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The use of layered double hydroxides as DNA vaccine delivery vector for enhancement of anti-melanoma immune response

Ang Li [a], Lili Qin [a], Wenrui Wang [a], Rongrong Zhu [a], Yongchun Yu [a], Hui Liu [b], Shilong Wang [a,*]

[a] Tenth People's Hospital, School of Life Science and Technology, Tongji University, 1239 Siping Road, Shanghai 200092, PR China
[b] Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, PR China

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ABSTRACT

Our previous studies have shown that Mg:Al 1:1 layered double hydroxides (LDH(R1)) nanoparticles could be taken up by the MDDCs effectively and had an adjuvant activity for DC maturation. Furthermore, these LDH(R1) nanoparticles could up-regulate the expression of CCR7 and augment the migration of DCs in response to CCL21. In current study, we have evaluated whether LDH(R1) as DNA vaccine delivery carrier can augment the efficacy of DNA vaccine immunization in vivo. Firstly, we found that LDH(R1) was efficient in combining DNA and formed LDH(R1)/DNA complex with an average diameter of about 80 −120 nm. Its high transfection efficiency in vivo delivered with a GFP expression plasmid was also observed. After delivery of $pcDNA_3$-OVA/LDH(R1) complex by intradermal immunization in C57BL/6 mice, the LDH(R1) induced an enhanced serum antibody response much greater than naked DNA vaccine. Using B16-OVA melanoma as tumor model, we demonstrated that $pcDNA_3$-OVA/LDH(R1) complex enhanced immune priming and protection from tumor challenge in vivo. Furthermore, we showed that LDH(R1) induced dramatically more effective CTL activation and skewed T helper polarization to Th1. Collectively, these findings demonstrate that this LDH(R1)/DNA plasmid complex should be a new and promising way in vaccination against tumor.

1. Introduction

DNA vaccination has been proven to be effective in small animal models for many years. Immunization with DNA vaccines results in humoral and cellular immune responses that protect against disease in preclinical models of infectious diseases, cancer, and autoimmunity [1]. Intramuscular (i.m.) injection has been the primary route of administration for DNA vaccines, and has been shown to elicit protective and therapeutic immune responses in many animal models. However, its efficacy in humans does not mirror these successes. Optimal antibody and cytotoxic T lymphocyte (CTL) responses were only induced with high doses of DNA in non-human primates and human volunteers [2–4]. Hence it's neceessary to investigate novel methods to improve its immunogenicity. Although it is difficult to quantitate the amount of plasmid DNA that enters cells or is degraded before it enters the nucleus and initiates gene expression, it is believed that more than 90% of DNA injected never gets into the cytoplasm and <10% of DNA that does enter the cell and 1% enters the nucleus where gene expression occurs [5,6]. Understanding the process of efficient gene delivery allows to devise methods to overcome these problems. Many of these efforts are focused on using viral or non-viral carriers to act as vehicles to transport genetic material into cells [7,8]. Although viral vectors are highly efficient in transfecing cells, they lack in targeting specificity. This presents several disadvantages in in vivo applications such as eliciting undesired effects on bystander cells, the need for larger doses and restriction to local administrations. There is, therefore, a clear need to induce effective immunity in humans with lower and fewer doses of DNA, as well as to increase the magnitude of the immune response [9]. Several groups have attempted to improve the delivery efficacy using cationic microparticles [10], chitosan [11], non-ionic block co-polymers [12] and cationic liposomes [13,14].

Layered double hydroxides (LDHs), also known as "anionic clay" and hydrotalcite-like compounds, are an unusual family of layered materials consisting of positively charged layers with charge-balancing anions between the layers. Due to their hydrolysis behavior in acidic media and their anionic exchange capacity, LDHs are already used as antacids [15] and in pH-sensitive controlled released for drug delivery [16]. Our previous reports have demonstrated Mg:Al 1:1 LDH(R1) nanoparticles could be taken up by the

* Corresponding author. Tel.: +86 21 65982595; fax: +86 21 65982286.
E-mail address: wsl@tongji.edu.cn (S. Wang).

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MDDCs effectively and had an adjuvant activity for DC maturation. Furthermore, these LDH(R1) nanoparticles could up-regulate the expression of CCR7 and augment the migration of DCs in response to CCL21. The NF-κB pathway was involved in the maturation of DCs and up-regulation of CCR7 on DCs treated with the LDH(R1) nanoparticles. These effects of LDH(R1) nanoparticles on MDDCs suggested that they would become effective antiviral or anticancer vaccine carriers by their excellent capacity to be taken-up and the ability to induce maturation of DCs [17].

