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The extent of the uptake of plasmid into the skin determines the immune responses induced by a DNA vaccine applied topically onto the skin

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Abstract

Objectives—Non-invasive immunization by applying plasmid DNA topically onto the skin is an attractive immunization approach. However, the immune responses induced are generally weak. Previously, we showed that the antibody responses induced by topical DNA vaccine were significantly enhanced when hair follicles in the application area were induced into anagen (growth) stage by hair plucking. In the present study, we further investigated the mechanism of immune enhancement.

Methods—Three different methods, hair plucking or treatment with retinoic acid (RA) or O-tetradecanoylphorbol-13-acetate (TPA), were used to induce hair follicles into anagen stage before mice were dosed with a β -galactosidase-encoding plasmid, and the specific antibody responses induced were evaluated.

Key findings—The hair plucking method was more effective at enhancing the resultant antibody responses. Treatment with RA or TPA caused more damages to the skin and induced more severe local inflammations than hair plucking. However, hair plucking was most effective at enhancing the uptake or retention of the DNA in the application area.

Conclusions—The uptake of plasmid DNA in the application area correlated with the antibody responses induced by a topically applied DNA.

Keywords

hair plucking; RNA Replicase; immunogenicity; inflammation; skin integrity

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Declarations

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

Introduction

Non-invasively applying a vaccine topically onto the skin is an attractive immunization modality. DNA vaccine is a promising new generation vaccine. The feasibility of topical immunization with plasmid DNA was proven, but the resultant immune responses are generally weak.^[1, 2] To enhance the immune responses, many approaches have been adopted, including physical disruption of the stratum corneum by tape-stripping or metal brushing,^[3, 4] co-administration of vaccine adjuvants (e.g., cholera toxin (CT))^[5] or skin permeation enhancer (e.g., dimethyl sulfoxide),^[6] or the delivery of DNA using carrier systems such as nanoparticles, liposomes, microemulsions, or nanoemulsions.^[7, 8] Unfortunately, all these approaches had only limited success.

Data from previous studies pointed out that hair follicles were the site for the uptake of topically applied DNA.^[9–13] It was reported that when plasmid was applied onto the skin, the expression of the gene of interest encoded by the plasmid was mainly in the keratinocytes in hair follicles.^[10] It was also known that only anagen stage hair follicles were open to penetration by foreign objects on the skin surface,^[14] and that the expression of a topically applied reporter gene in the skin was significantly enhanced when the hair follicles in the plasmid application area were induced into growth stage.^[11] Therefore, it was not surprising to see a report showing that normal hair follicles were required for topical DNA vaccine to induce immune responses.^[12] Based on these findings we hypothesized, and generated data to support, that applying a DNA vaccine onto a skin area wherein the hair follicles were induced into anagen-onset stage induced a stronger immune response than when the hair follicles in the application area were in telogen or resting stage.^[13] Using an anthrax protective antigen (PA63) protein-encoding plasmid, pGPA, we showed that the anti-PA antibody responses induced by topical pGPA were significantly enhanced when the hair follicles in the application area were induced into growth stage by hair plucking.^[13] The integrity of the skin in the application area was not significantly compromised at the time of DNA application. However, mild to moderate local dermal inflammation was evident in the plucking area at the time of the DNA application, and the plucking enhanced the expression of the PA63 gene in the skin.^[13]

In the present study, we sought to identify the extent to which the immune response enhancement was related to the local dermal inflammations and the enhanced antigen gene expression in the skin caused by the hair plucking. We reasoned that if the dermal inflammation associated with the anagen-induction method contributed significantly to the enhancement of the resultant immune responses, the immune responses would be stronger when the hair follicles in the application area were induced into anagen stage by treatment with RA or TPA, which were known to induce more severe skin inflammations than plucking.^[15] Because PA protein is strongly immunogenic, it may be difficult to identify differences in the resultant antibody responses when it was used as the immunogen. Therefore, we used two β -galactosidase-encoding plasmids, pCMV- β and pCMV-sin-LacZ (pSIN- β). In pCMV- β , the β -galactosidase gene was driven by a CMV promoter. The pSIN- β contained the sindbis virus non-structure protein genes (nsp 1–4) that encode the sindbis RNA replicase. In the pSIN- β , the nsp 1–4 genes were driven by a CMV promoter, but the lacZ gene was driven by a viral subgenomic promoter.^[16] Leitner *et al* showed that immunization using a similar replicase-based plasmid induced stronger immune responses than the conventional CMV promoter-based plasmid.^[17] Interestingly, our data showed that the extent of the uptake of plasmid in the skin, not the level of local dermal inflammations, determined the immune responses induced by topical DNA vaccine. This represents an alternative and novel approach demonstrating the importance of enhancing the penetration of plasmid DNA into viable skin cells in order to enhance the immune responses induced by topically applied DNA vaccines.

