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ARTICLE

Polysaccharide from Fruits of Physalis Alkekengi L. Enhances Antitumor Efficacy by a DNA Vaccine

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ABSTRACT

Physalis. alkekengi fruit has long been used in traditional Chinese medicine for tumor therapy. In the present study, using plasmids that encode ovalbumin (OVA) gene, we investigate the adjuvant activity of a polysaccharide fraction (PPSB) isolated from P.alkekengi fruit. Formulation by simple procedures of mixing of the OVA-encoding pCI-neo-sOVA plasmid with PPSB not only induced specific antibody responses, but also induced antigen-specific cytotoxic T lymphocyte (CTL) responses (Graph abstract). Furthermore, immunization using this vaccine prevented the growth of OVA-expressing B16-OVA tumor cell growth in the immunized mice. Thus, we provide evidence supporting the adjuvant activity of PPSB in DNA vaccine against tumor.

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1. Introduction

DNA vaccines are cost effective, heat-stable and safe and have been extensively investigated within the last two decades for the prevention or therapy of infectious diseases, allergy and tumors. Despite these advantages, the rather poor immunogenicity and inefficient delivery of DNA vaccines hindered its exploitation.[1] A promising strategy to augment potency of DNA vaccines is through improvement of adjuvants. Although a variety of chemical adjuvants or immunomudulatory molecules have been formulated into the DNA vaccines, different disadvantages with these adjuvants have also been observed.[2] In this study, we accessed the adjuvancity of the polysaccharide fraction (designated PPSB) isolated from fruit of Physalis alkekengi L. of the family of Solanaceae, which has been used historically in traditional Chinese medicine for its anti-inflammatory and anti-tumor activity.[3-5] This herbaceous perennial plant is native in southern Europe and Asia. The polysaccharide fraction isolated from P. alkekengi fruit has been characterized structurally and its anti-diabetic effect has also been evaluated by Tong etc. It is an acid heteropolysaccharide of 27kDa consisting of Ara, Gal, Glc and GalA in ratio of 2.6:3.6:2:1 and demonstrates potential of adjuvant in an anti-fungal vaccine formulation.[6] The present study was designed to test whether the polysaccharide molecule can enhance cellular immune responses as well. Moreover, we evaluated the anti-cancer potential of the molecule.

2. Materials and Methods

2.1 Preparation of Polysaccharide PPSB from Fruits of P. Alkekengi

The fresh fruits of P. alkekengi were identified by Prof. Dafang Cui of South China Agriculture University. The polysaccharide PPSB was isolated from fruits of P. alkekengi as described by Wang et al.[6] Briefly, fresh fruits of P. alkekengi were decocted in distilled water at 100◦C for 3 h. The crude polysaccharide (CP) was precipitated from the decoction with 85% ethanol. CP dissolved in distilled water was frozen at −20℃ and thawed for at least 3 times, during which the insoluble materials were removed by centrifugation. CP was further precipitated with 50% ethanol. The residue was discarded, and the supernatant was further precipitated with 70% ethanol to obtain precipitate (PPSA). The proteins in PPSA were removed by treatment with a combination of proteinase and followed by Sevag method. Then the PPSA was further purified through a Sepharose CL-6B column eluted with 0.15 mol/L NaCl, and the main polysaccharide fraction (PPSB) was collected, dialyzed and lyophilized. The solution of PPSB was filtered by 0.20 m filter. The endotoxin level was less than 10 pg/mL measured by Limulus amoebocyte lysate assay on a microbiology kinetic reader MB-80 (Goldstream, China).

2.2 Plasmids

The pCI-neo-sOVA plasmid, which encodes soluble chicken egg ovalbumin (OVA), was from Addgene (plasmid # 25098, Cambridge, MA). Plasmids were purified using a QIAGEN Midiprep kit according to the manufacturer's instruction (Valencia, CA). Large scale plasmid preparation was performed by Feiyang (Guangzhou, China).

2.3 DNA Immunization

The institute guidelines for animal use and care were followed in all animal studies. Female C57BL/6 mice, 6-8 weeks of age, were purchased from Medical Experimental Animal Center of Guangdong (Guangzhou, China). DNA immunization was completed under diethyl ether anesthesia. Plasmid DNA (pCI-neo-sOVA, 10 μg) mixed with or without 30 μg PPSB in 100 l of PBS was injected subcutaneously around the base of the tail. In the preliminary experiments, 30 μg PPSB as adjuvant resulted in the highest antibody titer among different doses and the peak of antibody titer occurred one week after the last immunization (data not shown). Thus, 30 μg PPSB/mouse was used in all animal experiments. Mice in the negative control group were left untreated. Mice were dosed three times, at an interval of 2 weeks.

