DNA vaccine expressing herpes simplex virus 1 glycoprotein C and D protects mice against herpes simplex keratitis

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Abstract

● AIM: To investigate whether DNA vaccine encoding herpes simplex virus 1 (HSV-1) glycoprotein C (gC) and glycoprotein D (gD) will achieve better protective effect against herpes simplex keratitis (HSK) than DNA vaccine encoding gD alone.

● METHODS: DNA vaccine expressing gD or gC combined gD (gD.gC) were constructed and carried by chitosan nanoparticle. The expression of fusion protein gD and gC were detected in DNA/nanoparticle transfected 293T cells by Western-blot. For immunization, mice were inoculated with DNA/nanoparticle for 3 times with 2wk interval, and two weeks after the final immunization, the specific immune responses and clinical degrees of primary HSK were evaluated.

● RESULTS: Fusion protein gD.gC could be expressed successfully in cultured 293T cells. And, pRSC-gC.gD-IL21 DNA/chitosan nanoparticle could effectively elicit strongest humoral and cellular immune response in primary HSK mice evidenced by higher levels of specific neutralizing antibody and sIgA production, enhanced cytotoxicities of splenocytes and nature killer cells (NK), when compared with those of gD alone or mocked vaccine immunized mice. As a result, gC-based vaccine immunized mice showed least HSK disease.

● CONCLUSION: gC-based DNA vaccine could effectively prevent the progress of primary HSK, suggesting that this DNA vaccine could be a promising vaccine for HSK treatment in the future.

● KEYWORDS: herpes simplex virus 1; keratitis; gC-based DNA vaccine; nanocarrier; immune response

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INTRODUCTION

Herpes simplex keratitis (HSK) is a chief worldwide blind-causing corneal disease that mainly caused by herpes simplex virus type 1 (HSV-1) infection[1]. After infection, HSV-1 virus will exist in host for a lifelong time[2]. Generally, in human there are 3 ongoing phases that finally contribute to the development of HSK, namely primary infection, latent phase and recurrent phase[2]. The antiviral medications, which is the main strategy for HSK management at present, just temporarily control symptoms of HSK, not eradicate the disease or virus[3]. As yet, no methods that can effectively control the spread of virus are available. Due to the ability of causing specific immune response against HSV-1, HSK vaccines are thought to be the most effective approach to deal with HSK[4-5]. Among them, DNA vaccine is getting particular attention. After being inoculated, the target genes of DNA vaccine are expressed and presented in host cells and could produce strong and sustained cellular and humoral immune responses[6-7]. In some fields such as Venezuelan equine encephalitis, cancer and human immunodeficiency virus infection, DNA vaccine have being designed or tested in clinical trials[8-10]. We previously constructed a DNA vaccine encoding glycoprotein D (gD) of HSV-1 and interleukin (IL)-21[11]. There, gD serves as major immunogen and IL-21 is an adjuvant molecular for the purpose of improving the entire immune outcome. After being tested, this DNA vaccine showed...
immunization with gC of HSV-1 could totally blocks C3b binding and reduces disease severity. However, gC reported on the surface of HSV-1 can disrupt the process of complement activation via binding complement C3b and blocking the C5 and properdin interaction with C3b, thereby benefiting virus infection[16-18]. Strategies to prevent gC mediated immune evasion may be required to develop successful HSV vaccines. Chang et al[9] reported that immunization with gC of HSV-1 could totally blocks C3b binding and reduces disease severity. However, gC is not very immunogenic and gC alone can’t induce sufficient immune protective effect against HSV-1 disease[20]. Recent studies focus on investigating whether combination gC and gD will have a better therapeutic effect to HSV disease, compared to gC or gD alone. It has been reported that immunizing mice with gD and gC proteins provided better protection than gD alone in preventing HSV-1 or HSV-2 associated infectious diseases[21-22]. However, DNA vaccine encoding gC and gD for HSK treatment have not been reported.