Materials and Methods

Materials

The pCMV- β plasmid was from the American Type Culture Collection (ATCC, Manassas, VA). Plasmid pSIN- β (pCMV-sin-LacZ) was constructed as previously described by cloning the lacZ gene from the pCMV- β plasmid and then inserting it into the pCMV-sin plasmid.^[16] We confirmed that the pSIN- β produced double stranded RNA when transfected into a tumor cell line, and that cells transfected with the pSIN- β expressed β -galactosidase. Small-scale plasmid was prepared using a Qiafilter plasmid Maxi kit (Qiagen, Valencia, CA). Large-scale plasmid DNA (5–10 mg) was prepared by GenScript USA Inc. (Piscataway, NJ). Horseradish peroxidase (HRP)-labeled goat anti-mouse secondary antibody was from Southern Biotechnology Associates, Inc. (Birmingham, AL). Sodium dodecyl sulfate (SDS), all trans-retinoic acid (RA), O-tetradecanoylphorbol-13-acetate (TPA), 3,3',5,5'-tetramethylbenzidine solution (TMB), β -galactosidase protein, bovine serum albumin (BSA), and Tween 20 were from Sigma-Aldrich (St. Louis, MO). 1,2-Dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were from Avanti Polar Lipids, Inc. (Alabaster, Alabama). CT was from List Biological Laboratories, Inc. (Campbell, CA). Lipofectamine[®] was from Invitrogen (Carlsbad, CA).

Preparation of liposomes and plasmid DNA-liposome complexes

A mixture of DOTAP (9.69 mg) and DOPE (10.31 mg) (1:1, m/m, in chloroform) was dried under nitrogen gas. The lipid film was rehydrated with 2 ml of water and extruded through a 0.1 μ m polycarbonated filter to make positively charged DOTAP/DOPE liposomes (10 mg/ml).^[18–20] Plasmid was complexed with the liposomes by mixing equal volumes of plasmid in solution and liposome suspensions. The complexes were allowed to stay in ambient conditions for at least 15 min before further use. The size and zeta potential of liposomes were measured using a Malvern Zetasizer Nano ZS (Westborough, MA) and were found to be 122.3 ± 0.3 nm and 59.9 ± 0.8 mV, respectively. The size and zeta potential of the pSIN- β /liposome complexes were 198.3 ± 1.4 nm and -62.9 ± 5.8 mV, respectively.

Application of plasmid DNA onto mouse skin

Female BALB/c mice, 6 weeks of age, were from Simonsen Laboratories (Gilroy, CA). National Institutes of Health guidelines for animal use and care were followed. Animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Oregon State University and the University of Texas at Austin. To pluck hair, the hair on the middle-dorsum of anesthetized mice was trimmed using a clipper and then plucked using a Veet[®] wax strip (Reckitt Benckiser, Parsippany, NJ) in an area of about 1.5 cm².^[13] Mice were anesthetized again 48 h later, and the plucked area was hydrated for 15 min using 0.05% SDS and paper-dried.^[13, 21] The plasmid, 'naked' or complexed with liposomes, with or without admixing with CT, was gently dripped on the area using a pipette tip. The applied area was then carefully covered with a piece of Tegaderm self-adhesive dressing film (3M, St. Paul, MN).^[13, 22, 23] For mice pre-treated with RA or TPA, the hair on the mid-dorsum was trimmed, and the trimmed area was treated with RA (50 μ l, 0.05%, w/v in ethanol) or TPA (50 μ l, 0.01%, w/v in ethanol) daily for 5 consecutive days before the application of the DNA.^[11] Mice in the positive control group were injected intramuscularly (IM) with 'naked' DNA in phosphate buffered saline (PBS, pH 7.4, 10 mM) (25 μ g per hind leg). DNA was not applied on mice in the negative control group. Mice were dosed on days 0, 14, and 28 and euthanized and bled on day 49 or where mentioned.

