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Research Paper

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 application of 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 are significantly enhanced when hair follicles in the application area are induced into the 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 mice hair follicles into the anagen stage before they 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 damage to the skin and induced more severe local inflammation 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; immunogenicity; inflammation; RNA Replicase; skin integrity

Introduction

Non-invasive application of a vaccine topically onto the skin is an attractive immunization modality. DNA vaccine is a promising new-generation treatment. The feasibility of topical immunization with plasmid DNA has been 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 have had only limited success.

Data from previous studies have indicated that hair follicles are the site for the uptake of topically applied DNA.[9–13] It has been reported that when plasmid is applied onto the skin, the expression of the gene of interest encoded by the plasmid is mainly in the keratinocytes in hair follicles.[10] It is also known that only anagen-stage hair follicles are open to penetration by foreign objects on the skin surface,[14] and that the expression of a topically applied reporter gene in the skin is significantly enhanced when the hair follicles in the plasmid application area are induced into the growth stage. [11] Therefore, it was not surprising to see a report showing that normal hair follicles are required for topical DNA vaccine to induce immune responses.[12] Based on these findings, we generated data to support a hypothesis that applying a DNA vaccine onto a skin area in which the hair follicles were induced into the anagen-onset stage induced a stronger immune response than when the hair

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follicles in the application area were in the 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 the 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 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 immune response enhancement is related to local dermal inflammation 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 contributes 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 the anagen stage by treatment with RA or TPA, which are known to induce more severe skin inflammation than plucking.[15] Because PA protein is strongly immunogenic, it would have been difficult to identify differences in the resultant antibody responses when PA 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 is driven by a cytomegalovirus (CMV) promoter. The pSIN-β contains the sindbis virus non-structure protein genes (nsp 1–4), which encode the sindbis RNA replicase. In the pSIN-β, the nsp 1–4 genes are driven by a CMV promoter, but the lacZ gene is 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 inflammation, determines 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, USA). 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, USA). Large-scale plasmid DNA (5–10 mg) was prepared by GenScript USA Inc. (Piscataway, NJ, USA). Horseradish peroxidase (HRP)-labeled goat anti-mouse secondary antibody was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). 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, USA). 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, AL, USA). CT was from List Biological Laboratories, Inc. (Campbell, CA, USA). Lipofectamine® was from Invitrogen (Carlsbad, CA, USA).

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. The lipid film was rehydrated with 2 ml of water and extruded through a 0.1 μm 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 the liposomes were measured using a Malvern Zetasizer Nano ZS (Westborough, MA, USA) and were found to be 122.3 $\pm$ 0.3 nm and 59.9 $\pm$ 0.8 mV, respectively. The size and zeta potential of the pSIN-β/liposome complexes were 198.3 $\pm$ 1.4 nm and −62.9 $\pm$ 5.8 mV, respectively.

Application of plasmid DNA onto mouse skin

Female BALB/c mice, 6 weeks of age, were obtained from Simonsen Laboratories (Gilroy, CA, USA). National Institutes of Health guidelines for animal use and care were followed. The animal protocols were approved by the Institutional Animal Care and Use Committee at Oregon State University and at 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, USA) in an area of about 1.5 cm^2.[13] Mice were anesthetized again 48 h later, and the plucked area was hydrated for 15 min using 0.05% SDS and then paper-dried.[13,21] The plasmid, 'naked' or complexed with liposomes, with or without admixing with CT, was gently dripped onto 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, USA).[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 μl, 0.05%, w/v in ethanol) or TPA (50 μl, 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 μg per hind leg). DNA was not applied to 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

The level of anti-β-galactosidase IgG in serum samples was determined using enzyme-linked immunosorbent

assay (ELISA).[24] Briefly, 96-well plates were coated with β-galactosidase (100 ng in 100 μl) 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 (5000-fold dilution) was added to the wells, followed by another 1 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, USA).