In this study, we developed a DNA vaccine construct pRSC-gC.gD-IL21 by fusing gC sequence of HSV-1 with gD and using IL-21 which is well-known adjuvant molecular. Considered the fact that the efficiency of DNA membrane associated transport is extreme low and DNA can be degraded easily without protection, therefore a suitable carrier is needed[23-24]. Chitosan, as a natural cationic polysaccharide, can easily combine with the negatively charged plasmid DNA and has the properties of being easily absorbed and biodegraded, allowing gene slow release and long lasting expression, low toxicity and cost, enhancing the immune response, have been widely used as DNA carrier[25-26]. Here, we embedded the plasmid DNA into chitosan to form DNA plasmid/chitosan nanoparticle for immunization.

Besides DNA carrier, the inoculation approach also matters. We previously demonstrated that topical administration of nanoparticle-loaded DNA vaccine could induce stronger ocular local immune response of mice than intramuscular inoculation[27]. What’s more, it has been noted that nanoparticle could benefit local inoculation by inducing strong local mucosal immunity[28-29]. Therefore, we adopted topical immunization in this study. Here, we reported that we successfully constructed gC-based vaccine termed pRSC-gC.gD-IL21 and investigated its effect against primary HSK. This study provided the experimental fundamentals for further investigating this vaccine in dealing with recurrent HSK which is the key point for HSK management.

MATERIALS AND METHODS

Mice, Cell Lines and Virus Balb/c background female mice (4-6wk) were purchased from the Animal Center of Yangzhou University for experimental use. And mice were maintained under pathogen free conditions with a 12h/12h light-dark cycle. Vero cells derived from African green monkey kidney were preserved in our lab. SP2/0 cells derived from Balb/c mice myeloma cells and YAC-1 cells derived from moloney leukemia-induced T cell lymphoma, were acquired from Cellular Institute of China in Shanghai. All the cells were cultured in RPMI1640 containing 10% fetal bovine serum, 100 units/mL penicillin G sodium and 100 g/mL streptomycin sulfate at 37℃ in 5% CO₂ atmosphere. HSV-1 strain F was obtained from the Institute of Virology in Wuhan, China. All the animal experiments were strictly in accordance with the guidelines of the Animal Research Ethics Board of Southeast University, China.

Plasmids and Primers Recombined pRSC-gD-IL21 plasmid were constructed and preserved in our lab. The gC gene (whole extracellular sequence from Gly25 to Gly480) was harvested by polymerase chain reaction (PCR) amplifying with a pair of specific primers to the gC gene (forward: 5'-TTTACAAGCTTGGGCTCGA-3', reverse: 5'-ATATGGGTACCGCCGCCACCATGGGGGGCGCC-3'), and HSV-1 gene was used as template. The gD (whole extracellular sequence from Met1 to Met340) was harvested by double time PCR. Briefly, pRSC-gD-IL21 was set as template for the first round amplifying with a pair of specific primers (forward: 5'-ATATGGGTACCGCCGCCACCATGGGGGGCGCC-3', reverse: 5'-CCACCCGAACCTCCACCTTCACCTTCACTTCCACCTTGTTTGC-3'). The product of first round PCR was digested by HindIII and NheI, gC DNA was connected with HSV-1 strain F and finally developed the plasmid pRSC-gC.gD-IL21 DNA, which was identified by the analysis.
of restriction endonuclease digestion, DNA sequence and target protein expression in 293T cells.

Transfection and Western Blot For transfection, the cultured 293T cells in 6-well plates were transfected with 20 μL mixture of pRSC-gC-gD-IL21 DNA/chitosan or mock plasmid (10 μg plasmid/1×10^6/cells) by using LipofectAMINETM 2000 reagent (Invitrogen, USA). After incubation for 4h, the transfected cells were washed with RPMI1640 to remove the free nanoparticle mixtures, and the cells were further cultured for 48h. For Western blot, the cell were harvested and homogenized in RIPA buffer containing Tris-HCl (50 mmol/L), NaCl (150 mmol/L), EDTA (1 mmol/L), Triton-X (1%), sodium deoxycholate (0.5%) and SDS (0.1%). Then, the homogenates were centrifuged at 13 000 r/min for 15min at 4℃ and the supernatant was acquired as extracted protein. After electrophoresis, proteins were transferred on PVDF membranes. The membranes were soaked in 5% milk/PBST for 2h at room temperature. Then, the membranes were incubated with anti-gD antibody for 1h. The following steps were performed according to the Western-Breeze Kits protocol (Invitrogen, USA).