Enzyme-linked immunosorbent assay (ELISA)

The level of anti- β -galactosidase IgG in serum samples was determined using ELISA.^[24] Briefly, 96-well plates were coated with β -galactosidase (100 ng in 100 μ l) overnight at 4°C. The plates were washed with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20) and blocked with 4% (w/v) of BSA in PBS/Tween 20 for 1 h at 37°C. Diluted serum samples were added to the wells following the removal of the blocking solution. The plates were incubated for an additional 3 h at 37°C and washed with PBS/Tween 20. HRP-labeled goat anti-mouse IgG (5,000-fold dilution) was added to the wells, followed by another h of incubation at 37°C. The plates were washed again and incubated in the presence of TMB for 30 min at room temperature. The reaction was stopped by the addition of sulfuric acid (0.2 N). The absorbance was read at 450 nm using a BioTek Synergy HT Multi-Mode Microplate Reader (Winooski, VT).

Histology and the measurement of trans-epidermal water loss (TEWL)

Skin samples were collected 48 h after the plucking or 24 h after the last treatment with RA or TPA, fixed in 3.4% formaldehyde, embedded in paraffin, sectioned vertically, and stained using hematoxylin and eosin (H&E). Slides were examined under a light microscope by a board-certified veterinary pathologist.^[13] TEWL was measured as described previously.^[13, 25] Briefly, skin samples were placed in a 60-mm culture dish, and the desired area (1.5 cm²) was covered using the mouth of a glass test tube. Pre-weighed melt wax was applied around the test tube. Upon the solidification of the wax at room temperature and the removal of the glass tube, an area of 1.5 cm² on the skin was then exposed to air. Water loss could occur only through the exposed area. The samples (dish with skin embedded in wax) were weighed every h for 8 h using a Mettler-Toledo analytical balance (readability of 0.01 mg). TEWL was reported as the loss of weight per unit area of skin per unit time.^[13] As a positive control, hair on another group of mice was trimmed. The mice were immediately euthanized, and the skin was abraded with sandpaper.^[26]

Quantification of plasmid in skin samples

Mice were euthanized 48 h after the plasmid application, and the skin in the application area was washed and collected. Total DNA was extracted from skin samples using a Qiagen DNeasy kit. Polymerase chain reaction (PCR) was carried out to semi-quantify the amount of plasmid remaining in the skin samples.^[13] The mouse β -actin gene was also amplified as an internal control. The primers for the β -actin were 5'-AGCCATGTACGTAGCCATCC-3' (forward) and 5'-CTCTCAGCTGTGGTGGTGAA-3' (reverse), which amplified a 228 bp fragment of the mouse β -actin gene. The primers for the β -galactosidase gene were 5'-GACGTCTCGTTGCTGCATAA-3' (forward) and 5'-CAGCAGCAGACCATTTTCAA-3' (reverse), which amplified a 399 bp fragment of the β -galactosidase gene. The primers for the sindbis virus nsp 4 gene were 5'-CCGGAATGTTCTCCTCACACTT-3' (forward) and 5'-GGAATGCTCTTTTGCTCTGG-3' (reverse), which amplified a 501 bp fragment of the nsp 4 gene in the pSIN- β . A 50 μ l PCR reaction contained 1U platinum taq DNA polymerase, 0.2 mM of each dNTP, 0.2 μ M of each primer, and 50 μ g DNA. PCR cycling conditions included an initial step of 5 min at 94°C followed by 30 cycles of 30 s at 94°C to denature the DNA, 30 seconds at 55°C for primer annealing, and 30 s at 72°C for extension. A final cycle with a further 5 min extension at 72°C concluded the reaction. The PCR product was analyzed using 1% agarose gel containing ethidium bromide and quantified by measuring the band intensity using the NIH ImageJ software (Bethesda, MD). The relative amount of plasmid remaining in each skin sample was arrived by dividing the band intensity of β -galactosidase gene or nsp 4 gene by that of the β -actin gene.

Statistical analysis

Statistical analyses were completed by performing analysis of variance followed by Fisher's protected least significant procedure. A p value of ≤ 0.05 (two-tail) was considered significant.

Results and Discussions

The hair follicle cycle modification approach to enhance the antibody responses induced by a topically applied DNA vaccine was applicable to an antigen other than the anthrax protective antigen protein

In our previous study, pGPA, a plasmid that encodes the *Bacillus anthracis* PA63 protein, was used to dose the mice.^[13] It is known that the PA protein is highly immunogenic.^[27, 28] Therefore, we tested whether our approach of enhancing the antibody responses induced by a topical DNA vaccine by modifying the hair follicle cycle was applicable to an antigen other than the PA. We replaced the pGPA with pCMV- β , a plasmid that encodes the β -galactosidase gene, and immunized mice similarly. Induction of the hair follicles in the application area into anagen stage by plucking also enhanced the resultant β -galactosidase-specific serum IgG titer (~50-fold increase, $p < 0.001$, anagen vs. telogen), demonstrating that the hair follicle cycle modification approach was applicable to antigens other than the anthrax PA protein.