Histology and the measurement of trans-epidermal water loss

Skin samples were collected 48 h after 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] Trans-epidermal water loss (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 cm^2) was covered using the mouth of a glass test-tube. Pre-weighed melt wax was applied around the test-tube. On solidification of the wax at room temperature and the removal of the glass tube, an area of 1.5 cm^2 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 1 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′-CCGGAATGTTCCTCACACTT-3′ (forward) and 5′-GGATGCTCTTTTGCTCTGG-3′ (reverse), which amplified a 501 bp fragment of the nsp 4 gene in the pSIN-β. A 50 μl PCR reaction contained 1 U platinum taq DNA polymerase, 0.2 mM of each dNTP, 0.2 μM of each primer, and 50 μg 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 s 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 analysed using 1% agarose gel containing ethidium bromide, and quantified by measuring the band intensity using NIH ImageJ software (Bethesda, MD, USA). The relative amount of plasmid remaining in each skin sample was calculated 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 less than 0.05 (two-tail) was considered significant.

Results and Discussions

The hair follicle cycle modification approach is 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 is applicable to an antigen other than PA. We replaced the pGPA with pCMV-β, a plasmid that encodes the β-galactosidase gene, and immunized mice in a similar way. Induction of the hair follicles in the application area into the anagen stage by plucking also enhanced the resultant β-galactosidase-specific serum IgG titer (~50-fold increase, $P < 0.001$, anagen versus telogen), demonstrating that the hair follicle cycle modification approach is applicable to antigens other than the anthrax PA protein.

An RNA-replicase-based plasmid DNA induces a strong antibody response when applied to a skin area where the hair was plucked

To test whether an RNA-replicase-based plasmid DNA is more effective than the conventional CMV-promoter-driven plasmid DNA in inducing antibody response after topical application onto a skin area where the hair follicles have been induced into the 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 Figure 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 enable transfected cells to produce double-stranded RNA, which is pro-apoptotic and immunostimulatory and is thought to be responsible for the enhanced immunity of the replicon vector DNA vaccine.[29–31] In this experiment, only a weak antibody response was

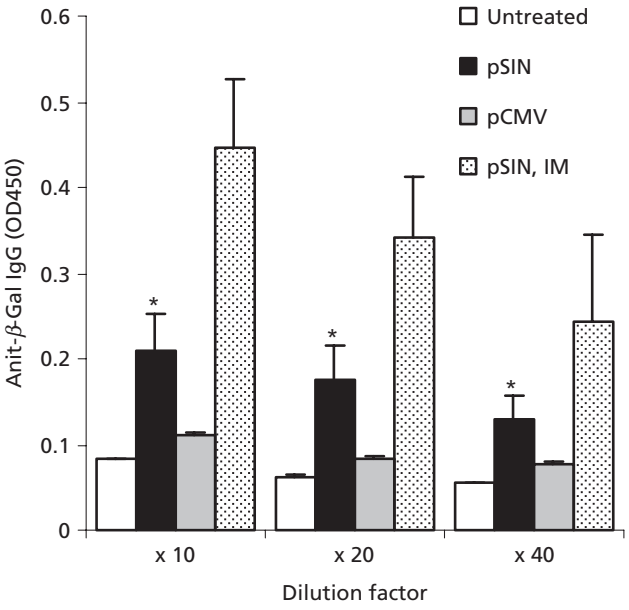

Figure 1 Induction of β-galactosidase-specific serum IgG using pCMV-β or pSIN-β. Mice were dosed with 'naked' pSIN-β ($n = 8$) or pCMV-β ($n = 9$) at 50 μg per mouse without CT on days 0, 14, and 28. Hair in the application area was plucked 48 h prior to the application. The IgG level was measured 42 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×, $P = 0.02$ at 20×, $P = 0.03$ at 40×). Data shown are mean ± SEM ($n = 5$ for untreated control and intramuscularly injected pSIN-β (pSIN, IM)).

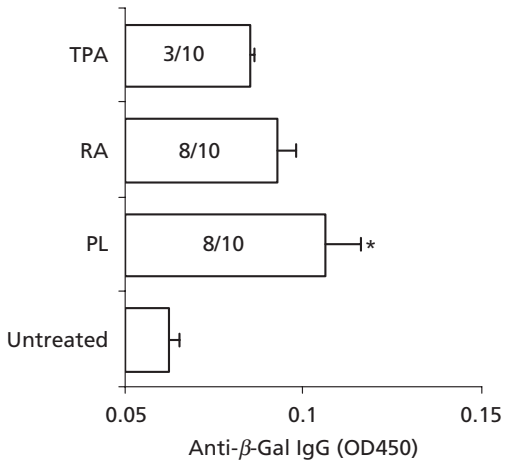

Figure 2 Different methods of hair growth induction have different effects on antibody responses. Hair in the DNA application area was trimmed and plucked, or the trimmed area was treated with RA or TPA. pSIN-β (100 μg per mouse), complexed with DOTAP/DOPE liposomes (100 μg per mouse) and then admixed 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 ($P = 0.006$).

induced, which may be beneficial in detecting any small differences in antibody responses in the following experiments.

