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

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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.

Keywords

Hair follicles; antibody response; cytokine release; and *in vivo* CTL; tumor prevention

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.* (1999) [1]. Fan *et al.* (1999) 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.* (2000) showed that the growth stages of the hair follicles significantly influences follicular transfection of plasmid DNA applied topically

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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 immuno-competent 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 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 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 $\sim 1.5 \text{ cm}^2$. 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 (3M, 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 μ l 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 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 μ l 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 x standard deviation (S.D.) (i.e., $OD_{450} + 2 \times S.D.$) 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 $\times$). Spleens were homogenized in RPMI1640 medium (Invitrogen, Carlsbad, CA) containing 2% fetal bovine serum (FBS, Sigma-Aldrich) by pressing through a sterile cell strainer with a syringe plunger to remove connective tissues and other debris. Red blood cells were removed by treating on ice for 5 min with Tris-NH₄Cl buffer (0.75% NH₄Cl and 0.205% KHCO₃ in H₂O, 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 μ g/ml of streptomycin (Invitrogen), and 40 μ M 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 μ g/ml of β -galactosidase (Spectrum Chemical, New Brunswick, NJ). After incubation at 37°C with 5% CO₂ for 94 h, 80 μ l 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% CO₂, 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 μ g/ml of β -galactosidase) over the OD490 value of un-stimulated cells (i.e., 0 μ g/ml of β -galactosidase). In addition, splenocytes (3×10^6 cells in 500 μ l, $n = 4$) were stimulated with 0 or 10 μ g/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 ($n = 4-5$) 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) = $\frac{1}{2}$ [length $\times$ (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 μ M SIINFEKL peptide (GenScript) in PBS for 45 min or left unpulsed. The pulsed cell population was labeled with 10 μ M of carboxyfluorescein diacetate succinimidyl ester (CFSE^{high}), while the unpulsed population was labeled with CFSE at a lower concentration of 1 μ M (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:

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

2.7. Statistical analysis

Except where mentioned, statistical analyses were completed by performing a one-way analysis of variance followed by pair-wise comparisons with Fisher's protected least significant difference procedure. A *p* value of ≤ 0.05 (two-tail) was considered significant.

3. RESULTS AND DISCUSSION

Previously, we have shown that TC immunization through the hair follicles by applying plasmid DNA onto a skin area wherein the hair was plucked by warm-waxing induced a strong antigen-specific antibody response [3]. It remains unknown whether this mode of TC immunization can induce antigen-specific cellular immune responses as well. Here we demonstrate that this mode of TC DNA immunization can elicit both humoral and cellular immune responses.

Mice were transcutaneously immunized using pCMV- β admixed with CT in a skin area where the hair was plucked and boosted twice with the same regimen. Intramuscular injection of the same amount of plasmid DNA, without CT, was used as a positive control. The level of serum anti- β -gal IgG detected in the TC group was not different from that in mice in the IM group (Fig. 1A, *p* = 0.38). However, the level of serum anti- β -gal IgG1 detected in the TC group was significantly higher than that in the IM group (*p* < 0.001), whereas the level of anti- β -gal IgG2a was significantly higher in IM group than in the TC group (*p* < 0.001) (Figures. 1B–C), indicative of a slightly IgG1-biased antibody response induced by TC DNA immunization following hair plucking. This response is independent of the antigen encoded by the plasmid because the antibody responses induced by TC immunization with the OVA-encoding pCI-neo-sOVA plasmid were slightly IgG1-biased as well (data not shown). In both IM and TC groups, antigen-specific IgE was not detected (data not shown), suggesting the lack of allergic responses. Moreover, strong anti-CT IgG was also detected in the serum of mice transcutaneously immunized with the pCMV- β mixed with CT (data not shown), suggesting that this mode of TC immunization following hair plucking may be applicable to subunit protein-based vaccines as well.

Splenocytes isolated from mice in the TC group secreted high levels of both IL-4 and IFN- γ after *in vitro* re-stimulation with β -galactosidase, whereas the ones isolated from mice in the

IM group only secreted IFN- γ , but not IL-4 (Figures. 1D-E). While IFN- γ secretion indicates the development of a type 1 CD4⁺ T helper cell (Th1) response, an elevation in IL-4 level indicates the development of a Th2 response. In addition, the splenocytes from mice in both TC and IM groups proliferated significantly after *in vitro* re-stimulation with β -galactosidase, to a larger extent in the TC group (Fig. 1F). Taken together, it appears that TC DNA immunization induced strong Th1 and Th2-balanced responses, whereas IM immunization induced a more Th1-biased response. This finding is in agreement with data from previous studies showing that DNA immunization onto the skin using 'gene gun' tends to induce a stronger Th2 response than IM injection of the DNA [20]. Generally, TC DNA immunization tends to favor the induction of a Th1/Th2 balanced immune response [14]. Accordingly, TC DNA immunization might inherently benefit immunotherapies targeting diseases that need strong Th1 and Th2 responses.