A RNA replicase-based plasmid DNA induced a strong antibody response when applied onto a skin area where the hair was plucked

To test whether a RNA replicase-based plasmid DNA was more effective than the conventional CMV promoter-driven plasmid DNA in inducing antibody response after topical application onto a skin area wherein the hair follicles were induced into growth stage by hair plucking, mice were topically dosed with pCMV- β or pSIN- β , and the resultant serum anti- β -galactosidase IgG level was measured. As showed in Fig. 1, both pCMV- β and pSIN- β induced β -galactosidase-specific serum IgG, but the anti- β -galactosidase IgG level in mice that received the pSIN- β was significantly higher than that in mice that received the pCMV- β . This finding is in agreement with data from previous studies by Leitner *et al.*^[29, 30] The RNA replicase genes (nsp 1–4) in the pSIN- β plasmid enabled transfected cells to produce double stranded RNA, which was pro-apoptotic and immunostimulatory and was thought to be responsible for the enhanced immunity of the replicon vector DNA vaccine.^[29–31] In this experiment, only a weak antibody responses was induced, which may be beneficial for us to detect any small differences in antibody responses in the following experiments.

Different methods of hair growth induction had different effects on the antibody responses induced by a plasmid DNA vaccine applied topically onto mouse skin

Three different methods known to induce hair growth, plucking or treatment with RA or TPA, were used to prepare mouse skin prior to dosing with pSIN- β , and the specific anti- β -galactosidase antibody response induced were evaluated. More mice (80%) in the group, in which the hair in the application area was plucked, responded (Fig. 2). In contrast, only 30% of the mice in the group, in which the DNA application area was pretreated with TPA, responded, and with a weak anti- β -galactosidase IgG level (Fig. 2). Eighty percent of mice in the group pretreated with RA also responded. The anti- β -galactosidase levels in groups pre-treated with RA or TPA were comparable (Fig. 2). Judged from the micrographs in Fig. 3, all three methods induced hair follicles into growth stage because the percent of hair follicles in growth stage in the treated groups at the time of the DNA application tended to be higher than in the untreated group. Therefore, although all three methods mentioned

above have induced hair follicles in the application area into anagen stage, the antibody responses induced by the same plasmid DNA applied onto the skin area received the three different pretreatments were different.

The uptake of the plasmid in the DNA application area on the skin correlated to the antibody responses induced by the topically applied plasmid DNA

Pretreatment of the skin with RA or TPA or by plucking the hair can induce hair follicles into anagen stage, but may also cause at least three different changes in the skin: physical damage to the integrity of the skin, local inflammation, and enhanced uptake of the plasmid in the skin.^[13] All three changes may have contributed to the enhancement of the resultant immune responses.^[13] Therefore, we examined the skin at the time of the plasmid DNA application. As shown in Fig. 3, hair plucking or pretreatment with RA or TPA all induced local cutaneous inflammation in the treated area at the time of the DNA application. However, compared to plucking, the inflammation induced by RA or TPA was much more severe as shown by epidermal hyperplasia (7–9 layers for the RA and TPA groups vs. 3–4 layers for the plucking group) and the more pronounced dermal infiltrates of inflammatory cells (Fig. 3). In fact, skin after plucking showed only mild epidermal hyperplasia in comparison to skin after simple hair trimming. In contrast, treatment with TPA resulted in diffuse, marked epidermal hyperplasia, mild orthokeratotic hyperkeratosis, multifocal to diffuse, mild to moderate, perivascular to interstitial, mixed-cellular, dermal infiltrates, and mild pustular epidermitis (not shown). Skin treated with RA showed marked, multifocal to coalescing serocellular crusts and dermal changes similar to those seen in skin treated with TPA (Fig. 3). Therefore, it seemed that the extent of local skin inflammations caused by the methods used to induce hair follicles into growth stage was not directly correlated to the immune responses induced.

The TEWL value in the skin area treated with RA was significantly higher than in the skin area treated with TPA or where the hair was plucked (Fig. 4), an indication of more physical damage to the integrity of the skin, and thus, an enhancement of skin permeability by treatment with RA. It is worth pointing out that although plucking and treatment with RA or TPA all caused increases in TEWL, the increases were significantly lower than that caused by the abrasion (Fig. 4). Again, it seemed that the TEWL associated with the methods used to induce hair follicles into growth stage was not directly correlated to the immune responses induced.

