European Journal of Pharmaceutics and Biopharmaceutics 82 (2012) 212–217

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Note

Transcutaneous DNA immunization following waxing-based hair depilation elicits both humoral and cellular immune responses

Gang Xiao, Xinran Li, Amit Kumar, Zhengrong Cui *

The University of Texas at Austin, College of Pharmacy, Austin, TX, USA

ARTICLE INFO

Article history:
Received 26 March 2012
Accepted in revised form 25 June 2012
Available online 3 July 2012

Keywords:
Hair follicles
Antibody response
Cytokine release
In vivo CTL
Tumor prevention

ABSTRACT

Previously, we showed that transcutaneous (TC) DNA immunization by applying plasmid DNA onto a mouse skin area wherein the hair follicles were induced into growth stage by plucking the hair using warm waxing induced strong and functional antigen-specific antibody responses. In the present study, using plasmids that encode β-galactosidase gene or ovalbumin (OVA) gene, we showed that this mode of TC DNA immunization not only induced specific antibody responses, but also induced antigen-specific cytotoxic T lymphocyte responses. In fact, TC DNA immunization using a plasmid that encodes OVA gene prevented the growth of OVA-expressing B16-OVA tumor cells in the immunized mice. Moreover, we provided additional evidence supporting that hair follicles are essential for this mode of TC DNA immunization.

1. Introduction

Transcutaneous (TC) immunization has attracted much attention in recent years as a feasible route of immunization due to the abundance of antigen presenting cells (APCs) in the skin. In addition, as a non-invasive approach, TC immunization avoids the risks and complications that are associated with immunization by the traditional hypodermic needle injection, and promises to be cost-effective. Although TC immunization represents an attractive immunization modality, the difficulties in overcoming the stratum corneum barrier keep it a challenge. The feasibility of TC immunization with plasmid DNA was first confirmed by Shi et al. [1]. Fan et al. then reported that a successful TC DNA immunization is dependent on the presence of normal functional hair follicles [2]. Latest research reports confirmed the feasibility of enhancing the immune responses induced by TC DNA immunization by targeting the plasmid DNA into the hair follicles [2–11]. Whether hair follicles are 'open' or 'close' to penetration and uptake of topically applied substances into the skin is influenced by both hair growth and sebum flow [11]. Data from a previous study by Domashenko et al. showed that the growth stages of the hair follicles significantly influences follicular transfection of plasmid DNA applied topically onto the skin [12]; induction of hair follicles into growth stage by wax-based hair plucking significantly increases the transfection of cells in the hair follicles [12]. A typical hair cycle is divided into anagen (growth), catagen (a transitional period), and telogen (resting) stages. The highest uptake of substances applied topically onto the skin happens at the anagen stage [12]. The hair follicles in mice of 35–75 days of age are predominately in the second telogen stage, and they can be induced into growth stage by hair plucking [13]. Previously, we showed that naked plasmid DNA applied onto a mouse skin area wherein the hair follicles had been induced to growth stage by warm waxing-based plucking significantly enhanced the antibody responses against the antigen encoded by the plasmid [3]. Over the past two decades, data from numerous studies have documented that plasmid DNA vaccine can elicit both humoral and cellular immunities [14]. The present study was designed to test whether TC DNA immunization onto a skin area following warm waxing-based hair plucking can elicit cellular immune responses as well. Moreover, in order to further confirm the importance of functional hair follicles in TC DNA immunization, we evaluated the antibody responses induced in immunocompetent hairless SKH-1 mice. The hair on the SKH-1 mice is normal to 10 days of age, but is gone by 20 days of age [15].

2. Materials and methods

2.1. Plasmids

The pCMV-β plasmid (7164 bp), which encodes β-galactosidase (β-gal), was from the American Type Culture Collection (Manassas, VA). The pCI-neo-sOVA plasmid (6772 bp), which encodes soluble chicken egg OVA, was from Addgene (plasmid # 25098, Cambridge,

* Corresponding author. The University of Texas at Austin, Dell Pediatric Research Institute, 1400 Barbara Jordan Boulevard, Austin, TX 78723, USA. Tel.: +1 512 495 4758; fax: +1 512 471 7474.
E-mail address: Zhengrong.cui@austin.utexas.edu (Z. Cui).

0939-6411/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejpb.2012.06.012

MA). Plasmids were purified using a QIAGEN Midiprep kit according to the manufacturer's instruction (Valencia, CA). Large scale plasmid preparation was performed by GenScript (Piscataway, NJ).

2.2. TC DNA immunization by applying plasmid DNA onto a mouse skin area wherein the hair was depilated by warm-waxing

National Institutes of Health guidelines for animal use and care were followed in all animal studies. Animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Female C57BL/6 mice and SKH1-Elite mice were from Charles River Laboratories (Wilmington, MA). TC immunization was completed as previously described [3]. Mice (n = 5–10/group) were anesthetized, and the hair in the mid-dorsum was plucked with 'warm' wax (GiGi® Honee, American International Industries, Los Angeles, CA) in an area of ~1.5 cm^2. Two days after the plucking, mice were anesthetized again, and the plucked area was cleaned with 70% ethanol, hydrated for 20 min with warm water, and paper-dried. Plasmid DNA (pCMV-β or pCI-neo-sOVA, 50 μg) admixed with 10 μg cholera toxin (CT) (List Biological Laboratories, Campbell, CA) was gently dripped onto the hydrated area using a pipette tip. The applied area was allowed to air-dry and then carefully covered with a piece of Tegaderm™ self-adhesive dressing film (3 M, St. Paul, MN). Mice in the control group were injected intramuscularly (IM) with 50 μg of plasmid in phosphate buffered saline (PBS, pH 7.4, 10 mM). Mice in the negative control group were left untreated. Mice were dosed three times, around 2 weeks apart. Cholera toxin (CT) from *Vibrio cholera*, a heat-labile enterotoxin, is widely used experimentally as an adjuvant in mucosal and transcutaneous immunization [16]. It was used in the present study because data from a previous study of ours showed the CT enhances the immune responses induced by plasmid DNA applied topically onto the skin [17].

