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Synthetic vaccine nanoparticles target to lymph node triggering enhanced innate and adaptive antitumor immunity

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ABSTRACT

In this study, synthetic vaccine nanoparticles (SVNPs) that efficiently targeted lymph nodes, where immune responses against foreign antigens are primed, were developed to enhance antitumor immunity. The size (20–70 nm) and surface character (amination) of poly(γ-glutamic acid)-based SVNPs were selected for effective loading and delivery (*i.e.*, migration and retention) of model tumor antigen (OVA) and toll-like receptor 3 agonist (poly (I:C)) to immune cells in lymph nodes. Antigen-presenting cells treated with SVNP-OVA and SVNP-IC showed higher uptake of OVA and poly (I:C) and higher secretion of inflammatory cytokines (TNF-α, IL-6) and type I interferon (IFN-α, IFN-β) than those treated with OVA and poly (I:C) alone. *In vivo* analysis revealed higher levels of activation markers, inflammatory cytokines, and type I IFNs in the lymph nodes of mice immunized with SVNP-IC compared to those of mice in other groups. SVNP-IC-treated mice showed significantly greater *in vivo* natural killer cell expansion/activation ($NK1.1^+$ cells) and $CD8^+$ T cell response ($CD8^+$ INF-γ^+ cells) in innate and adaptive immunity, respectively. Both preventive and therapeutic vaccination of EG7-OVA tumor-bearing mice using the simultaneous injection of both SVNP-OVA and SVNP-IC induced higher antitumor immunity and inhibited tumor growth.

1. Introduction

The tuning of physicochemical properties of nanoparticles (NPs) to modulate the immune response has received considerable research impetus [1–3]. Size and shape dictate the interaction of NPs with antigen-presenting cells (APCs), and as such, the rational design and synthesis of NPs are prerequisites for modulating the immune response against a target disease [4–9]. On injection of NPs containing antigens and immunomodulatory compounds, APCs surrounding the injection point are recruited and programmed, followed by their migration into lymph nodes to deliver antigenic information to T cells and B cells and consequent induction of an adaptive immune response [10–12]. The generation of an adaptive immune response relies on the efficient drainage or trafficking of the antigens to lymph nodes for the subsequent processing and presentation of foreign molecules to T and B lymphocytes. Lymphatic vessels have evolved to drain pathogens into lymph nodes, thus enabling the immune system to rapidly mount a response. Lymph nodes have thus become critical targets for the delivery of vaccines and immunotherapeutic agents, because the direct delivery of vaccine components into APCs residing in lymph nodes can induce another arms of adaptive immune response, in addition to that induced by migrated APCs. However, most of the commercialized vaccination strategies resulted in the inefficient and transient delivery of antigen and

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adjuvants to the lymphoid tissue, because the delivery carriers in vaccine were not optimized for direct migration through lymphatic vessels [13–15]. Thus, new approaches to improve the targeting and retention of vaccine components in the lymph nodes are envisaged to have significant impact on the potency and efficiency of new vaccines. Three major approaches have been proposed to improve the vaccine targeting efficiency: size tuning, hitch-hiking on albumin, and PEGylation. Previous studies have shown that the kinetics of NP migration through lymphatic vessels is highly dependent on NP size [13–15]. NPs of less than 5-nm diameter can easily enter the bloodstream, whereas those of over 100-nm diameter remain at the injection site and do not move into lymphatic vessels. Typically, larger particles with diameters in the range of 500–2000 nm are carried into lymph nodes by dendritic cells (DCs). It has been reported that 15–70-nm diameter NPs are optimal for rapid entry into lymphatic vessels and migration into lymph nodes [13,14]. As a novel strategy for developing lymph node-targeting vaccines, liposomes containing synthetic peptide antigens and immunostimulatory adjuvants were designed and fabricated to hitch-hike on albumin proteins, which migrate into lymph nodes [16]. In addition, PEGylated hydrogel that can dramatically improve *in vivo* lymphatic drainage and efficiently target multiple immune cell subsets have been proposed [17]. Concerning the effect of vaccine retention on the immune response, rapid clearance of vaccines would limit the quality and duration of the generated immune memory because the adaptive immune response can be maximized by sustained antigen/inflammation over several days [18].

In this study, we designed and developed synthetic vaccine NPs (SVNPs) that are expected to improve the targeting and retention of cancer vaccines (*i.e.*, tumor antigens and immunostimulatory adjuvants) in lymph nodes and are expected to induce a strong and effective immune response for enhanced cancer immunotherapy. The size and surface characteristics of SVNPs were tuned for the effective loading and delivery of antigens and immunostimulatory materials into lymph nodes and for the induction of efficient migration through lymphatic vessels. To fabricate SVNPs (20–70 nm in diameter) and facilitate loading of negatively charged protein tumor antigen (OVA) and toll-like receptor 3 (TLR3) agonist (polyinosinic-polycytidylic acid, poly (I:C)), the carboxyl-terminated surfaces of poly(γ-glutamic acid) (γ-PGA) NPs were further modified with amine moieties (Fig. 1A). Poly (I:C) is a synthetic analog of double-stranded RNA and is known to interact with TLR3 expressed on the membrane of B-cells, macrophages, and DCs [19,20]. The poly (I:C)-loaded SVNPs are expected to stimulate innate immune system, which senses invading viral pathogens and initiates signaling pathways and induces protective genes, including those encoding type I IFNs and pro-inflammatory cytokines that directly limit viral replication and help direct subsequent adaptive immune responses [21]. The lymph node-targeting SVNPs were developed to trigger the protective mechanism in lymph nodes and thus enhance both innate and adaptive antitumor immunity (Fig. 1B).