Importantly, TC DNA immunization on a mouse skin area wherein the hair was plucked by warm waxing also induced antigen-specific CTL responses (Fig. 2), and the OVA-specific CTL responses were strong enough to prevent the growth of OVA-expressing B16-OVA tumor cells in the immunized mice (Fig. 3). Therefore, TC DNA immunization via growth stage hair follicles can induce antigen-specific cellular immune responses as well. Cytotoxic CD8⁺ T cells are considered the principle effectors for stopping intracellular pathogens and in tumor immunotherapy. This method of TC DNA immunization may potentially be used to elicit immunity against intracellular pathogens or tumor cells.

In the present study, we also further confirmed the importance of normal hair follicles in the induction of immunity by TC immunization onto a skin area wherein the hair was plucked by warm waxing by using the immuno-competent SKH-1 hairless mice. As shown in Fig. 4, TC immunization using pCMV- β plasmid admixed with CT failed to induce an anti- β -gal IgG response in SKH-1 mice, whereas SKH-1 mice that were injected (IM) with the same plasmid developed a strong anti- β -gal IgG response. Hair follicles as the main portals of entry for topically applied DNA have been well established [2, 12, 21]. The data from the SKH-1 hairless mice further confirm that the hair follicles play a pivotal role in this mode of TC DNA immunization strategy.

It is important to note that our warm waxing-based hair plucking procedure only presents one of the methods to enhance the delivery of a (DNA) vaccine to hair follicles. Another reported method to target vaccines to the hair follicles is the cyanoacrylate skin surface stripping (CSSS) [5–8, 11, 22]. The CSSS involves painting razor-shaved skin area with cyanoacrylate superglue, followed by the application of an adhesive tape. After the hardening of the glue for 20 min, the tape and glue are removed [6]. The CSSS removes approximately 30% of the stratum corneum, including mild stratum corneum barrier disruption [6]. It also removes cellular debris and sebum from the hair follicles, increasing the number of hair follicles available for penetration [6, 11]. The CSSS method has proven to be rather effective, and recent clinical trial data using inactivated influenza vaccine are promising [5, 6]. Our method of hair plucking by warm waxing is different from the CSSS. In our method, before the warm waxing, mouse hair is intact. A small quantity of the GiGi[®] Honee wax is applied to the hair in the desired area. The area is then covered by a muslin strip. After a few second to allow the wax to cool, the strip is rapidly pulled away. Due to the thick coat of hair on mice, if the waxing is not repeated in the same area, the wax never directly touches the skin. In a previous study, we have investigated the mechanisms underlying the enhancement of immune responses induced by TC DNA immunization following the warm waxing-based hair plucking [3]. The plucking did not physically damage the skin. The transepidermal water loss (TEWL) value in the skin area at the time of the DNA application (i.e., 2 days after the hair plucking) was not different from that in the skin area where the hair was simply trimmed using an electric clipper 2 days earlier [3].

However, mild to moderate local inflammation was induced by the plucking, as demonstrated by the increased thickness of the epidermis and the increased cellularity in the dermis in the plucked area [3]. Furthermore, we have shown that the hair plucking significantly increased the uptake or retention of the plasmid DNA in the application area [3], and also significantly increased the expression of the antigen gene encoded by the plasmid in the interfollicular and follicular epidermis [3]. It is unclear whether the plucking has removed any sebum from the hair follicles. We do not believe that the physical removal of the stratum corneum layers by the plucking, if any, was extensive, because the warm wax-based plucking on immuno-competent hairless mouse skin did not help subsequently topically applied plasmid DNA to induce detectable specific antibody responses (Fig. 4). Therefore, it is very likely that when plasmid DNA is applied onto a skin area wherein the hair has been plucked by warm waxing, the DNA enters the skin and accesses live cells in the epidermis mainly through the hair follicles. Concerning follicular penetration, the size of the penetrating substances is of prime importance [7, 9]. There is little evidence that particles with a size of larger than 100 nm penetrate intact skin [9, 23], but it was reported that the CSSS procedure makes it possible for particles as large as ~290 nm to penetrate hair follicles [9]. The plasmids used in the present study were 6–7 kb. When dissolved in water, plasmid DNA can stretch into various geometric shapes, including linear fibers and closed circles [24]. It was shown that circular plasmid DNA is stretched closest to a theoretical size of 34 nm per 100 kb [24]. Assuming that the pCMV- β and pCI-neo-sOVA plasmids formed closed circles, theoretically, their geometric sizes should be around 2.0–2.5 nm. Therefore, they should be able to readily penetrate ‘open’ hair follicles.