Preparation and Examination of Chitosan/DNA Nanoparticle Chitosan was dissolved in NaAc (5 μmol/L, pH5.5) solution at a ratio of 0.02% (w/v). Plasmid DNA (0.8 μg) was diluted in Na2SO4 (5 mmol/L) solution. After bathing in water (55℃) for 10min, chitosan solution and DNA solution were mixed by vortex at equal volume, and the mixture was stored for experimental use. For the purpose of examining the loading efficiency, the naked plasmid DNA, precipitation of chitosan/DNA nanoparticle and supernatant of chitosan/DNA nanoparticle were subjected to agarose gel electrophoresis. DNA bands were visualized by EtBr. To determine the protective effect of chitosan on plasmid DNA, the naked plasmid DNA and chitosan/DNA nanoparticle were digested by Dnpi and the products were subjected to agarose gel electrophoresis, DNA bands were visualized by EtBr.

Vaccine Immunization and Virus Challenge in Mice Mice (n=12) were randomly divided into 4 groups: pRSC-gC-gD-IL21/chitosan group; pRSC-gD-IL21/chitosan group; pRSC/chitosan group and chitosan group. The plasmid DNA/chitosan (20 μL containing 100 μg DNA) or chitosan were dropped into conjunctival sacs of each mice. The total immunization (20 μL containing 100 μg DNA) or chitosan were dropped into conjunctival sacs of each mice. Two weeks after final immunization, the mice were sacrificed and spleen homogenates were centrifuged at 13 000 r/min for 15min at 4℃ and the supernatant was acquired as extracted protein. After electrophoresis, proteins were transferred on PVDF membranes. The membranes were soaked in 5% milk/PBST for 2h at room temperature. Then, the membranes were incubated with anti-gD antibody for 1h. The following steps were performed according to the Western-Breeze Kits protocol (Invitrogen, USA).

RESULTS Identification of Constructed pRSC-gC-gD-IL21 DNA Vaccine To validate whether the pRSC-gC-gD-IL21 DNA vaccine shows promise for clinical application, 4 groups of mice were immunized with pRSC-gC-gD-IL21 DNA vaccine, chitosan/chitosan group, pRSC-gD-IL21/chitosan group, pRSC/chitosan group and chitosan group, respectively. The LD50 of HSV-1 was calculated as the reciprocal of the dilution which resulted in 50% reduction in virus titer.
The vaccine was constructed successfully, the plasmid were sequenced. The gC sequences we detected are 99.12% consistent with the data existed in the Genebank (only 4 amino acids are different, including P132S, F299L, H306R, I383H), and the gD sequences are 98.22% with only 7 different amino acids, including L50P, Q52R, N71D, T217A, P295Q, V361G, P374R. We consider that these rare mutated sites are due to the different strains of HSV-1 and couldn't affect its function. For further confirmation, the pRSC-gC.gD-IL21 were digested by HindIII/NheI or Hind/NheI and the products were subjected to agarose gel electrophoresis. We observed a 2454 bp DNA band which is equal to the length of gD+gC and a 1393 bp gC DNA band in the gel (Figure 1A).

To test the packaging efficiency of chitosan to plasmid DNA, the mixture of pRSC-gC.gD-IL21 DNA/nanoparticle was centrifuged and DNA content in the supernant and precipitation were detected by ultraviolet spectrophotometer. The ratio of pRSC-gC.gD-IL21 DNA in chitosan nanoparticle was near 97% and this was further confirmed by agarose gel electrophoresis. We observed a 2454 bp DNA band which is equal to the length of gD+gC and a 1393 bp gC DNA band in the gel (Figure 1A).

The Levels of HSV-1 Neutralizing Antibody and sIgA in Immunized Mice To examine the humoral immune responses in immunized mice, we detected the levels of HSV-1 neutralizing antibody and sIgA. Results showed that the levels of HSV-1 neutralizing antibody in pRSC-gC.gD-IL21 DNA/chitosan immunized mice were near ten folds of the mice immunized with pRSC-gD-IL21 DNA/chitosan and the pRSC/chitosan or chitosan immunized mice showed the lowest levels of HSV-1 neutralizing antibody. In addition, the levels of sIgA in tear of pRSC-gC.gD-IL21 DNA/chitosan immunized mice is highest, with intermediate levels of sIgA in tear of pRSC-gD-IL21 DNA/chitosan immunized mice and relatively lower levels in tear of pRSC/chitosan or chitosan immunized mice (Figure 2). These results demonstrated that pRSC-gC.gD-IL21 DNA/chitosan vaccine could effectively induced humoral immune responses in mice.

