Vesicle size, polydispersity index and zeta potential of liposomes were determined by dynamic light scattering (Malvern Instruments, Malvern, UK). Liposomes were stored at 4 °C under argon.

Animal immunization and splenocyte collection

BALB/c mice (10 per group) were immunized with different liposomal formulations three times at two-week intervals subcutaneously. The liposome dose of 5 μmol per mouse was used for each injection. Free P5 peptide (10 μg/mouse) and HEPES-dextrose buffer were used as control groups.

Two weeks after the last booster, the mice (four per group) were sacrificed and their splenocytes aseptically collected to evaluate cellular immune responses.

Enzyme-linked immunospot (ELISpot) assays

ELISpot assays were carried out using mouse ELISpot kits from U-cytech (Utrecht, The Netherlands) according to the manufacturer's instruction. Briefly, one day before mice sacrifice, ELISpot 96-well plates were coated with anti-IL-4 and anti-IFN-γ antibodies and incubated overnight at 4 °C. Splenocytes were cultured in triplicate wells in a final volume of 200 μl with medium containing P5 peptide (10 μg/ml) in precoated plates. Splenocytes were incubated for 24 h at 37 °C in tissue culture incubator. When spots appeared, counting was done with Kodak 1D image analysis software (Version 3.5, Eastman Kodak, Rochester, New York).

Intracellular cytokine assay via flow cytometric analysis

Splenocytes (10^6 cells/ml) in medium containing GolgiPlug™ (1 μl/ml) was stimulated with PMA/ionomycin cocktail (2 μl/ml) for 4 h at 37 °C. After stimulation, 10^5 splenocytes were transferred into flow cytometry tubes and washed two times with stain buffer (2% FCS in PBS). Splenocytes were stained with 1 μl anti-CD8a-PE-cy5 antibody and 1 μl anti CD4-PE-cy5 antibody in separate tubes for 30 min at 4 °C. The cells were washed with stain buffer and fixed using Cytofix/Cytoperm™ solution. Fixed cells were washed two times with Perm/Wash™ buffer and then stained with 1 μl anti-IFN-γ- FITC antibody for 30 min at 4 °C. CD4 cells were also stained with 1 μl anti-IL-4-PE antibody. The cells were washed with Perm/Wash™ buffer and suspended in 300 μl stain buffer for flow cytometric analysis (BD FACSCalibur™, BD Biosciences, San Jose, USA).

In vitro CTL assay

Two weeks after the last booster, splenocytes were isolated from four mice per group and re-stimulated in vitro with P5 peptide (10 μg/ml) and recombinant IL-2 (20 U/ml) for 5 days. After stimulation, Splenocytes, as effector cells, were transferred to U-bottomed plates in triplicate wells. TUBO tumor cells in DMEM-20% were incubated with 12.5 μM Calceine AM at 37 °C for one hour in the dark [21]. After removing the excess dye, TUBO cells (2×10^4), as target cells, were added to splenocytes and incubated at 37 °C for 4 hours in the dark. Culture medium only and medium

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