Different methods of hair growth induction have different effects on antibody responses

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 responses induced were evaluated. More mice (80%) responded in the group in which the hair in the application area was plucked (Figure 2). In contrast, only 30% of the mice responded in the group in which the DNA application area was pretreated with TPA, and with a weak anti-β-galactosidase IgG level (Figure 2). Eighty per cent 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 (Figure 2). Judging from the micrographs in Figure 3, all three methods induced hair follicles into the growth stage because the percentage of hair follicles in the 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 induced hair follicles in the application area into the anagen stage, the antibody responses induced – by the same plasmid DNA applied onto a skin area receiving the three different pretreatments – were different.

Uptake of plasmid in the DNA application area correlates to antibody responses

Pretreatment of the skin with RA or TPA or by plucking the hair can induce hair follicles into the 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 Figure 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 is much more severe, as shown by epidermal hyperplasia (seven to nine layers for the RA and TPA groups versus three to four layers for the plucking group) and the more pronounced dermal infiltrates of inflammatory cells (Figure 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 (Figure 3). Therefore, it seems that the extent of local skin inflammations caused by the methods used to induce hair follicles into the growth stage is not directly correlated to the immune responses induced.

The TEWL value of the skin area treated with RA was significantly higher than for the skin area treated with TPA or

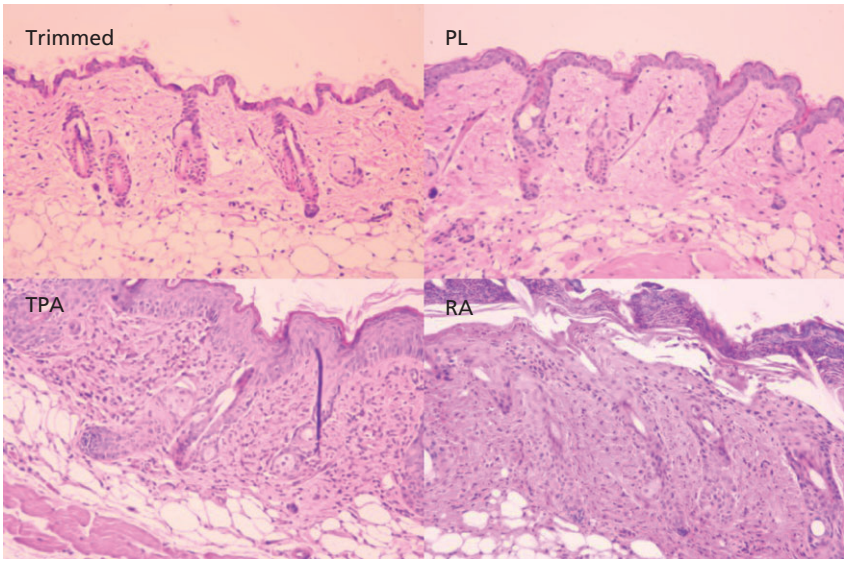

Figure 3 Micrographs comparing hair growth induction methods. H&E staining shows the different extent of local cutaneous inflammation caused by hair plucking or treatment with TPA or RA. The photographs were taken at ×20 magnification.

