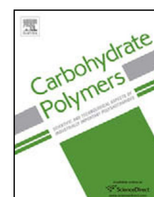
Carbohydrate Polymers 155 (2017) 1–10

Contents lists available at ScienceDirect

Carbohydrate Polymers

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



Hyaluronic acid-supported combination of water insoluble immunostimulatory compounds for anti-cancer immunotherapy

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ARTICLE INFO

Article history:
Received 18 March 2016
Received in revised form 1 August 2016
Accepted 13 August 2016
Available online 16 August 2016

Keywords:
Cancer immunotherapy
Powder-form adjuvant
Immunostimulation
Hyaluronic acid
Cellular immunity

ABSTRACT

A novel powder-form combination adjuvant system containing two immunostimulatory compounds was firstly developed and evaluated as a therapeutic intervention for cancer immunotherapy. With the help of hyaluronic acid (HA), water insoluble monophosphoryl lipid A (MPL), QS21 and imiquimod (R837), could be easily dispersed in aqueous solution and lyophilized as powder-form, which have an advantage in room-temperature storage stability compared with those conventional liquid formulation that requires cold storage. Two kinds of HA-based combination vaccine adjuvants (HA/MPL/QS21, HMQ and HA/MPL/R837, HMR) contributed to the increase of both humoral and cellular immunity, which is very important for efficient cancer immunotherapy. Through the challenge experiments in EG7-OVA (mouse lymphoma-expressing OVA) tumor-bearing mice model, we found out that the immunostimulatory effects of HMQ and HMR were successful in the inhibition of tumor proliferation. Taken together, both HA-based powder-form combination adjuvant systems are expected to be used as potent prophylactic and therapeutic cancer vaccine.

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1. Introduction

Immunotherapy has emerged as an alternative and innovative therapeutic intervention that can overcome the side effect and limited efficacy of conventional chemotherapy against chemo-resistant and relapsing tumors (Dougan & Dranoff, 2009; Mellman, Coukos, & Dranoff, 2011). The major progress for cancer immunotherapy has involved the development of dendritic cells (DCs)-based therapy (Tacken, de Vries, Torensma, & Figdor, 2007) or T-cell adoptive transfer therapy (Hutchinson, 2011; Itzhaki et al., 2013; Kaluza & Vile, 2013; Vacchelli et al., 2013). However, these cell-based therapeutic strategies require significant cost, labor, and time in the isolation, activation and proliferation of these immune cells before they are re-injected into the patient. An alternative strategy that overcomes the limitation of *ex vivo* cultured immune cell-based cancer immunotherapy is to activate and proliferate immune cells *in vivo* using various immunomodulatory materials (Kim & Mooney, 2011; Li & Mooney, 2013). Several immunomodulatory molecules such as cytokines (Kurzrock, Feinberg, Talpaz, Saks, & Gutterman, 1989), chemokines (Chen, Guo, Yang, Zhu, & Cao, 2011), and toll-like receptors (TLRs) agonists (Adams, 2009) have been enthusiastically investigated both in animal experiments and clinical studies (Ali, Huebsch, Cao, Dranoff, & Mooney, 2009; Kim, Noh, Heo, Cho, & Lim, 2012; Makkouk & Abdelnoor, 2009; Scheiermann & Klinman, 2014). Monophosphoryl lipid A (MPL) is a chemically detoxified lipid A moiety extracted from lipopolysaccharides (LPS) of the Re595 strain of *Salmonella minnesota* and was demonstrated to act through TLR 4 (Cui et al., 2014; Hu, Liu, & Zhu, 2013; Patil et al., 2014; Romero et al., 2011). MPL have shown excellent properties in the activation of immune cells such as dendritic cells and macrophages, also induced effective humoral and cellular immunity. As a TLR 7 agonist, Imiquimod (R837) has been widely used to treat diseases of the skin and activated Langerhans cells, natural killer cells, macrophages, and B-lymphocytes (Bernstein, Miller, & Harrison, 1993; Craft et al., 2005; Jimenez-Sanchez et al., 2015; Zhang et al., 2014). Both MPL and R837 stimulated immune system by the recognition of common pathogen-associated molecular-patterns (PAMPs) and induced a cellular immune response. QS-21 is also an immunomodulatory molecule that is extracted from the bark of the Quillaja *saponaria* Molina tree, and can induce humoral and cellu-

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http://dx.doi.org/10.1016/j.carbpol.2016.08.040
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lar immunity (Mbawuike, Zang, & Couch, 2007; Ragupathi, Gardner, Livingston, & Gin, 2011). However, the main problem with the use of these immunomodulatory molecules is their poor water solubility and possible toxicity for *in vivo* use. Especially, both MPL and R837 molecules are poorly water-soluble compounds that are difficult to be dispersed in aqueous solution and need the aid of various solubility-enhancing materials for biological applications. Due to the surfactant-like membrane destabilizing character of QS21, it should be assembled with other materials to minimize any toxic properties (Brito, Malyala, & O'Hagan, 2013; Mbawuike et al., 2007; Pfaar, Cazan, Klimek, Larenas-Linnemann, & Calderon, 2012; Ragupathi et al., 2011). Although various low molecular weight surfactants and lipids were adopted as solubility-enhancing materials to formulate MPL, QS21 and R837 as emulsions or liposomes, their protocols required complicated fabrication process and had a limitation in quality control (Brito et al., 2013; Pfaar et al., 2012).