Finally, it is also important to note that water hydration, alcohol cleaning, the occlusion by the Tegaderm patch used in the TC immunization procedure are all expected to have played a role in assisting the plasmid DNA to induce a strong immune response. In addition, the CT as an adjuvant is important for the TC DNA to induce a strong immune response [3, 17]. Unfortunately, intact CT is unlikely to find a place as an adjuvant for human use, even topically on the skin, because of its high toxicity. However, various researches continue to divorce the adjuvant activity and toxicity of CT. The use of nontoxic B subunit of CT alone (CTB), B subunit containing a trace amount of toxin, and chemically modified or mutated CT are a few of the options, which can retain the adjuvant property of CT while avoiding or minimizing its toxicity [25–28]. In fact, we have evaluated and compared CT and CTB as adjuvants in TC DNA immunization following hair plucking. The CTB also significantly improved the antibody responses (i.e., anti-OVA IgG) induced by plasmid DNA (i.e., pCI-neo-sOVA) applied onto a mouse skin area wherein the hair had been plucked by warm waxing, although the CTB was not as potent as the whole CT (data not shown). Increasing the dose of the CTB in future studies may help.

CONCLUSIONS

In the present study, we showed that TC DNA immunization onto a skin area wherein the hair was plucked by warm waxing induced not only antibody responses, but also cellular immune responses. Furthermore, using immuno-competent hairless SKH-1 mice, we provided additional evidence supporting the importance of normal and functional hair follicles in TC DNA immunization without physically or chemically disrupting the stratum corneum. Future studies may include the testing of the feasibility of this mode of TC DNA immunization in farm animals, such as sheep or chicken, and ultimately in humans. In addition, convenient methods that can reliably induce the hair follicles in the desired area into growth stage without disrupting the stratum need to be developed.

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Fig. 1. Immune responses after TC immunization with pCMV-β plasmid
 (A–C) Anti-β-gal total IgG (A), IgG1 (B), and IgG2a (C) induced by pCMV-β plasmid applied onto a skin area where the hair was plucked by warm waxing. Mice in control groups were intramuscularly (IM) injected with pCMV-β or left untreated (UT). (D–E) Secretion of IL-4 (D) and IFN-γ (E) by splenocytes isolated from mice immunized with pCMV-β after *in vitro* re-stimulation. (F) Proliferation of splenocytes isolated from mice immunized with pCMV-β after *in vitro* re-stimulation. Mice were dosed on days 0, 13, and 28, and euthanized on day 93 to collect blood and spleen. Splenocytes were incubated in the presence of 0 or 10 μg/ml of β-galactosidase for 48 h before measuring the concentrations of IL-4 and IFN-γ in the supernatant, and for 96 h before measuring the number of splenocytes (UT, untreated; IM, intramuscular injection; TC, transcutaneous immunization). Immune responses were detected in 4 of 6 mice in the TC group, and 6 of 10 mice in the IM group. Data reported in A–C are mean ± S.D. (n = 4–6). Data in D–E are mean ± S.D. from 5 different measurements, whereas data in F are from 3 different measurements. (* p < 0.05, TC vs. IM after stimulation with β-galactosidase).

Fig. 2. TC immunization with pCI-neo-sOVA induced OVA-specific CTL responses

Mice were immunized on days 0, 17, and 30, CTL activity was measured on day 57. Shown are typical histograms. Numbers are the mean of percent of cell lytic activities determined from 3-5 mice per group. Before the CTL assay, mice were bled to collect serum sample. Only those mice that showed anti-OVA antibody responses were used for the CTL assay.

Fig. 3. TC immunization with pCI-neo-sOVA prevented the growth of OVA-expressing B16-OVA tumor cells

C57BL/6 mice were immunized for 3 times with pCI-neo-sOVA transcutaneously (TC) or by IM injection on days 0, 13, and 27. Mice in the control group were left untreated. Five weeks after the last immunization, mice ($n = 4-5$) were injected subcutaneously with B16-OVA cells. Before tumor cell injection, mice were bled to collect serum sample. Only those mice that showed anti-OVA antibody responses were used for the tumor prevention study. Data shown are mean $\pm$ S.D.

Fig. 4. TC DNA immunization by applying pCMV-β onto SKH-1 hairless mice failed to induce β-galactosidase-specific antibody response

SKH-1 mice ($n = 5$) were immunized for 3 times with pCMV-β transcutaneously (TC) or by IM injection on days 0, 16, and 30. Mice in the control group were left untreated. For the TC groups, the hair in one group of mice was simply trimmed (Trimming), while the hair in the other group was plucked by warm waxing (Plucking), 48 h before dosing with plasmid DNA. Data (mean $\pm$ S.D.) are after the serum samples collected on day 48 were diluted 100-fold. (*, $p < 0.05$, IM vs. UT).