2.4 Enzyme-linked Immunosorbent Assay (ELISA)

OVA-specific antibodies (IgG, IgG1, and IgG2b) in serum samples of the immunized mice were measured by an indirect ELISA as previously described.[7] Briefly, EIA/RIA flat bottom, medium binding, polystyrene, 96-well plates (Corning-Costar, Corning, NY) were coated with 5 g/ml of OVA proteins in 100 μl carbonated buffer (0.1 M, pH 9.6) at 4°C overnight. The plates were washed with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20) twice and then blocked with 4% (w/v) bovine serum albumin (BSA) in PBS/Tween 20 for 1 h at 37°C. Serum samples were diluted two or ten-fold serially in 4% BSA/PBS/Tween 20 and added to the plates in triplicate wells following the removal of the blocking solution. The plates were incubated for an additional 4 h at 37°C. The serum samples were removed, and the plates were washed 5 times with PBS/Tween 20. Horseradish peroxidase-labeled goat anti-mouse immunoglobulin (IgG and IgG1 from Shrbio, Nanjing, China, or IgG2a from Bio-Tc, Luoyang, China, 5000-fold dilution in 1% BSA/PBS/Tween 20) was added into the wells and the plate was incubated for another hour at 37°C. Plates were again washed four times with PBS/

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Tween 20, and 100 ml of 3, 3', 5, 5'-tetramethyl benzidine solution (TMB, Maibio, Shanghai, China) was added in each well at room temperature, followed by termination with the addition of 0.2 M sulfuric acid. The plate was read at 450/630 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT). Antibody titers were expressed as Log2 value of the highest dilution of serum that gave an absorbance value which exceeded an optical density value plus 2x standard deviation (S.D.) of the untreated mice.

2.5 Splenocyte Stimulation and Measurement of Cytokine Production

Two weeks after the last immunization, mice were sacrificed, and single-cell suspensions of splenocytes were prepared as previously described.[7] Briefly, spleens from each group of mice were collected aseptically and pooled together into 5 ml of HBSS (Hank's Balanced Salt Solution, 1x). Spleens were homogenized in RPMI1640 medium (Invitrogen, Carlsbad, CA) containing 2% fetal bovine serum (FBS, Thermo Scientific) with the plunger of a 1-mL syringe and followed by passing through a sterile 70 mm cell strainer for removing connective tissues and other debris. Red blood cell lysis was achieved by treating the cell suspension on ice for 5 min with Tris-NH4Cl buffer (0.75% NH4Cl and 0.205% KHCO3 in H2O, pH 7.2), followed by washing. The suspension was centrifuged at 800 rpm for 4 min at 4°C. After pouring off the supernatant, the cell pellet was re-suspended in of RPMI1640 medium supplemented with 10% FBS, 100 units/ml of penicillin (Invitrogen), 100 mg/ml of streptomycin (Invitrogen), 1% insulin-transferrin-selenium medium (Invitrogen) and 40 M of 2-mercaptoethanol (Sigma-Aldrich). Prepared splenocytes (5×106/well) were seeded into a 48-well plate in triplicate (n=3) and stimulated with 0 or 0.2 μM of SIIFEKL peptide (Genscript). After incubation at 37°C with 5% CO2 for 94 h, 80 μl of 5 mg/ml MTT (Thiazolyl blue tetrazolium bromide, Sigma-Aldrich) solution was pipetted into each well. After further 3 h of incubation at 37°C with 5% CO2, the plate was read at 490 nm. The cell proliferation index was reported as the ratio of the OD490 value of the stimulated cells (i.e. 0.2 μM of SIIFEKL peptide) over the OD490 value of un-stimulated cells. In addition, splenocytes (3 x 106 cells in 500 μl, n=4) were stimulated with 0 or 0.2 μM of SIIFEKL peptide for 48 h. The cells were spun down, and the supernatant was harvested for detectiong of IL-4 and IFN-levels using ELISA kits from Raybiotech (Atlanta, GA).

2.6 Tumor Prevention Assay

C57BL/6 mice were immunized with pCI-neo-sOVA plasmid s.c. for 3 times at an interval of 2 weeks as described above. One week after the last immunization, mice (n=5) were grafted s.c. in the right flank with 5 x 105 of OVA-expressing B16-OVA melanoma cells. Tumor volume was monitored every two days. Tumor size was calculated using the following formula: Tumor volume (mm3)= ½ [length x (width)2].

