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Research Paper

Novel thermal-sensitive hydrogel enhances both humoral and cell-mediated immune responses by intranasal vaccine delivery

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ABSTRACT

A novel thermal sensitive hydrogel was formulated with N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC) and α, β-glycerophosphate (α, β-GP). A serial of hydrogels containing different amount of GP and HTCC with diverse quarternize degree (QD, 41%, 59%, 79.5%, and 99%) were prepared and characterized by rheological method. The hydrogel was subsequently evaluated for intranasal vaccine delivery with adenovirus based Zaire Ebola virus glycoprotein antigen (Ad-GPZ). Results showed that moderate quarternized HTCC (60% and 79.5%) hydrogel/antigen formulations induced highest IgG, IgG1, and IgG2a antibody titers in serum, as well as mucosal IgA responses in lung wash, which may attributed to the prolonged antigen residence time due to the thermal-sensitivity of this hydrogel. Furthermore, $CD8^+$ splenocytes for IFN-γ positive cell assay and the release profile of Th1/Th2 type cytokines (IFN-γ, IL-2, IL-10, and IL-4) showed that hydrogel/Ad-GPZ generated an overwhelmingly enhanced Th1 biased cellular immune response. In addition, this hydrogel displayed low toxicity to nasal tissue and epithelial cells even by frequently intranasal dosing of hydrogel. All these results strongly supported this hydrogel as a safe and effective delivery system for nasal immunization.

1. Introduction

The advantages of mucosal vaccination over conventional intramuscular (IM) or subcutaneous immunization make it an efficient way to induce both system and mucosal immune responses [1]. Firstly, may the mostly attractive, mucosal vaccines can efficiently induce local mucosal secretory immunoglobulin A (sIgA) antibody response [2]. The most promising route is intranasal immunization which can generate sIgA antibody responses not only in nasal cavity but also in other mucosal sites because of the common mucosal immune system (CMIS), resulting in the total mucosal protection [3,4]. In practical use, nasal vaccines can be administrated by needle-free devices, which make the intranasal vaccination more acceptable and more suitable for the use of self-vaccination.

In spite of these advantages, the fast mucosal self-clearance and tight arrangement of nasal epithelial cells lead to the short residence time of antigen in nasal cavity, and accordingly the low antigen penetration through nasal mucosal surface. To solve this problem, many researches focused on bioadhesive cationic polysaccharides [5,6] due to their excellent mucoadhesion and biocompatibility [7–10]. Typically, cationic chitosan salts can not only strongly bind to negatively charged mucosal surfaces but also loosen the tight conjugation between epithelial cells. These cationic chitosan salts have been widely investigated and formulated into solutions [11] and particles [12] or powders by spray drying [13] as nasal antigen vehicles. Although promising, they still have to face the hurdles of poor antigen uptake by antigen presenting cells (APCs) and low stimulation of T and B cells under mucosal surface, since they could not provide the vaccine formulations sufficient time in the nasal cavity for desired responses [9]. Antigens that co-administrated with cationic solutions could be still freely drained away from the mucosal surface with the cationic chains solely bound on the surface. Besides, as the natural targets for mucosal clearance, the particulate vehicles were not the ideal candidates as mucoadhesive units yet because they could not provide enough surface adhesion with mucosa, and the complicated preparation process of particles also hindered their step on the way to clinical use.

Previously, we developed a N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC)-based thermal sensitive

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hydrogel with preferable sol–gel transition feature and net-work structure [14]. HTCC/GP system was solution state and low viscosity under room temperature, while it could turn to an un-flowing hydrogel after heated at 37 °C. Inspired by this advantage, we firstly tried this positive charged thermal-sensitive hydrogel in nasal mucosal vaccination. It would, in principle, provide the nasal antigens a shield from being removed off the nasal mucosal surface, which might be much more superior to other nasal formulations in prolonging the antigen residence time and inducing strong antigen-specific immune responses.

Ebola virus is one of the most critical pathogens [15] that can transmit by mucosal exposure like small-particle aerosol dispersion [16,17]. Adenovirus expressing the Zaire Ebolavirus (ZEBOV) envelope glycoprotein (Ad-GPZ) antigen was proved safe and immunogenic in healthy adults by IM route [18,19]. However, the pre-existing immunity against the adenovirus itself greatly hinders its development as antigen vectors. Fortunately, recent findings proved that mucosal delivery of adenovirus-based vaccines could effectively circumvent the pre-existing immunity [20,21], which opened a promising perspective for the adenovirus as antigen vector. To develop a new generation of needle-free mucosal vaccine, we used Ad-GPZ antigen as a prototype and testified the feasibility of thermal-sensitive hydrogel vehicle for the intranasal delivery. The ZEBOV glycoprotein specific cellular and humoral immune responses and the toxicity of this thermal-sensitive HTCC hydrogel were evaluated in details.