Cellular Immune Response to DNA Vaccine To determine cellular immune response in immunized mice, we investigated the cytotoxic activities of NK cells or splenocytes. Results showed that pRSC-gC.gD-IL21 DNA/chitosan immunized mice exhibited the highest NK and splenocytes activities, followed by pRSC-gD-IL21 DNA/chitosan immunized mice, and the lowest were pRSC/chitosan and chitosan immunized mice (Figure 3). These results indicated that pRSC-gC.gD-IL21 DNA/chitosan vaccine conferred the most robust cellular immune response to gD on mice compared with that of pRSC-gD-IL21 DNA/chitosan or mocked plasmid vaccine.

Clinical Keratitis of Vaccine Immunized Mice After Virus Challenge The vaccine immunized mice were inoculated with HSV-1 virus and the pathological changes of corneas were monitored daily for 15d. After virus challenge, pRSC-gC.gD-IL21 DNA/chitosan immunized mice showed the least severe herpes keratitis and got a total recovery by day 5, while the mocked plasmid/chitosan or chitosan exhibited the most severe...
herpes keratitis with a recovery in day 15. And the severity of herpes keratitis in pRSC-gC.gD-IL21 DNA/chitosan immunized mice ranked from pRSC-gC.gD-IL21 DNA/chitosan to mocked plasmid/chitosan or chitosan immunized mice (Figure 4). These results demonstrated that pRSC-gC.gD-IL21 DNA/chitosan vaccine could effectively protect mice against herpes keratitis caused by HSV-1 infection when compared with pRSC-gD-IL21 DNA/chitosan vaccine.

DISCUSSION

It has been reported that gC play a critical role in mediating the immune evasion of HSV-1 and the strategy targeting to gC represents a promising approach to control the spread of virus\textsuperscript{[19,31-32]}. Several studies demonstrated that gC could effectively enhance gD induced immune protection against HSV disease\textsuperscript{[21-22]}. In present study, we developed a gC based DNA vaccine called pRSC-gC.gD-IL21 DNA vaccine by fusing gD sequence of HSV-1 to gC of HSV-1, and we demonstrated that pRSC-gC.gD-IL21 DNA vaccine could
induced strongest humoral and cellular immune response to protect mice against primary HSK, when compared with pRSC-gD-IL21 DNA vaccine.

First of this study, we examined whether our gC based DNA vaccine was developed successfully. Endonuclease digestion experiments revealed that our vaccine constructed successfully. Given low efficiency of cell entry for naked DNA, we loaded DNA vaccine in chitosan to form the DNA/chitosan nanoparticle. After being tested, the chitosan nanoparticle could effectively carry the DNA vaccine, with 97 percent of loading efficiency and protect the DNA vaccine from DnpI. Furthermore pRSC-gC.gD-IL21 DNA/chitosan nanoparticle transfected cells could effectively produce fusion protein gD plus gC. These results demonstrated that gC based DNA vaccine was developed successfully.

It is known that a successful vaccine should be able to induce both high level of humoral and cellular immune response. In this study, we found that chitosan nanoparticle containing pRSC-gC.gD-IL21 DNA immunized mice produced more HSV-1 neutralizing antibody in serum and sIgA in tear when compared with that of pRSC-gD-IL21 DNA vaccine or mock DNA vaccine immunized mice. What’s more, cellular immune responses reflected by splenocytes and NK activities in pRSC-gC.gD-IL21 DNA/chitosan immunized mice were the strongest among 4 groups. Above results were consistent with the observations that the corneal pathological grade of pRSC-gC.gD-IL21 DNA/chitosan nanoparticle transfected cells could effectively produce fusion protein gD plus gC. These results demonstrated that gC based DNA vaccine was developed successfully.

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