2.7 In Vivo Cytotoxic T Lymphocyte (CTL) Assay

An in vivo CTL assay was carried out as previously described[8] with a little modification. Briefly, C57BL/6 mice were immunized as described above. One week after the last immunization, splenocytes isolated from naïve C57BL/6 mice were pulsed with 0.2 μM SIINFEKL peptide (GenScript) in PBS for 45 min or left unpulsed. The pulsed cells were labeled with 10 μM of CFSE (CFSEhigh), while the unpulsed population was labeled with CFSE at a lower concentration of 1 μM (CFSElow). The two populations of cells were pooled together at a 1:1 ratio, from which, ten million cells were injected intravenously into the immunized mice. Mice were euthanized 16 h later, and the splenocytes were prepared and analyzed with a flow cytometer (BD, FACSCalibur) to determine the relative frequences of CFSEhigh and CFSElow populations. Specific lytic activity was calculated using the following equation:

$$\%\ \text{specific cell lytic activity} = \left(1 - \frac{\frac{CFSE^{low}_{untreated}}{CFSE^{high}_{untreated}}}{\frac{CFSE^{low}_{treated}}{CFSE^{high}_{treated}}}\right) \times 100$$

2.8 Statistical Analysis

Unless otherwise indicated, statistical significance was determined with a one-way analysis of variance followed by pair-wise comparisons with Fisher's protected least significant difference procedure. A p value of < to 0.05 (two-tail) was considered significant (*, p <0.05; **, p <0.01).

3. Results and Discussion

3.1 PPSB Augmented Both Antibody and Cellular Responses against OVA in Mice Immunized with OVA-encoding pCI-neo-sOVA Plasmid

Wang group has shown previously the adjuvant effect of PPSB in a DNA vaccine against systemic candidiasis which elicited a strong specific antibody response against a 47-kDa antigen, a breakdown product of heat shock protein 90 (HSP90). Thus, the protective efficacy against systemic candidiasis was also elevated by PPSB.[4] Whether this approach can enhance antigen-specific CD8+ T cell responses as well remains unresolved. We show herein that PPSB can augment both antibody and CTL responses. Mice were immunized subcutaneously with OVA-en-

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coding pCI-neo-sOVA plasmid with PPSB as adjuvant (designated plasmid/PPSB) as described above and boosted twice with the same regimen. Mice immunized with OVA-encoding pCI-neo-sOVA plasmid without PPSB (designated plasimid) or left untreated (UT) were used as controls. Blood samples were collected two weeks after the last immunization. The serum OVA-specific total IgG titier detected in the plasmid/PPSB group was significantly higher than that in the plasmid group (Figure 1A). Both IgG1 and IgG2a levels in mice serum were significantly elevated in plasmid/PPSB group compared with those in plasmid group (Figure 1B, 1C). This adjuvant effect is independent of the type of antigen encoded by the plasmid because the similar effect was also observed when PD-HSP90C plasmid with PPSB was immunized as previously reported.[4] No specific IgE antibody was detected (data not shown), indicating the lack of allergic responses.

To investigate whether PPSB affects the cellular immune responses induced by pCI-neo-sOVA immunization, splenocytes isolated from the immunized mice were re-stumulated in vitro with SIINFEKL peptide. Significant enhancement of both IFNg and IL-4 levels in culture supernatant of plasmid/PPSB group were detected in comparison with those of plasmid group (Figure 1D, 1E). In the classical Th1/Th2 paradigm, while IFNg elevation suggests the development of a type 1 CD4+ T helper cell (Th1) response, an elevation in IL-4 level is generally thought to indicate a Th2 response. But this useful paradigm is not able to account for results of many studies and thus cause controversial.[9] In addition, while IFNg plays a role in the activation of monocyte/macrophages, IL-4 is also a B cell growth and differentiation factor. When re-stimulated in vitro with SIINFEKL peptide, the proliferation of splenocytes from the mice of plasimid/PPSB group was significantly augmented compared with that of plasmid group (Figure 1F). Taken together, PPSB as adjuvant enhanced both the specific antibody and cellular responses in the present DNA vaccine formulation with pCI-neo-sOVA plasmid.