In the present study, we have evaluated the capacity of LDH(R1) nanoparticles to enhance DNA-based tumor vaccines in the setting of B16-OVA melanoma. We detect whether LDH(R1) nanoparticles improve the efficiency of this DNA vaccine, and then plasmid DNA was nanoencapsulated using LDH(R1) to formulate a DNA-LDH(R1) nanoparticle complex (DLNP). The physicochemical structure of the DLNPs, release characteristics for the DNA, and the protection of encapsulated DNA from nuclease degradation were investigated in vitro. Importantly, we examined efficiency of immunization with DLNP in vaccine-mediated immune priming and therapeutic efficacy in vivo. Finally, the possible mechanisms by which LDH(R1) nanoparticles serve as an adjuvant augmenting Th1 response and class I peptide restricted CTL response were confirmed.

2. Materials and methods

2.1. Animals, reagent, plasmid DNA and cell line

Six-to-eight-week-old female C57BL/6 mice were purchased from the Laboratory Animal Center of Chinese Academy of Science and housed in the pathogen-free animal facility of Tongji University. A C57BL/6-derived thymoma cell line, EL-4, was obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium (GIBCO) supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine at 37 °C in 5% CO_2 (complete RPMI medium). The B16-OVA cell line, a B16 cell line that is stably transfected with cDNA of OVA to allow endogenous production of OVA with an H-2K^b-restricted CTL epitope was maintained in complete RPMI medium supplemented with 400 μg/mL G418 (Sigma Aldrich Co., UK) at 37 °C in 5% CO_2. We obtained the following antibodies commercially: rat anti-mouse IgG, IgG1, IgG2a and PE labeled anti-mouse CD11c. Recombinant human IL-2 with a specific activity of 18×10^6 IU/mg was used to culture specific T cell. All recombinant cytokines were purchased from Peprotech Inc. (Rocky Hill, NJ).

2.2. Preparation of LDH(R1) nanoparticles and LDH(R1)/DNA complex

The pristine layered double hydroxides (LDH(R1)s) were prepared by coprecipitation under N_2 atmosphere to avoid or at least to minimize the contamination by atmospheric CO_2 following the conventional route. The mixed solution of 1 M Mg$(NO_3)_2 \cdot 6H_2O$ and Al$(NO_3)_3 \cdot 9H_2O$ (Mg:Al ratio of 1:1) was prepared in sealed conical flasks with continuous stirring under nitrogen gas flow. Final solution pH was then adjusted to 10.0 ± 0.2 by adding 1 M NaOH solution dropwise and was stirred vigorously at 80 °C for 5 h. Resulting precipitates were filtered, washed thoroughly with decarbonated water, and dried overnight under a vacuum at 60 °C. The supercoiled pEF-eGFP or pcDNA_3-OVA plasmid was prepared using the Qiagen maxiprep kit and diluted in deionized water at a final concentration of 1 μg/μL. The DNA:LDH(R1) weight ratio in the DNA-LDH(R1) nanohybrid suspensions was fixed at 1:50. For example, to make DNA-LDH(R1) suspension with DNA:LDH(R1) weight ratio of 1:50, 100 μL of LDH(R1) suspension (0.80 wt%), 16 μL of plasmid DNA (1 μg/μL), 50 μL of sterile water and 34 μL of sterile Mg–Al buffer solution were mixed and shaken at 37 °C for 4 h in a sealed sterile tube.

2.3. Physical property characterization for the LDH(R1)/DNA complex

Powdered sample X-ray diffraction (XRD) patterns were recorded on a Rigaku Diffractomer Model Miniflex using CuKα radiation ($\lambda = 1.54060$ Å, 40 kV, 40 mA, step of 0.0330°). Zeta potential distribution of nanoparticles was analyzed by Nano ZS, Malvern Instrument. The mean particle size (z-average size) and size distribution were measured using photon correlation spectroscopy (LS230 Beckman Coulter) at 25 °C under a fixed angle of 90° in disposable polystyrene cuvettes. The measurements were recorded using a He–Ne laser of 633 nm.

2.4. LDH(R1)/DNA complex determination by agarose gel electrophoresis and treatment with DNase I enzymes

In order to demonstrate DNA combine with LDH(R1) and the ability of LDH(R1) to protect DNA from endonuclease attacks, the nanobiocomposite was assayed by agarose gel electrophoresis and subjected to DNase I treatment. Nanobiocomposite samples were incubated with 3 mL of DNase I enzyme for 30 min at 37 °C. The mixture was then centrifuged at 2500 rpm for 2 min. The DNA was revered from the inorganic host by acidificaton and incubated at pH 2. Then an appropriate amount of DNA loading buffer was added to each sample. Gel electrophoresis of the supernatant was carried out on 1% agarose gel (TAE buffer, 1% ethidium bromide) at 100 V for 45 min and gels were subsequently imaged using a kodak gel documentation system.