2. Materials and methods

2.1. Materials

Chitosan (MW 7.8×10^5, degree of deacetylation (DD) is 95%) was purchased from Putian Zhongsheng Weiye Co. Ltd. (Fujian, China). The α-β-GP was provided by Kaiyuan Pharmaceutical & Chemical Co. Ltd. (Shanxi, China). All other reagents were of analytic reagent grade. The following reagents were ordered from the suppliers indicated: FITC-, PE-, APC-, or PerCP-Cy5.5-labeled antibodies to CD8, CD4, and CD3 (BD Biosciences; San Jose, CA); Cy™ 5 Mono NHS Ester (GE Healthcare); goat anti-mouse IgA, IgG total, IgG1, and IgG2a antibodies (LifeSpan Biosciences; Seattle, WA); ELISA kits for IL-4, IFN-γ, IL-2 and IL-10 (eBioscience; San Diego, CA); Peptide (LYDRLASTVI) was synthesized by GL Biochem (Shanghai) Ltd. Peptide Complete medium was prepared with RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES buffer, and 55 mM 2-mercaptoethanol.

2.2. Construction of adenoviral vectors

The recombinant replication-deficient adenovirus expressing the glycoprotein of ZEBOV (Ad-GPZ) was produced as previously described [22]. Briefly, the appropriate open reading frame for the gene encoding the glycoprotein was generated by PCR, cloned into the adenoviral shuttle plasmid pDC316. The shuttle plasmid and the adenoviral backbone plasmid (pBHGlox_E1, 3Cre) were cotransfected into HEK293 cells using Lipofectamine™ Reagent (Invitrogen, USA) following the manufacturer's instruction. Transfected cells were maintained until adenovirus related cytopathic effects were observed. The adenoviruses were harvested and confirmed by PCR. Positive recombinant adenovirus was reamplified in HEK293 cells and purified by ion exchange (SOURSE 15Q) and size exclusion. The viruses were titrated on HEK293 cells by Adeno-XTM Rapid Titer Kit (Clontech, USA) for plaque forming units (pfu).

2.3. Synthesis of HTCC and formation of HTCC/GP hydrogel

The HTCC was prepared by a modified method proposed by our previous work [23]. Firstly, a certain amount of chitosan was dispersed in isopropanol (30 ml), and then glycidyltrimethylammonium chloride (GTMAC) dissolved in 5.3 ml deionized water was dropped to the chitosan suspension. After reaction for 9 h at 80 °C, the reacting solution was cooled to room temperature before pouring into cold acetone, after that, the mixture was stirred overnight at 4 °C. After filtration, more purified HTCC was obtained by washing with hot ethanol using a Soxhlet extractor for 24 h and then dried at 60 °C. Quarternize degree (QD) of HTCC was determined by a titration method described by our previous report [23]. Quarternized HTCC with different QD (30%, 41%, 50%, 60%, 79.5%, and 99%) were synthesized by adjusting the mol ratio of GTMAC to amino groups of chitosan.

The HTCC/GP hydrogel was prepared by the following steps. Briefly, a required amount of HTCC was dissolved in 5.0 ml 0.1 M aqueous lactic acid solution at room temperature. And then a solution of α-β-GP in 3.0 ml deionized water was dropped into the stirring HTCC solution in an ice bath. The obtained solution was stirred for 30 min with an extra addition of 2.0 ml deionized water to get a homogeneous mixture for the detection of gelating time and viscosity. Ad-GPZ antigen solution was added to replace the 2.0 ml deionized water to make a constant component of hydrogel.

2.4. Rheological characterization of HTCC hydrogel

The gelating time and viscosity of hydrogel solutions were measured using a rheometer (Bohlin Gemini 200, Malvern). Gelating time was determined by the transition from a prevalently viscous state ($G'' > G'$) to one that is prevalently elastic ($G' > G''$). For the detection of gelating time at 37 °C, 40 mm diameter and 4° cone plate was used, and the strain sweep measurements at 1 Hz allowed the determination of the linear viscoelastic region (LVR). For the detection of viscosity at 20 °C, 40 mm diameter flat plate was chosen with a pre-shearing time of 90 s and equilibrium time of 150 s, and the clamp distance was set as 800 μm.

2.5. Mice immunization with hydrogel/Ad-GPZ vaccine

We intranasally and intramuscularly immunized 8 weeks aged Balb/c mice ($n = 6$) with HTCC hydrogel carrying adenovirus expressed Ebola membrane protein vaccine (hydrogel/Ad-GPZ). Mice were lightly anesthetized with diethyl ether prior to vaccination, 30 μl containing 5×10^9 viral particles per mouse and hydrogel solution was pre-mixed for nasal immunization on day 0. 100 μl containing 5×10^9 viral particles per mouse was used for the intramuscular immunization in the mouse's right limb. Mice in control group were vaccinated with PBS. On day 28 mice were given a boost immunization with the same composition of hydrogel/Ad-GPZ or PBS/Ad-GPZ as first vaccination. On week 6, bloods were collected and centrifuged to generate serum samples, and then stored at −70 °C. Spleen cells were harvested in sterile cabinet and dispersed by filtering through a 70-mm nylon mesh strainer, and then treated with ACK, a red blood cell lyses buffer. The experiments on animals were carried out in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC) and approved by Experimental Animal Ethics Committee in Beijing.

2.6. Antibody assays (ELISA)

Blood was collected from the tail vein and serums were obtained by centrifugation at 5000*g* for 10 min. The nasal and lung wash samples were collected by flushing the lung and nose with