3.2 PPSB Enhanced the Antigen-specific CTL Responses and Prolonged the Survival of Tumor-bearing Mice

It is well accepted that cellular immune responses, especially CD8+ response, are major players in the anti-tumor defense. The present DNA vaccine also induced antigen-specific CTL responses. Cytotoxic CD8+ T cells are considered the principle effectors in protective immune responses against intracellular pathogens and tumor. Significantly higher CTL activity was detected in mice of plasmid/PPSB group than that of plasmid group (Figure 2).

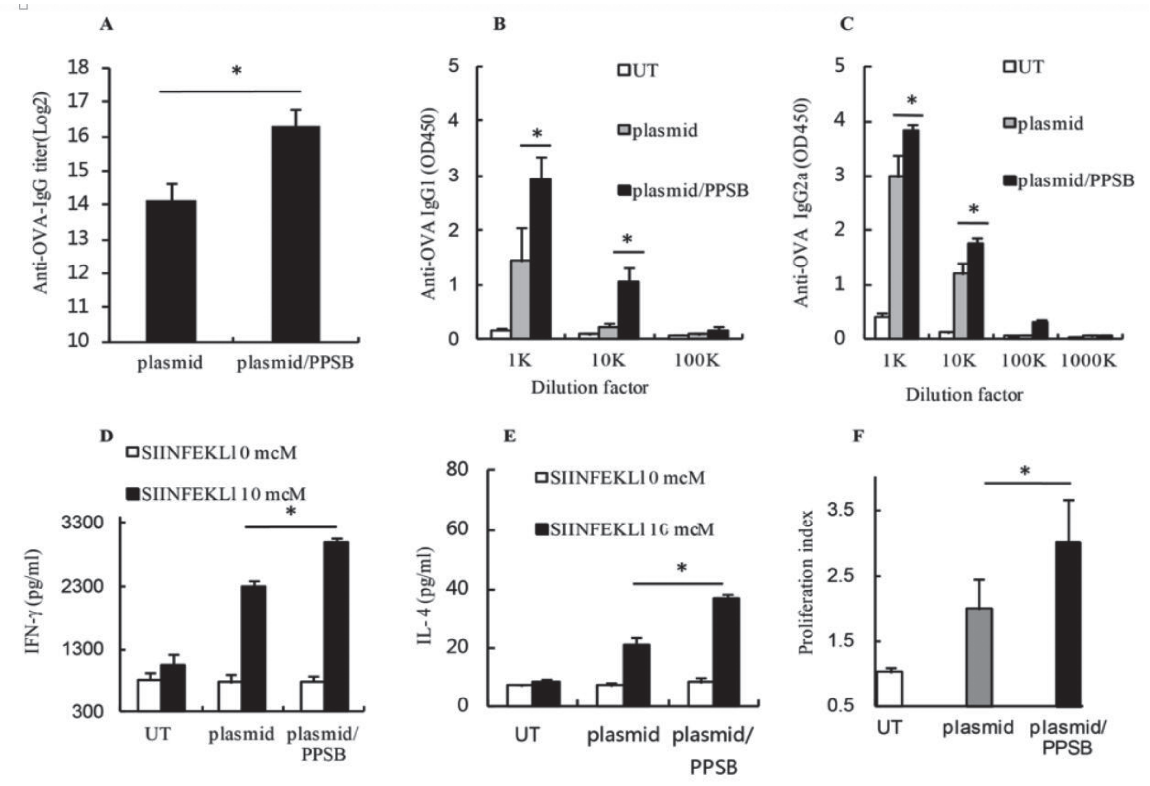

Figure 1

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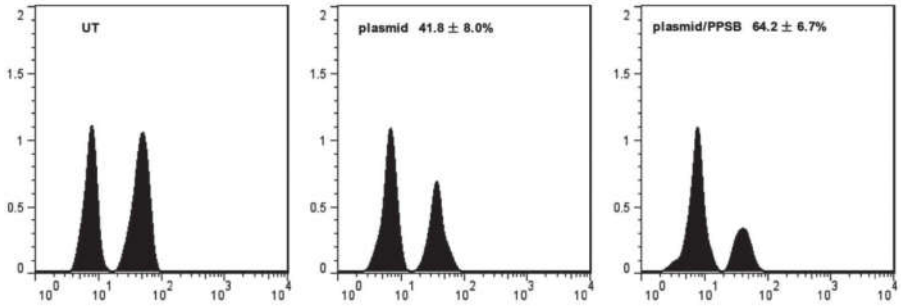

