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Transcutaneous DNA immunization following waxing-based hair depilation

Brian R. Sloat [a], Kaoru Kiguchi [b], Gang Xiao [a], John DiGiovanni [b], Wendy Maury [c], Zhengrong Cui [a,*]

[a] The University of Texas at Austin, College of Pharmacy, Pharmaceutics Division, Austin, Texas, 78712, United States
[b] The University of Texas at Austin, College of Pharmacy, Pharmacology & Toxicology Division, Austin, Texas, 78712, United States
[c] University of Iowa, Carver College of Medicine, Department of Microbiology, Iowa City, IA 52242, United States

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ABSTRACT

Transcutaneous DNA immunization is an attractive immunization approach. Previously, we reported that transcutaneous immunization by applying plasmid DNA onto a skin area wherein the hair follicles had been induced into growth stage by 'cold' waxing-based hair plucking significantly enhanced the resultant immune responses. In the present study, using a plasmid that encodes the *Bacillus anthracis* protective antigen (PA63) gene fragment, it was shown that the anti-PA63 antibody responses induced by applying the plasmid onto a skin area where the hair was plucked by 'warm' waxing were significantly stronger than by 'cold' waxing, very likely because the 'warm' waxing-based hair depilation significantly i) enhanced the uptake (or retention) of the plasmid in the application area and ii) enhanced the expression of the transfected gene in the follicular and interfollicular epidermis in the skin. The antibody response induced by transcutaneous DNA immunization was hair cycle dependent, because the plasmid needed to be applied within 5 days after the hair plucking to induce a strong antibody response. The antibody responses were not affected by whether the expressed PA63 protein, as an antigen, was secreted or cell surface bound. Finally, this strategy of enhancing the immune responses induced by transcutaneous DNA immunization following 'warm' waxing-based hair depilation was not limited to the PA63 as an antigen, because immunization with a plasmid that encodes the HIV-1 *env* gp160 gene induced a strong anti-gp160 response as well. Transcutaneous DNA immunization by modifying the hair follicle cycle may hold a great promise in inducing strong and functional immune responses.

1. Introduction

Transcutaneous immunization onto the skin has become a favorable route for vaccine administration over the traditional use of needles and syringes. The feasibility of transcutaneous immunization using plasmid DNA was proven in the 1990s, but the resultant immune responses were generally weak [1,2]. Several approaches have been taken to enhance the immune responses from transcutaneous DNA immunization, including physical or chemical disruption of the stratum corneum [3,4], the use of vaccine adjuvants or skin permeation enhancers [5,6], or formulating the plasmid into carrier systems such as nanoparticles or emulsions [7,8]. However, all these approaches have had only limited success at enhancing the immune responses.

Data from several studies have shown that the main portals of entry for plasmid DNA applied onto the skin are the hair follicles, and the transfected gene expression was mainly confined to the hair follicles [9–13]. The penetration or uptake of substances applied onto the skin via the hair follicles is reported to be dependent on whether the hair follicles are in growing or resting stages [14]. Hair follicles are open for penetration by foreign objects when in the growth (or anagen) stage, and closed when in the resting (or telogen) stage [14]. Domashenko et al. (2000) reported that the stage of the hair follicle cycle at the time of DNA application is critical for the expression of transfected genes [10]; the highest expression occurred when the plasmid was applied during the anagen stage when cells in the hair follicle are proliferating [10]. Fan et al. (1999) reported that functional hair follicles were required for a topically applied DNA vaccine to induce immune responses [11]. Based on the aforementioned findings, we hypothesized, and provided data to support, that transcutaneous immunization by applying plasmid DNA onto a skin area wherein the hair follicles were induced into growth stage by hair plucking elicited a stronger antibody response than when the hair follicles in the application area were in the resting stage [15].

In our previous study, the hair in the application area was plucked by 'cold' waxing. In the present study, the immune responses induced by plasmid DNA applied onto a skin area wherein the hair had been plucked using a 'warm' waxing technique were evaluated. 'Cold' waxing comes in the form of a pre-waxed strip, which is placed over the desired, hair-trimmed skin area and is rapidly removed, resulting in significant breakage of the hair at or below the surface of the skin. In contrast,

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

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in 'warm' waxing, wax is applied directly onto the hair in the desired skin area in a viscous liquid form; and the warmth of the wax is expected to allow the hair follicle pores to expand and the complete removal of the hair from the follicles. Therefore, we reasoned that the 'warm' waxing-based hair depilation would allow plasmid DNA applied onto the skin to induce a stronger immune response than the 'cold' waxing-based hair depilation, because the former not only induces the hair follicles into growth stage, but is also expected to lead to the better removal of hair from the hair follicles, and thus, leaves the hair follicles open for penetration by the topically applied plasmid DNA.

Although mouse hair cycling is dependent on genetic background (strain), sex as well as environmental and nutritional factors, in general, after the removal of hair on the dorsal skin of 6–7 week old mice, days 0 through 2 are the early anagen stage, where upregulated genes were mainly related to dermal inflammation [16,17]. Days 3 through 6 are the mid-anagen stage where up-regulated genes mainly functioned in cell proliferation [16]. Late anagen and early catagen stages start around day 7, and the telogen stage starts around day 25 [17]. To identify the optimal time to apply the plasmid DNA after hair plucking, the dependence of time, at which the plasmid DNA was applied onto the skin following 'warm' waxing-based hair depilation, on the resultant immune responses was evaluated. The *Bacillus anthracis* PA63 protein encoding plasmid, pGPA, was used in the aforementioned studies. The pGPA construct has a signal sequence of human plasminogen activator fused to the N-terminus of the PA63 protein, and thus, resulting in PA63 secretion by transfected cells [18]. To determine the extent to which the secretion of the PA63 protein by the transfected cells contributed to the resultant immune response, pCPA, another PA63 plasmid that does not allow the secretion of the PA63 by transfected cells, was used to immunize mice [19]. Finally, to test whether the 'warm' waxing-based hair depilation approach of enhancing the immune response induced by transcutaneous DNA immunization is applicable to an antigen other than the PA63, the immune responses induced by a plasmid that encodes the HIV-1 full-length gp160 *env* gene (p96ZM651gp160-opt) were evaluated as well [20].