In this research, we suggested an easy and robust protocol for the preparation of powder-form vaccine adjuvant formulation that contained water-insoluble immunostimulatory compounds (MPL, QS21, and R837) and showed their potent applications in cancer immunotherapies. We developed novel powder-form adjuvant systems by combining hyaluronic acid (HA) with MPL/QS21 (HMQ; HA/MPL/QS21) and MPL/R837 (HMR; HA/MPL/R837), respectively (Scheme. 1). Since dry powder formulation enhances the stability of an adjuvant even at elevated temperatures and reduces the cold-chain requirement or the addition of preservatives, the HA-based powder-form adjuvant systems developed in this study, can be stored at room temperature and be combined with various tumor-associated antigens just before administration, in order to increase both humoral and cellular immunity (Scheme. 1).

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA; Mw = 500–1300 kDa) was purchased from Bioland Corporation (Cheonan, Korea). 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). QS-21 was purchased from Desert King International (San Diego, CA, USA). Imiquimod was purchased from Tokyo Chemical Industry Corporation (Tokyo, Japan). Chicken egg ovalbumin (OVA) was purchased from the Boryung Pharmaceutical Co. (Ansan, Korea).

2.2. Fabrication of the hyaluronic acid (HA)-based immunostimulatory complex

MPL (1 mg, 0.57 mmol) dissolved in 200 μL of Dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, Mo) was added to HA (13.3 mg) dissolved in 13.3 mL of deionized water (DW) by vigorous stirring at room temperature for 5 min. After the reaction, the solution was incubated at 25 °C for 24 h while being shaken. The solution was dialyzed with a Slide-A-Lyzer Dialysis Cassettes (MWCO = 10 kDa, Thermo Fisher Scientific Inc.) in DW. After 48 h of dialysis, QS-21(1 mg) dissolved in 200 μL of 20% tetrahydrofuran (THF; Sigma-Aldrich) solution or R837 dissolved in 200 μL of DMSO is then added by vigorous stirring at room temperature for 5 min. The solutions were incubated for 2 h while being shaken, and then THF was evaporated at 25 °C overnight. Solutions were lyophilized to obtain the final product as a powder.

2.3. Characterization of the HA-based immunostimulatory complex

Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR) measurements were performed using a Bruker IFS66\S (Bruker, Germany) in a wavelength range of 4000–600 cm^{-1} with 110 scans/s rate. In case of MPL and QS-21, they were dissolved in DMSO and analyzed through ATR (diffuse reflection system, reflection and ATR unit), and spectrum of DMSO was subtracted from spectrums of MPL and QS-21 as the reference solution. Next, antigen caring ability of HA-based immunostimulatory complex was determined using Fluorescence Scanning. FITC conjugated OVA (FITC-OVA; Life Technologies, CA, USA), dissolved in PBS (10 μg/mL) was mixed with HA with 1:1.33, 1:10, 1:100 ratios, then the final concentration of FITC-OVA was 1 μg/mL in all samples. Samples were agitated for 1 h at room temperature. After finished reaction, samples were analyzed with fluorescence photometer (LS55, Perkin Elmer, Massachusetts, USA). When analyzing excitation spectrum, the emission was administered at 530 nm, and analyzing emission spectrum, the excitation was administered at 480 nm. The scan speed was 0.5 nm/s.

2.4. Mice and cell lines

C57BL/6 and BALB/c mice (Female, 6–8 weeks old) were purchased from KOATECH (Pyeongtaek, Korea) and were maintained under pathogen-free conditions. All of the experiments employing mice were performed in accordance with the Korean NIH guidelines for care and use of laboratory animals. EG7-OVA (EL-4 thymoma cells transfected with chicken albumin cDNA (American Type Culture Collection, Manassas, VA, USA)) and RAW 264.7 cells (Mouse leukemic monocyte/macrophage cell line, ATCC) were cultured in RPMI medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich), 50 IU/mL penicillin and 50 μg/mL streptomycin (Life Technologies).

2.5. Generation of bone marrow derived murine dendritic cells from mice

Bone marrow derived murine dendritic cells (BMDCs) were generated from the bone marrow cells of C57BL/6 ($H-2^b$) mice. Briefly, bone marrow was collected from the tibias and femurs. Red blood cells were depleted by red blood cell lysing buffer (Sigma-Aldrich). The bone marrow cells (2×10^6 cells) were collected and cultured in a 100 mm Petri dishes containing 10 mL of RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 50 IU/mL penicillin, 50 μg/mL streptomycin and 20 ng/mL recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ, USA). After 7 days, non-adherent and loosely adherent cells (imDCs) were harvested, washed and used for *in vitro* experiments.

2.6. In vitro cell proliferation assay

BMDCs and RAW 264.7 cells were tested for *in vitro* proliferation after treatment with various concentrations of HA-based immunostimulatory complex. 1×10^4 cells in 100 μL media were seeded per well in a 96 well plate (Corning Costar, Cambridge, MA, USA). 100 μL of sample dispersed in cell culture media was added and incubated for 24 and 48 h at 37 °C and 5% CO_2. After incubation, 20 μL of MTS solution-Cell Titer 96 Aqueous One Solution Kit (Promega, Madison, WI, USA) was added to each well and incubated for 2 h. The absorbance of the samples was taken at 490 nm (VersaMax, Molecular Devices, Sunnyvale, CA, USA) and normalized with respect to the absorbance of untreated cells.

2.7. In vitro cytokine and maturation assay

BMDCs were cultured in 6-well plates at a density of 1×10^6 per well (1 mL) and allowed to adhere overnight. HA-based immunos-

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