2. Materials and methods

2.1. Materials

Poly(γ-glutamic acid) (γ-PGA, Mw = 50 kDa) was provided by BioLeaders Corporation (Daejeon, Korea). Cholesteryl chloroformate (97%), 1,1′-carbonylbis-1H-imidazole, 1,2- ethanediamine, polyinosinic-polycytidylic acid sodium salt (low molecular weight poly (I:C)), and chicken egg ovalbumin (OVA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The endotoxin content was analyzed with the Limulus amoebocyte lysate assay (QCL-1000; Lonza, Walkersville, MD, USA) as per the manufacturer's protocols and was found to be 0.6 endotoxin units/mg of soluble OVA [22]. Fluorescein isothiocyanate-conjugated ovalbumin (FITC-OVA) was obtained from Thermo Scientific (Waltham, MA, USA). IRDye800-labeled OVA was prepared using the IRDye800 NHS ester (Li-COR, Lincoln, NE, USA). Rhodamine-labeled poly (I:C) was prepared using the 5′ EndTag™ Nucleic Acid Labeling System (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions.

2.2. Preparation of SVNPs

Cholesteryl chloroformate (2.25 g, 5 mmol) was slowly added to a solution of 1,2-ethanediamine (16.7 mL, 250 mmol) in toluene (anhydrous, 250 mL) in an ice bath. The reaction was carried out at 25 °C for 16 h, followed by washing with deionized water, drying over anhydrous magnesium sulfate, and evaporation in a rotary evaporator. The residue was dissolved in dichloromethane (20 mL) and then added to methanol (20 mL). The resulting suspension was filtered to remove biscarbamate by syringe-filtration (1 μm, polytetrafluoroethylene). The filtrate was evaporated in a rotary evaporator to obtain a white solid. To synthesize γ-PGA-CH, 500 mg γ-PGA (3.876 mmol) in DMSO (10 mL) and 46 mg cholesterol-amine (0.097 mmol) in tetrahydrofuran (10 mL) were reacted in the presence of 1,1′-carbonyldiimidazole (63 mg, 0.3876 mmol) at 40 °C for 18 h. The reaction mixture was evaporated in a rotary evaporator to remove tetrahydrofuran. The reaction mixture was then poured into acetone. After centrifugation, the precipitate was collected, dried, and mixed with $NaHCO_3$ solution and stirred for 4 h. Amberlite IR-120H beads were treated by ion exchange. After filtration with the beads, the reaction mixture was dialyzed through a 10–12 kDa molecular weight cut-off membrane against deionized water for 2 days. The solution was freeze-dried. Next, the γ-PGA-CH conjugate was aminated in the presence of ethylene diamine in DMSO. For amination, γ-PGA-CH (100 mg, 0.773 mmol) and ethylene diamine (10.3 mL, 155 mmol) were slowly mixed in DMSO (20 mL) containing 1,1′-carbonyldiimidazole (752.8 mg, 4.63 mmol) under constant stirring for 12 h. The reaction mixture was poured into acetone. After centrifugation, the precipitate was collected, dried, and mixed with $NaHCO_3$ solution and stirred for 4 h. The reaction mixture was dialyzed through a 10–12 kDa molecular weight cut-off membrane against deionized water for 2 days. The solution was freeze-dried. Elemental analysis of γ-PGA-CH-NH_2 was performed to determine the ratio of C, N, and H (Table 1). To generate the complexes of γ-PGA-CH-NH_2-OVA (SVNP-OVA) and γ-PGA-CH-NH_2-poly (I:C) (SVNP-IC), γ-PGA-CH-NH_2 NP solution was added to the solution of poly (I:C) and OVA at a specified mass ratio of 0.5:1 (γ-PGA-CH-NH_2: OVA) and 1:2 (γ-PGA-CH-NH_2: poly (I:C)) and reacted for 2 h to form a stable complex.

2.3. Characterization of SVNPs

The size distribution and zeta potential of γ-PGA-CH and γ-PGA-CH-NH_2 NPs in aqueous phase were analyzed by dynamic light scattering (DLS) using an electrophoretic light scattering photometer (ELS-Z, Otsuka Electronics, Osaka, Japan). The surface morphology and size of the SVNPs were analyzed using a high-resolution transmission electron microscope (JEOL Ltd., Japan). The samples were stained with 2% uranyl acetate (Sigma-Aldrich).

2.4. Mice and cell lines

C57BL/6 mice (female, 6–8 weeks old) were purchased from Orient Bio (Seongnam, Korea) and maintained under pathogen-free