Interestingly, the PCR data in Fig. 5A showed that the amount of pSIN- β plasmid recovered from the skin area where the pSIN- β was applied differed significantly depending on the methods of hair growth induction. Based on the relative band intensities of either the β -galactosidase gene fragment (Fig. 5B) or the sindbis virus nsP4 gene fragment (Fig. 5C), plucking of the hair more effectively helped the uptake (or the retention) of the pSIN- β plasmid in the skin. Therefore, the enhanced plasmid DNA uptake/retention in the skin by hair plucking seemed to be predominately responsible for the enhancement of the antibody responses induced by the topically applied DNA vaccine. Our findings suggested that the level of the immune responses induced by a topically applied DNA vaccine was controlled to a large extent by the amount of the DNA that can reach the live cells in the skin.

The uptake of the plasmid DNA was likely via the hair follicles. Data from previous studies showed that topically applied plasmid DNA was detectable only in the hair follicles, and normal hair follicles were needed for topically applied plasmid DNA to induce immune responses.^[12] The penetration or uptake of topically applied substances via the hair follicles was reported to be dependent on whether the hair follicles were growing or resting.^[14] Active hair follicles with hair growth are open to penetration, while inactive hair follicles without hair growth were closed to penetration. Resting hair follicles were filled with plug,

but the plug on the top of the closed follicles can be opened by peeling and/or stripping.^[9, 32, 33] The wax-based plucking we used may have generated the same hair follicle opening effect. In contrast, treatment with RA or TPA may not be able to open the closed hair follicles, and there were claims in the dermatology field that treatment with RA makes the hair follicle pore size smaller.^[34]

Conclusions

We have shown that the approach of inducing the hair follicles in the application area into growth stage to enhance the immune responses induced by a topically applied plasmid DNA vaccine was applicable to an antigen other than the anthrax PA protein, and thus was not unique to the highly immunogenic PA protein. A RNA-replicase-based plasmid was more effective than the conventional CMV promoter-driven plasmid in inducing specific antibody responses when applied topically onto a skin area wherein the hair follicles were induced into growth stage by hair plucking. Finally, multiple methods were able to induce hair follicles into growth stage, but the physical hair plucking method was more effective at enhancing the immune responses induced by a topically applied plasmid DNA, which was likely due to that hair plucking not only induced hair follicles into growth stage, but also significantly increased the uptake of the topically applied plasmid DNA in the skin.

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Fig. 1.

Mice were dosed with 'naked' pSIN-β (n = 8) or pCMV-β (n = 9) at 50 μg per mouse without CT on days 0, 14, and 28. Hair in the application area was plucked 48 h prior to the application. The IgG level was measured 42 days after the first immunization. The asterisks (*) indicate that the values of the pSIN (pSIN-β) and the pCMV (pCMV-β) are different from each other (p = 0.02 at 10 x, p = 0.02 at 20 x, p = 0.03 at 40 x). Data shown are mean ± SEM (n = 5 for untreated control and intramuscularly injected pSIN-β (pSIN, IM)).

Fig. 2.

Hair in the DNA application area was trimmed and plucked, or the trimmed area was treated with RA or TPA. pSIN-β (100 μg per mouse), complexed with DOTAP/DOPE liposomes (100 μg per mouse) and then admixed with CT (10 μg per mouse), was applied on days 0, 14, and 28. The IgG level was measured 49 days after the first dosing. Serum samples were diluted 25-fold (PL, plucking; TPA or RA, pre-treatment with TPA or RA). Data shown are the mean ± SEM of mice responded, which are shown as the ratios within the bars. The values of the TPA and the RA groups are not different ($p = 0.1$). * The values of the PL and TPA groups are different ($p = 0.006$).

Fig. 3. H&E staining showed different extent of local cutaneous inflammation caused by hair plucking or treatment with TPA or RA. The photographs were taken at a $\times 20$ magnification.

Fig. 4.

Hair was trimmed and plucked, or the trimmed area was treated with RA or TPA. The TEWL values ($n = 5$) were measured at the time of DNA application (48 h later after hair plucking or 24 h after the last treatment with TPA or RA). For the abrasion group, mice were euthanized immediately after hair trimming, and the skin was abraded with sandpaper (Abrasion). * The value of RA is different from that of the PL ($p = 0.0001$) and that of TPA ($p = 0.0006$).

Fig. 5. (A). The PCR products of a β -galactosidase gene fragment and a β -actin gene fragment. (B). Band intensity ratios (β -gal/ β -actin). (C). Band intensity ratios (nsp 4/ β -actin). In B and C, the value of the TPA is different from that of the PL ($p = 0.004$ and 0.001 , respectively). UT indicates trimmed but not treated with plasmid. Data reported are mean $\pm$ SEM ($n = 3$).