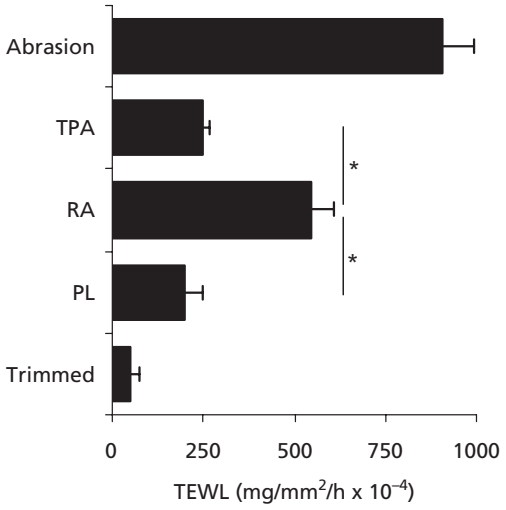

Figure 4 TEWL values after different pre-treatments. Hair was trimmed and plucked, or the trimmed area was treated with RA or TPA. The TEWL values ($n = 5$) were measured at the time of DNA application (48 h later after hair plucking or 24 h after the last treatment with TPA or RA). For the abrasion 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 ($P = 0.0006$).

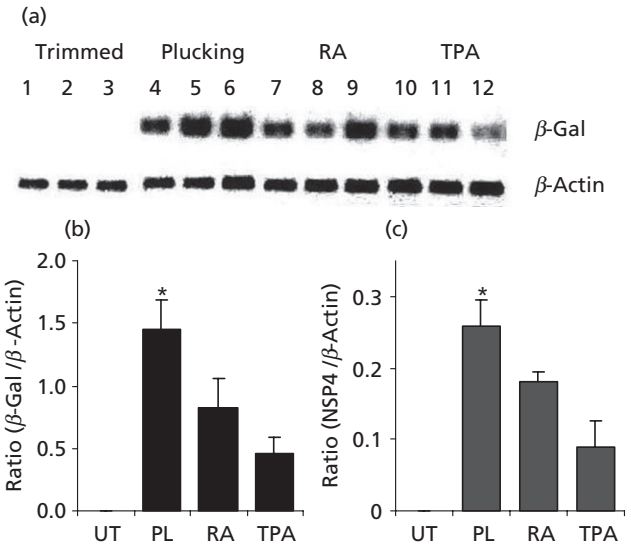

Figure 5 Plasmid DNA uptake or retention. A, The PCR products of a β-galactosidase gene fragment and a β-actin gene fragment. B, Band intensity ratios (β-gal/β-actin). C, Band intensity ratios (nsp 4/β-actin). In B and C, the value of the TPA is different from that of the PL ($P = 0.004$ and 0.001, respectively).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

where the hair was plucked (Figure 4), an indication of more physical damage to the integrity of the skin, and thus an enhancement of skin permeability from 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 abrasion (Figure 4). Again, it seems that the TEWL associated with the methods used to induce hair follicles into the growth stage is not directly correlated to the immune responses induced.

Interestingly, the PCR data in Figure 5a show 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 (Figure 5b) or the sindbis virus nsp 4 gene fragment (Figure 5c), plucking of the hair is more effective in helping the uptake (or the retention) of the pSIN-β plasmid in the skin. Therefore, the enhanced plasmid DNA uptake/retention in the skin caused by hair plucking seems to be predominately

responsible for the enhancement of the antibody responses induced by the topically applied DNA vaccine. Our findings suggest that the level of the immune responses induced by a topically applied DNA vaccine is controlled to a large extent by the amount of DNA that can reach live cells in the skin.

The uptake of plasmid DNA is likely to be via hair follicles. Data from previous studies show that topically applied plasmid DNA is detectable only in hair follicles, and that normal hair follicles are needed for topically applied plasmid DNA to induce immune responses.[12] The penetration or uptake of topically applied substances via hair follicles is reported to be dependent on whether the hair follicles are growing or resting.[14] Active hair follicles with hair growth are open to penetration, while inactive hair follicles without hair growth are closed. Resting hair follicles are 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 are 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 the growth stage to enhance the immune responses induced by a topically applied plasmid DNA vaccine is applicable to an antigen other than the anthrax PA protein, and thus is not unique to the highly immunogenic PA protein. An RNA-replicase-based plasmid is more effective than the conventional CMV-promoter-driven plasmid in inducing specific antibody responses when applied topically onto a skin area where the hair follicles have been induced into the growth stage by hair plucking. Finally, multiple methods are able to induce hair follicles into growth stage, but the physical hair-plucking method is more effective at enhancing the immune responses induced by a topically applied plasmid DNA, which is likely due to the hair plucking not only inducing hair follicles into the growth stage, but also significantly increasing the uptake of the topically applied plasmid DNA into the skin.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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