2.5. Immunizations and tumor challenge

6-week-old female C57BL/6 mice were anesthetized using acepromazine maleate (i.p.) and the hairs covering the areas of abdomen skin were removed with a shaver. Then we intradermally injected LDH(R1)/DNA complex, the same amount of DNA or LDH(R1)/DNA complex in combination with 10 mL CpG adjuvants (10 mg; Shanghai Biotec) in sterile DI water (200 mL) into the right flank region of C57BL/6 mice, and the same immunization was repeated twice at 1 week interval. Tumor challenge was initiated by subcutaneous injecting 5×10^4 viable B16-OVA melanoma cells into the rear leg of the immunized mice 1 week after the final immunization. Tumor occurrence was observed every other day. The length and width of tumor mass were measured with caliper every other day, and tumor area was expressed as length×width. Eight mice in each group were observed for their survival period. Mice becoming moribund were killed. All experiments were performed three times using individual treatment groups of five mice. Data are representative of three experiments performed. Tail vein blood samples of immunization groups were collected 2, 4, 6, 8, 10 weeks after immunization, and spleens were isolated at the end of the experiment for immunological assays. Serum was prepared from clotted blood and stored at −20 °C until analysis.

2.6. Quantification of antigen-specific IgG and subtypes by ELISA

The antibody responses (IgG, IgG1 and IgG2a) to OVA were determined based on a previously reported method [18]. Plates (corning, flat bottom plates, CA) were coated overnight with 1.0 mg/mL OVA protein in 0.1 M sodium carbonate buffer (pH 9.6), washed and afterwards blocked with a 2% (w/v) bovine serum albumin solution (Sigma Aldrich Co., UK). Plates were again washed and sera were tested at 1:64 dilutions. Sera obtained from naive mice were used as a control. Horseradish peroxide conjugate goat anti-mouse IgG, IgG1 and IgG2a (diluted 1:1000) were applied as secondary antibody (Sigma, Pool Dorset, UK). Finally, dissolved 2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) [ABTS] (Sigma Aldrich Co., UK) in citrate buffer, along with H_2O_2 was used to develop the plates, the colors reaction was stopped after 15 min by adding 5 N H_2SO_4 to the wells, and absorbance was read at 405 nm. The titres reported are the reciprocal of serum dilutions that gave an optical density 5% higher than the strongest negative control reading.

2.7. GFPpositive cells migration assay

Mice of 6–8 weeks old were treated with a single intradermal injection by the LDH(R1)/pEF-eGFP complex or the same amount of DNA. The cells of lymph nodes were isolated at 24 h after immunization. These cell samples were resuspended in a Hank's balanced salt solution (HBSS Sigma, St. Louis, MO) and then passed through a fine steel mesh to obtain the suspension of homogenized cells. The erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation, the pelleted cells were washed three times with PBS and resuspended in a complete medium (RPMI 1640 supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-mercaptoethanol, 100 IU/mL streptomycin, 100 mg/mL penicillin, and 10% FBS). Lymphocytes were counted and aliquoted in 10^7 per mL in RPMI 1640 medium containing 2% (v/v) fetal calf serum. The cells were analyzed by flow cytometry (FACSCanto, BD, CA).

2.8. Proliferation assays

Spleen cells from immunized C57BL/6 mice were harvested, and the cell suspensions were passed through nylon wool to get purified T cell. The enriched T cells (about 90% purity) were used as responder cells. The DCs prepared as previously [19] were pulsed with OVA protein(2.5 μg/mL or 5 μg/mL) for 60 min, and then used as antigen-presenting cells. 1×10^5 responder cells and pulsed DCs (1×10^4 cells) were cultured on flat-bottomed 96-well microtiter plate (Corning) for 48 h in the presence of 20 U/mL recombinant human IL-2. During the last 5 h of the culture, 5 mg/mL MTT reagent was added. DMSO (dimethyl sulfoxide) was then added to the wells to dissolve formazan crystals before being analyzed under a plate reader at 570 nm. Results are expressed as a stimulation index (SI) calculated as the ratio of (OD of cells cultured in the presence of OVA)/(OD of cells cultured in the absence of OVA). All samples were assayed in triplicate and the values were shown as the means ± SD.