Figure 2

To determine the protective effects of the DNA vaccine combining pCI-neo-sOVA plasmid and PPSB, the immunized mice were challenged with B16-OVA tumor cells. The PPSB containing DNA vaccine formulation induced immune responses strong enough to prevent the growth of OVA-expressing B16-OVA cells (Figure 3). Thus, PPSB as adjuvant may potentially be used to elicit protective immunity against intracellular pathogens or tumors. Each time when the blood samples were collected, complete blood count was performed on XT-1800i heamatology analyzer (Sysmex, Japan), blood urea nitrogen and alanine aminotransferase were detected on Cobas c 702 chemistry analyzer (Roche, Switzerland) and no significant difference was found among groups of animals (data not shown), indicating good tolerability of the vaccine.

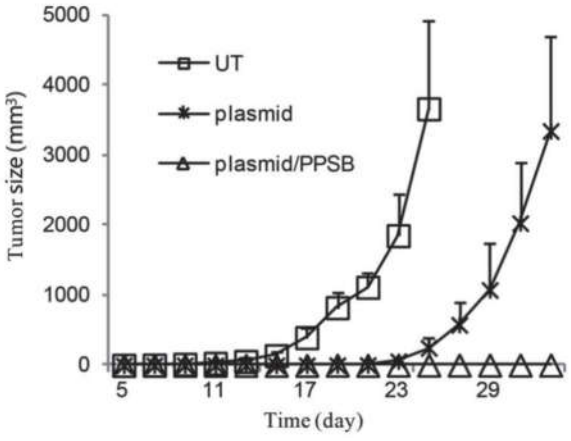

Figure 3

4. Discussion

DNA vaccines differ from other approaches of gene therapy in that they elicit tumor-specific immune responses, rather than directly kill tumor cells. Over the last decades, intensive preclinical studies have been performed using the DNA-based vaccine preparations. However, while most DNA-based antitumor vaccine formulations are well tolerated by animals and cancer patients, they often fail to generate therapeutically satisfied clinical responses.[10] And no DNA vaccine has been approved for human use by FDA so far. Improvement of adjuvant in the DNA vaccine formulations might be an attractive approach. Unfortunately, limited number of choices of adjuvant is available currently. Adjuvants often fail because of manufacture difficulties, lack of stability or effectiveness, tolerability or safety concerns. Adjuvants currently licensed for human use in the US and/or Europe includes aluminum salts, oil-in water emulsions (MF59, AS03 and AF03), virosomes and monophosphoryl lipid A (MPL, AS04). The inappropriate selection of adjuvant may render the vaccine inadequate. The classic aluminum hydroxide adjuvant is generally not optimal for eliciting cellular responses.[11] The oil-in-water emulsion preparations are not adequate for DNA vaccines. T cell responses are not optimally induced by these most commonly used adjuvants approved for use in human.[12] Thus, novel adjuvants appropriate for DNA vaccines are urgently needed. It is essential to avoid using undefined components in adjuvant formulations during vaccine development. Natural polysaccharides such as glucan from fungi or plants have been reported to be immunostimulatory.[13] And they are generally well-tolerated.[14] P. alkekengi grows in countries include China, Russia and Japan and has long been used in traditional Chinese medicine and as food in China. A polysaccharide PPSB isolated from P. alkekengi has been characterized by Wang group.[6] We are interested in the adjuvant activity of PPSB also because of the easy availability of the plant and low manufacturing cost of PPSB. Moreover, simple formulation procedures with PPSB are enough to create a vaccine which elicits considerable humoral and cellular immune responses. In the case of event of pandemic, large number of doses of vaccine is needed to cover the target population. For example, it is estimated approximately 1 billion doses of pandemic influenza vaccine could be produced currently, which is insufficient to cover the worldwide population.[15] In the future study, we plan to test the adjuvant activity of PPSB paired with protein antigen to

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explore its ability to increase manufacturing capacity by reducing the amount of antigen needed to induce immune responses desired.

5. Conclusion

The present study provides direct evidences for the adjuvant effect of PPSB which elevated both the humoral and cellular immune responses elicited by DNA vaccines. Antigen-specific CTL activity was enhanced by PPSB and the survival time of tumor-burden mice was significantly prolonged. In conclusion, PPSB is a potential adjuvant for DNA vaccines against tumor.

Abbreviations

OVA: ovalbumin;
CTL: cytotoxic T lymphocyte;
PPSB: polysaccharide isolated from fruits of P. alkekengi.

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