2. Experimental section

2.1. Plasmids

The pGPA plasmid, constructed by inserting the gene fragment encoding amino acids 173–764 of the *B. anthracis* PA gene into pJW4304, was kindly provided by Dr. Dennis Klinman [18]. This portion of the PA protein represents the protease-cleaved fragment (PA63) of the full-length protein that is active in vivo [18]. The pCPA plasmid was constructed following a previously published method with slight modifications [19]. Briefly, the gene fragment encoding amino acid 173–764 of PA gene was amplified from the pGPA plasmid using polymerase chain reaction (PCR) (primers, 5′-ACAAGTCTCGAGCCTACGCTTCCA-3′ and 5′-CCTAGATCTAGATTATCCTATCTCATAGCC-3′). The PCR product was digested with *XhoI* and *XbaI*, ligated into the pCI mammalian expression vector (Promega, Madison, WI), and transferred into *Escherichia coli* DH5α. The HIV-1 full-length gp160 *env* gene-encoding plasmid, p96ZM651gp160-opt, was from the NIH AIDS Research & Reference Reagent Program (Germantown, MD). The p96ZM651gp160-opt was constructed by ligating codon-optimized full-length gp160 gene into the pcDNA3.1(−) expression vector [20]. The pCMV-β was from the American Type Culture Collection (Manassas, VA) [21]. Plasmid was 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. Application of plasmid DNA onto mouse skin

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 BALB/c mice (n = 5–10), 6 weeks of age, were from Charles River Laboratories (Wilmington, MA). The 6-week old mice were used because the hair follicles on the dorsal skin of mice of 35–75 days of age are predominately in the second telogen phase so that they can be induced into growth stage by plucking [22,23]. The hair in the mid-dorsum of anesthetized mice was plucked with 'warm' wax (50–55 °C, GiGi® Honee, American International Industries, Los Angeles, CA) in an area of ~1.5 cm^2. As controls, hair was either trimmed with an electric clipper, or trimmed and then plucked with 'cold' wax (Veet® wax strips, Reckitt Benckiser, Parsippany, NJ). At predetermined time points (0, 2, 5, or 10 days later), the hair plucked area of anesthetized mice was cleaned with 70% ethanol swab, hydrated for 20 min with warm water, and paper-dried. Plasmid DNA (pGPA, pCPA, or p96ZM651gp160-opt, 50 μg) was admixed with cholera toxin (CT, 10 μg, List Biological Laboratories, Campbell, CA) and gently dripped onto the hydrated area using a pipette tip. CT was added into the plasmid DNA solution because data from one of our previous studies showed that CT significantly improved the antibody responses induced by plasmid DNA vaccine applied topically onto mouse skin [5]. 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) to keep the plasmid DNA in the application area for an extended period of time [24–26]. Mice in the positive control groups were either injected intramuscularly with 50 μg of plasmid in phosphate buffered saline (PBS, pH 7.4, 10 mM) or subcutaneously injected with protective antigen protein (PA, 5 μg/mouse, List Biological) in 50 μL of PBS admixed with incomplete Freund's adjuvant (IFA, 100 μL, Sigma-Aldrich, St. Louis, MO). Mice in the negative control group were left untreated. Mice were dosed on days 0, 14, and 28 on three different areas on the dorsal skin, and euthanized and bled on day 49 or where mentioned.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The levels of anti-PA and anti-gp160 in serum samples were determined using ELISA [15]. Briefly, EIA/RIA flat bottom, medium binding, polystyrene, 96-well plates (Corning-Costar, Corning, NY) were coated with 100 ng of PA (List Biological) or the gp140 protein (NIH AIDS Research & Reference Reagent Program) 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 serially (or as indicated) in 4% BSA/PBS/Tween 20, added to the plates following the removal of the blocking solution, and incubated for 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 immunoglobulins (IgG, IgG1, or IgG2a, 5000-fold dilution in 1% BSA/PBS/Tween 20, Southern Biotechnology Associates Inc., Birmingham, AL) were added into the wells, followed by another hour of incubation at 37 °C. Plates were again washed five times with PBS/Tween 20. The presence of bound antibody was detected following a 30 min incubation at room temperature in the presence of 3, 3′, 5, 5′-tetramethyl benzidine solution (TMB, Sigma-Aldrich), followed by the addition of 0.2 M sulfuric acid as the stop solution. The absorbance was read at 450 nm. Antibody titers were derived by comparing the OD450 nm values of the samples with the OD450 nm plus 2 x S.D. of the untreated mice.

2.4. Anthrax lethal toxin neutralization activity assay

To evaluate the functionality of the anti-PA antibodies against anthrax lethal toxin, a toxin neutralization assay was performed as previously described [27]. Briefly, confluent J774A.1 cells (1×10^4) were plated into sterile, 96-well, clean-bottom plates and incubated at 37 °C, 5% CO_2 for 24 h. A solution (50 μL) containing PA (400 ng/mL) and lethal factor (LF, List Biological, 100 ng/mL) was mixed with