2.3. Enzyme-linked immunosorbent assay (ELISA)

Antibody responses in mice serum samples were determined by ELISA as previously described [3]. Briefly, EIA/RIA flat bottom, medium binding, polystyrene, 96-well plates (Corning-Costar, Corning, NY) were coated with 100 ng of β-gal or OVA proteins dissolved 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) and blocked with 4% (w/v) bovine serum albumin (BSA) in PBS/Tween 20 for 1 h at 37 °C. Serum samples were diluted two-fold or ten-fold serially in 4% BSA/PBS/Tween 20 and added to the plates 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, IgG1, or IgG2a, 5,000-fold dilution in 1% BSA/PBS/Tween 20, Southern Biotechnology Associates Inc., Birmingham, AL) was added into the wells, followed by another hour of incubation at 37 °C. Plates were again washed five times with PBS/Tween 20, and 100 ml of 3,3′,5,5′-tetramethyl benzidine solution (TMB, Sigma–Aldrich, St. Louis, MO) was added in each well, followed by the addition of 0.2 M sulfuric acid as the stop solution. The plate was read at 450 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT). Antibody titers were determined by comparing the optical density (OD) values of the samples with the OD value plus 2 × standard deviation (S.D.) (i.e., OD450 + 2 × S.D.) of the untreated mice.

2.4. Splenocyte proliferation and cytokine release from splenocytes

Two weeks after the last immunization, mice from each group were euthanized, and spleen cells were isolated as previously described [18]. Briefly, spleens removed aseptically from each group of mice were pooled together and placed into 5 ml of HBSS (Hank's Balanced Salt Solution) (1×). Spleens were homogenized in PRMI1640 medium (Invitrogen, Carlsbad, CA) containing 2% fetal bovine serum (FBS, Sigma–Aldrich) by pressing through a sterile cell strainer with a syringe plunge to remove connective tissues and other debris. Red blood cells were removed by treating on ice for 5 min with Tris–NH_4Cl buffer (0.75% NH_4Cl and 0.205% $KHCO_3$ in H_2O, pH 7.2). The suspension was spun down at 800 rpm for 4 min at 4 °C. After pouring off the supernatant, the cell pellet was re-suspended in 15 ml of RPMI1640 medium supplemented with 10% FBS, 100 units/ml of penicillin (Invitrogen), 100 μg/ml of streptomycin (Invitrogen), and 40 μM of 2-mercaptoethanol (Sigma–Aldrich). Isolated splenocytes (5×10^6/well) with three replicates (n = 3) were seeded into a 48-well plate and stimulated with 0 or 10 μg/ml of β-galactosidase (Spectrum Chemical, New Brunswick, NJ). After incubation at 37 °C with 5% CO_2 for 94 h, 80 μl of 5 mg/ml MTT (thiazolyl blue tetrazolium bromide, Sigma–Aldrich) solution was pipetted into each well. After an additional 3 h of incubation at 37 °C with 5% CO_2, the OD value at 490 nm was measured. The cell proliferation index was reported as the ratio of the OD490 value of the stimulated cells (i.e., 10 μg/ml of β-galactosidase) over the OD490 value of un-stimulated cells (i.e., 0 μg/ml of β-galactosidase). In addition, splenocytes (3×10^6 cells in 500 μl, n = 4) were stimulated with 0 or 10 μg/ml of β-galactosidase for 48 h. The cells were spun down, and the interleukin-4 (IL-4) and interferon (IFN)-γ levels in the supernatant were measured using ELISA kits from Raybiotech (Atlanta, GA).

2.5. Tumor prevention assay

C57BL/6 mice were immunized with pCI-neo-sOVA plasmid by TC or IM routes for 3 times at an interval of 2 weeks as described above. Two weeks after the last immunization, mice (n = 5) were injected subcutaneously in the right flank with 5×10^5 of OVA-expressing B16-OVA melanoma cells (kindly provided by Dr. Edith M. Lord and Dr. John Frelinger from the University of Rochester Medical Center) [19]. Tumor growth was monitored every other day. Mice were euthanized when tumor size reached 15 mm (longest diameter). Tumor size was reported based on the following equation: Tumor volume (mm^3) = ½ [length × $(width)^2$].

2.6. In vivo cytotoxic T lymphocyte (CTL) assay

In vivo CTL assay was carried out as previously described [18]. Briefly, C57BL/6 mice were immunized as described above. On day 42, 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 cell population was labeled with 10 μM of carboxyfluorescein diacetate succinimidyl ester ($CFSE^{high}$), while the unpulsed population was labeled with CFSE at a lower concentration of 1 μM ($CFSE^{low}$). 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 applied to a flow cytometer (Milipore Guava easycyte 8HT, Billerica, MA) to determine the relative abundance of $CFSE^{high}$ and $CFSE^{low}$ populations. Specific lytic activity was calculated according to the following equation: