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Original Article

Topical application of plasmid DNA to mouse and human skin

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Abstract Gene expression following direct injection of naked plasmid DNA into the skin has been demonstrated in the past. Topical application of plasmid DNA represents an attractive route of gene delivery. If successful, it would have great prospects in skin gene therapy since it is painless and easy to apply. In this study, we analyzed the expression of plasmid DNA in vivo and in vitro following topical application of plasmid DNA in various liposomal spray formulations. Therefore, different concentrations of plasmid DNA expressing enhanced green fluorescent protein (pEGFP-N1) were sprayed onto mouse or human skin once daily for three consecutive days and compared with direct injection. Gene expression was assessed 24 h after the final topical application of various liposomal DNA formulations. The results showed that EGFP mRNA and protein were detectable by RT-PCR and Western blot, respectively. However, epicutaneously applied EGFP plasmid DNA did not lead to microscopically detectable EGFP protein, when assessed by confocal laser microscopy or fluorescence-activated cell sorting in contrast to about 4% of fluorescent keratinocytes following intradermal injection. In an in vivo mouse model, the application of pEGFP-N1 DNA led to the generation of GFP-specific antibodies. These results indicate that topical spray application of pEGFP-N1 liposomal DNA formulations is a suitable method for plasmid DNA delivery to the skin, yielding limited gene expression. This spray method may thus be useful for DNA vaccination. To increase its attractiveness for skin gene therapy, the improvement of topical formulations with enhanced DNA absorption is desirable.

Keywords Topical application - Plasmid DNA - Naked DNA - Liposome - DNA vaccination - Antibody synthesis - Skin gene therapy

Introduction

Significant interest has been generated in DNA vaccination and gene therapy via the skin. The skin is one of the most attractive target tissues for gene therapy due to its easy accessibility [1]. In recent years, skin gene therapy has been extensively researched at the cellular and molecular level, allowing increased expression [2–6] and the understanding of DNA absorption and transport [7].

Although multiple approaches have been developed to deliver plasmid DNA to the skin, most gene transfer studies have focused on ex vivo approaches. In addition to in vitro gene delivery, several methods have been developed to deliver genes to the skin in vivo via direct injection of naked DNA [2, 3]. Alternative methods such as the gene gun [8], magnetofection [9] or using an electric pulse [10] have also been reported. Polyplexes containing a variety of ligands (RGD peptide [11, 12]; anti-CD3 Ab [13]; Fab antibody fragments [14]; transferrin [15, 16]; and galactose [17, 18]) coupled to polyethylenimine have achieved targeted endocytosis.

Common to these gene transfer protocols is their potential for genetic vaccination, where local expression of protein antigens may suffice to elicit humoral and cellular reactions [19]. Cui and Mumper [20] reported limited success of delivering DNA vaccines to epidermal dendritic cells by a nanoengineered genetic vaccine targeted to dendritic cells. Recently, significant progress of DNA vaccines targeting dendritic cells has been achieved [21].

Topical application of plasmid DNA has the potential for treating large body surface areas. Yu et al. [22] topically applied naked plasmid DNA onto mouse skin. Fan et al. [23] topically applied both naked and liposome-entrapped plasmid vectors encoding the hepatitis B surface antigen (HBsAg), which resulted in antigen-specific cellular immune responses. Li and Hoffman [24] detected the *lacZ* gene product in hair follicles after topical application of a *lacZ*-expression plasmid combined with non-cationic liposome complexes onto mouse skin. However, most of these previous experiments were limited by low gene transfer efficiency.

Given the increasing knowledge of skin biology, the characterization of DNA uptake and gene expression by keratinocytes [7] and of the cutaneous immune system, the skin represents an attractive site for topical gene therapy [25]. Therefore, we attempted to develop a novel spray application with increased potential for DNA vaccination.

Materials and methods

Plasmid DNA and animal handling

The plasmid EGFP-N1 encoding the enhanced GFP gene was used to study gene expression (Clontech, Heidelberg, Germany). GFP, a 27 kDa protein with a quantum efficiency of 0.85 that absorbs light at 395 and 470 nm and emits green fluorescence at 509 nm, has been frequently used in molecular biology, especially in studies of protein tracking, localization and expression analysis [26]. The purity of plasmid DNA was confirmed on a 1–2% agarose gel. Liposome/pEGFP-N1 (50 μg) was topically applied to an area of 2 cm^2 on the back of immunodeficient BALB/c nude mice that are devoid of anagen follicles, BALB/c and

C57BL6 mice or healthy human skin from surgical resections maintained in organ culture [3]. The hair was epilated and the skin was slightly rubbed with fine sandpaper five times before treatment. Mice were treated twice daily for a total of 3 days (uptake and visualization studies) and once weekly for immunization studies. For DNA application, we used a spray technique with rapidly evaporating aqueous liposome solutions. Liposomal preparations containing 50 μg of Cy5-labeled pEGFP-N1 were prepared upon the manufacturer's recommendation (Mirus, Wisconsin, USA) and were used for tracking studies to analyze the penetration of plasmid DNA assessed by confocal laser microscopy. Empty liposomes or liposomes containing an unrelated plasmid (pCMV β-Gal) were used as controls. For expression studies, the same liposome preparation containing unlabeled pEGFP was used. Following three applications interspersed by 24 h each, the skin was excised and immediately transferred into liquid nitrogen. As positive controls, skin was injected intradermally with 50 μg of naked pEGFP-N1. Animals were housed in an accredited facility with all regulatory study documents being approved (AZ 50.05-230-6/02; G 658/01).

Liposomal DNA preparation

The liposome/DNA spray was prepared according to the patent EP 0 704 206 B1. Briefly, soybean lecithin was dissolved in isopropanol and propylene glycol at a tenfold stock concentration. The plasmid DNA was prepared in 0.016 M phosphate buffer containing 0.02% citric acid. The pH was adjusted to 6.8. For preparing the respective liposomal spray formulations, the DNA-containing solution was added to the appropriate amount of soybean lecithin at the concentrations indicated using a magnetic stir. Distilled water was used for dilution. Finally, the solution was filtered through an extrusion filter (100 nm, Millipore) and aliquoted in disposable spray containers (5 ml). For determining the optimal transfection solution, DNA concentrations varied between 0.025 and 0.2% and the soybean lecithin between 1.5 and 5%, respectively (Table 1). For control purposes, transfection of normal human keratinocytes (three to four passages) was performed using lipofectin (Sigma, Neunkirchen) as described [4].

Table 1 Composition of various spray formulations

Spray preparation[a]	Plasmid DNA concentration (%)	Soybean lecithin concentration (%)
#1	0.025	2
#2	0.025	3
#7	0.06	1.5
#8	0.06	5.5
#13	0.2	1.5
#14	0.2	5.5

[a]The buffer contained isopropanol, propylene glycol, 0.02% citric acid and 0.016 M phosphate-buffered saline, pH 6.8

Confocal laser scanning fluorescence microscopy

After the mice were sacrificed, the skin was excised and placed in optimum cutting temperature compound (OCTC) and shock frozen prior to storage at –20°C. The frozen tissue blocks were cut into 7 μm sections and were placed on glass slides. The fluorescence was visualized using a confocal laser scanning fluorescence microscope (Carl Zeiss LSM510, Axiovert 100 M, Jena, Germany). Each experiment was repeated between eight and ten times.

DNase treatment and reverse transcription PCR analysis

To determine mRNA expression following application of the respective DNA preparation, total RNA was extracted using the TRIzol Reagent (Life Technologies, Invitrogen). The isolated RNA was digested by treatment with DNase Grade I (Amersham Pharmacia Biotech Inc.) to remove contaminating DNA. The absence of DNA in the RNA preparation was confirmed by performing an RT-PCR reaction on 1 μg RNA without reverse transcriptase.

Total RNA was reverse-transcribed using Superscript RNase H-Reverse Transcriptase (Life Technologies, Invitrogen). The reverse transcription reaction was performed at 37°C for 50 min. cDNA (50 ng) was subjected to 30 cycles of amplification using hot start Taq polymerase (Roche). The annealing temperature was 60°C. The forward primer was ACAAGTTCAGCGTGTCCGGC, and the reverse primer was TTGTGGCTGTTGTAGTTGTACTCCAG. As a control, the β-actin gene was amplified to confirm the quality of RNA using the forward primer TGACGGGGTCACCCACACTGTGCCCATCTA and the reverse primer CTAGAAGCATTTGCGGTGGACGATGGAGGG. Each experiment was repeated between five and ten times.

Fluorescence-activated cell sorting

Injected or topically treated mouse skin was freshly obtained and incubated in dispase (2.4 U/ml) for 12 h at 4°C to separate the epidermal layer from the dermis. Upon extensive rinsing in PBS, the tissue was incubated at 37°C, in a solution of 0.05% trypsin containing 0.53 mM EDTA for 15 min to allow cell dissociation. After trypsin neutralization with 10% fetal calf serum, the cell suspension was obtained by aspiration through a 20-gauge needle. The suspension was washed with PBS three times prior to analysis using a FACS (fluorescence-activated cell sorting) sorter.

Detection of EGFP by Western blot

Skin specimens were collected after treatment and stored in liquid nitrogen. After homogenization for 1 min at 12,500 rpm in a homogenizer (Braun Biotech International), the proteins were extracted using the lysis buffer (25 mM HEPES, pH 9.0, 50 mM NaF, 1% Triton X-100, 5 mM EDTA, 100 mM NaCl). Subsequently, the specimens were centrifuged at 12,000×*g* for 15 min. The protein concentration in the supernatant was determined by the advanced protein assay (Cytoskeleton Inc., USA). Protein (20 μg) was loaded onto polyacrylamide gels (NuPAGE, 4–12%, BT Gel, Invitrogen) and electrophoresed at 80 V for 2 h. After blotting onto polyvinylidene fluoride membranes at 25 V for 1 h, the membrane was blocked with 2% milk powder in TBST buffer overnight at 4°C. Then, the membrane was incubated with a rabbit anti-EGFP antibody (dilution 1:2,000, Living Colors A.V.) and a secondary peroxidase-labeled anti-rabbit antibody (dilution 1:20,000, Amersham-Pharmacia,

Freiburg, Germany), respectively. A chemiluminescence detection kit (Amersham) was used for visualization of bound antibodies. Recombinant EGF protein (27 kDa; Sigma) was used as positive control and an isotype antibody as negative control. Each individual preparation was tested in at least five separate experiments.

Results

Quality control of the plasmid preparation

Since the plasmid was applied as a spray, the integrity of the DNA was analyzed prior to and after spraying through a fine nozzle of the spray bottle. The various spray preparations contained different amounts of lecithin (1.5–5.5%) and DNA (0.025–0.2%) (Table 1). Supercoiled, nicked and some linearized plasmid could be detected in the control as well as in the various spray preparations (Fig. 1). For this experiment, the formulations with the highest DNA concentration (i.e. 0.2%) were used since shearing is most likely observed with more concentrated DNA. The lack of significant shearing upon spraying indicated the stability of plasmids and supported the use of the spray formulation.

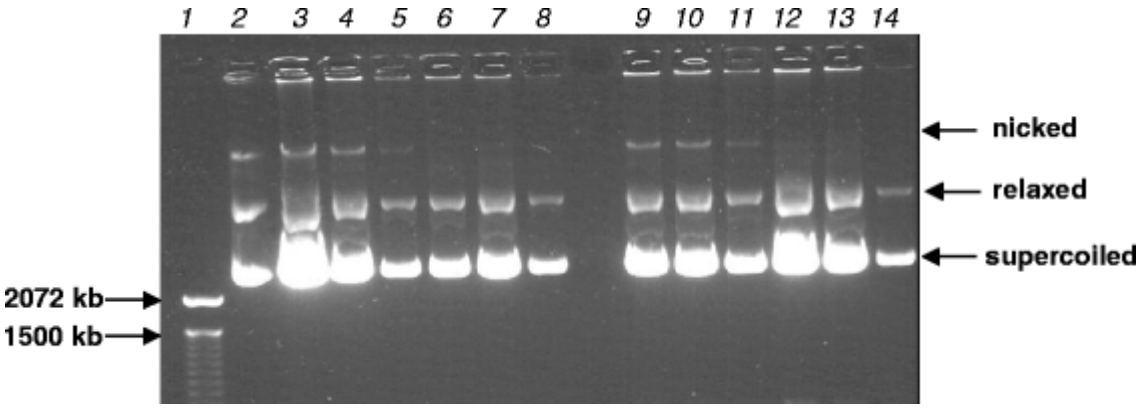

Fig. 1 Plasmid stability was assessed prior to and following spraying. Plasmid DNA was collected and analyzed by gel electrophoresis. No increased amounts of nicked or linearized plasmid were detected. *1* DNA ladder. *2* Undigested pEGFP (each preparation #13), 2 μg; *3* spray 5 μg; *4* spray 3 μg; *5* spray 1 μg. Repeat experiment: *6* spray 5 μg; *7* spray 15 μg; *8* spray 20 μg; *9* (each preparation #14) spray 5 μg; *10* spray 3 μg; *11* spray 1 μg. Repeat experiment: *12* spray 20 μg; *13* spray 15 μg; *14* spray 5 μg. Spray=spray experiment

The stability of the preparation was analyzed by storing the various preparations for up to 10 months at 37°C. As seen in Fig. 2, no autocatalytic degradation was detected, which confirmed the stability of the tested DNA preparations (Fig. 2; shown is preparation #1, since degradation is most likely to occur in the least concentrated DNA solution).

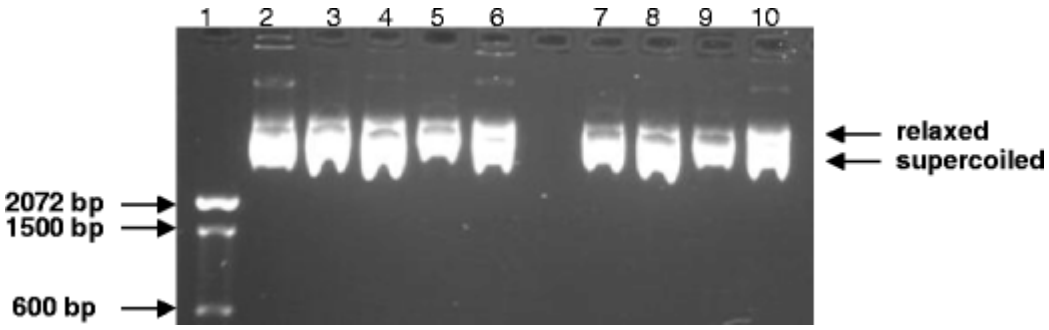

Fig. 2 Plasmid stability upon storage of the liposomal preparation. The preparations were stored at 37°C for up to 10 months and assessed for stability of the plasmid. Each 1 μg of DNA has been loaded. No increased amounts of nicked or linearized plasmid were detected. *1* DNA ladder; *2* pEGFP (each preparation #1), 1 μg; *3* 5 days; *4* 9 days; *5* 5 weeks; *6* 5 months; Repeat experiment: *7* 5 days; *8* 9 days; *9* 5 weeks; *10* 5 months

Epicutaneous detection of fluorescently labeled plasmid DNA

Cy5-labeled plasmid DNA (shown is preparation #8, containing a medium DNA and high lipid content) was detected epicutaneously and intraepidermally using confocal laser microscopy proving plasmid delivery to the epidermis (Fig. 3a,e). However, detectable levels of GFP were not detected following three applications onto mouse skin regardless of the spray formulation used (Fig. 3b,f), indicating the lack of ample amounts of GFP. The same experiment was performed with organ-cultured human skin, which again did not allow the detection of GFP (Fig. 3f). Human skin showed less superficial and intraepidermal red staining, indicating the presence of covalently labeled plasmid DNA (Fig. 3e). This is consistent with the higher number of epidermal cell layers in human skin as compared with mouse skin. Likewise, cell suspensions of epicutaneously applied liposomal formulations did not show detectable GFP signals using a FACS sorter in contrast to intraepidermally injected EGFP-N1 plasmid DNA, where up to 4% of cells were transfected (n=5; data not shown). These visualization experiments provide evidence for both intrafollicular and follicular uptake of Cy5-labeled plasmid DNA.

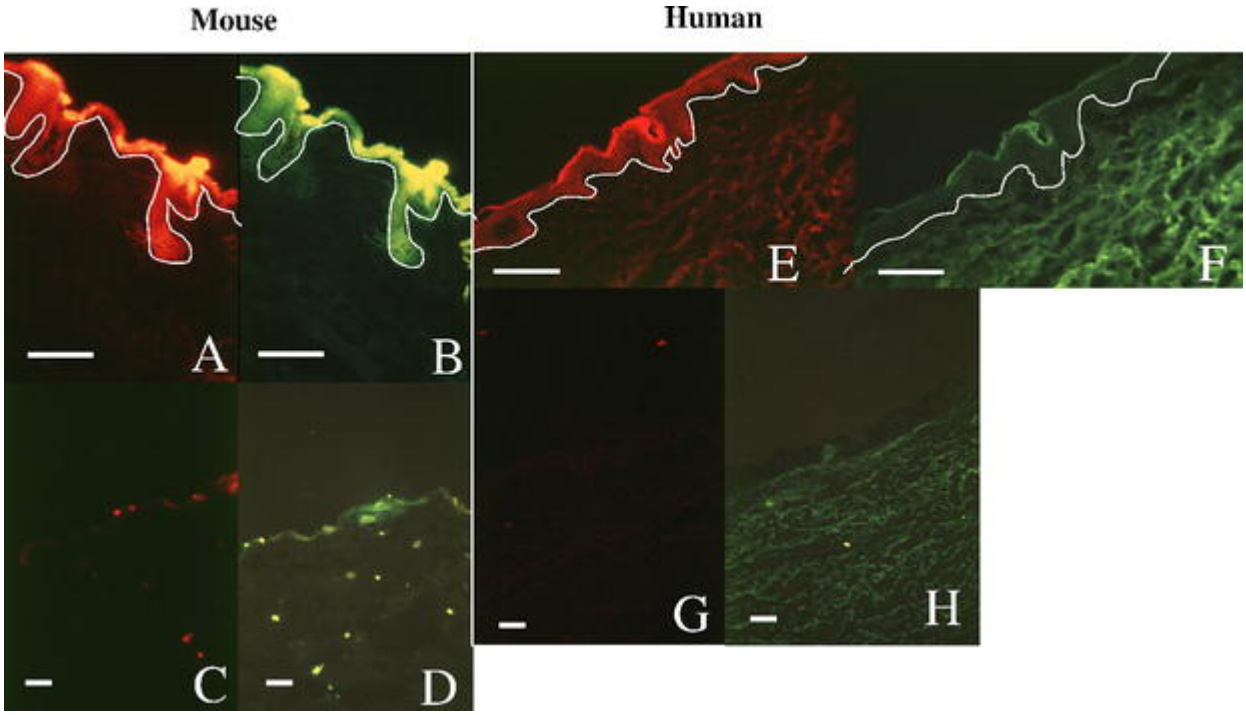

Fig. 3 Plasmid visualization following topical application of liposomal spray formulations. Cy5-labeled EGFP plasmid DNA (preparation #8) was detected epicutaneously and intraepidermally using confocal laser microscopy following three applications onto BALB/c-nude, BALB/c and C57BL6 mouse skin in vivo (*n*=15; **a**) and human skin organ cultures maintained at the air liquid interface (*n*=10; **e**), respectively. Panels **a**–**d** refer to BALB/c skin and panels **e**–**h** to human skin in organ culture. While the plasmid (*red*) could be visualized in both, follicular and interfollicular epidermis (**a** and **e**), GFP was not detectable with immunofluorescence (**b** and **f**). The negative controls (liposomal formulation with pCMVβ-Gal) show some background fluorescence (**c**, **d**, **g**, and **h**). The green fluorescence at the exact same location like in the red channel represents unspecific signals. Interfollicular delivery was also shown with a comparable uptake pattern observed in nude mice that are devoid of anagen follicles (data not shown)

Expression of topically applied plasmid DNA

Since upon topical application of the plasmid-encoding EGFP no gene product could be detected on confocal microscopy, we opted not to perform quantitative assessment. Instead, we addressed the question whether GFP-specific RNA was present. As shown in Fig. 4, specific GFP-RNA could be detected in plasmid-treated mouse and human skin upon topical spray application of all different preparations (Fig. 4). Importantly, DNAse treatment of plasmid DNA had to be performed in order to destroy any remaining plasmid DNA, which would have been amplified during the PCR reaction. As an additional control for every RNA sample, an RT-PCR reaction was performed without reverse transcriptase, confirming the absence of contaminating DNA in the respective samples (data not shown). Therefore, we conclude that small amounts of RNA are detectable upon topical application of plasmid DNA.

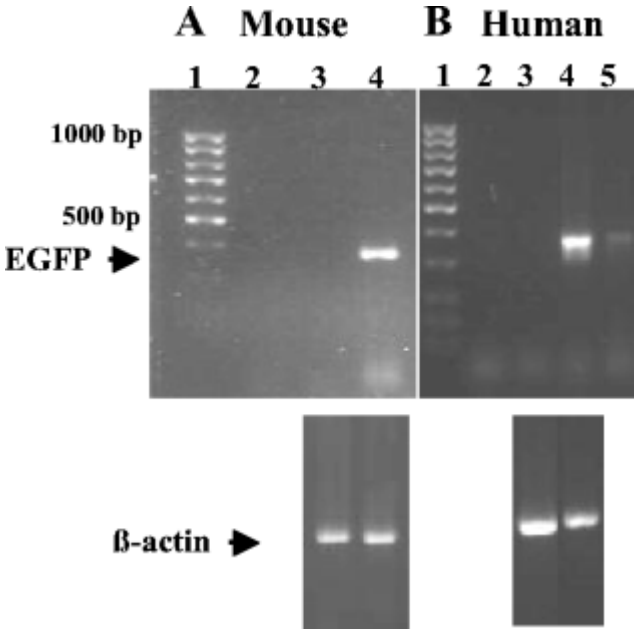

Fig. 4 RNA expression following topical application of liposomal spray formulation. RNA was extracted after three spray applications of the respective liposomal preparations. Results of preparation #8 (containing 0.06% DNA and 5.5% soybean lecithin) are shown for mouse and human skin. The other preparations showed similar results (data not shown). For control reasons, keratinocytes transfected with lipofectin/pEGFP-N1 and β-actin were also included. Topically sprayed pEGFP revealed a strong band (384 bp) both in ten of 13 mouse skin and eight of ten human skin specimens, respectively. *1* DNA ladder; *2* DEPC-water (negative control); *3* control-treated mouse/human skin that received pCMV β-Gal; *4* liposomal formulation sprayed on mouse/human skin; *5* normal human keratinocytes transfected with pEGFP-N1

Immunization of mice using a topical DNA spray

As only small amounts of protein were detected, topical application of plasmid DNA might be suitable for DNA vaccination, where minute amounts of intraepidermally produced protein may be processed and presented by epidermal Langerhans cells. This technology would represent major progress in vaccinology avoiding needle penetration of the skin. To test this exciting application, liposomal formulations containing pEGFP were applied once weekly for three consecutive weeks onto the skin of BALB/c mice. Preimmune sera and sera at 4 weeks (i.e. after three vaccinations) were used for Western blotting. While mice treated with irrelevant DNA and empty liposomes showed no specific GFP antibodies, vaccinated mice mounted a humoral immune response against GFP following vaccination with the corresponding plasmid DNA (Fig. 5). Immunization was comparable in both strains of mice analyzed (data not shown). Currently, an animal model is under study for the protection of genital herpes infection using topical spray immunization.

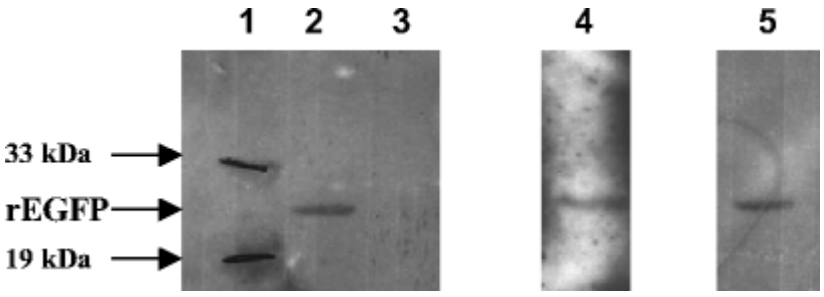

Fig. 5 Humoral immune response following topically applied liposomal preparations. Three spray applications (preparation #14, containing 0.2% DNA) were performed onto back skin of BALB/c or C57BL6 mice at 1-week intervals. At week 4, mouse serum was harvested and analyzed by Western blot against recombinant EGFP. *1* Protein ladder with molecular weights; *2* rec. EGFP positive control yielding a 27-kDa band; *3* normal mouse serum; *4* 1:100 dilution of mouse serum following treatment with preparation #14; *5* 1:10 dilution of mouse serum following treatment with preparation #14. The less concentrated samples (DNA and lecithin content) showed lower immunization efficiency

In conclusion, plasmid DNA applied in a liposome spray formulation led to the expression of the corresponding gene in mouse and human skin. Moreover, vaccination seemed possible following topical application onto mouse skin. Therefore, this vaccination strategy can advance conventional vaccinology by painless application of plasmid DNA encoding the desired gene. This may also be particularly important for proteins that cannot be produced in sufficient quality by recombinant technologies.

Discussion

The ability to deliver DNA vectors containing desired genes via topical application would be advantageous with regard to feasibility and patient acceptance. Although plasmid DNA drugs are effective when given systemically, topical DNA vaccination has only been performed against HIV [27]. A needle-free delivery is particularly attractive due to high patient acceptance and lack of pain.

Few other organ systems have received such attention in recent years due to its excellent accessibility [28]. The successful development of topical gene therapy depends on a two-step drug delivery process. The first step involves the delivery of biologically active plasmid DNA through the stratum corneum. The stratum corneum, composed of dead epithelial cells and lipids, represents the principal barrier to penetration of macromolecules. The second step consists of the delivery of plasmid DNA across the membrane of these cells. These two steps affect transfection efficiency in vivo. Recently, we have characterized this process in more detail showing that several forms of endocytosis (e.g. pinocytosis) seem to be involved [7]. Previous work has demonstrated that topically applied plasmids can access cellular targets via the hair follicles [23, 24]. Whether intercellular or transcellular routes of transportation play a role has not yet been resolved. Fan et al. [23] have shown that topical application of naked DNA in an aqueous solution to unaltered skin can induce a specific immune response, and that this effect is dependent on the presence of normal hair follicles which has also been shown for other hydrophilic drugs such as acyclovir [29]. Skin lacking normal hair follicles and without pretreatment failed to take up plasmid vectors [23, 30]. Elegant work from Domashenko et al. [31] in a human scalp xenograft model has demonstrated that DNA uptake

and expression by lipoplexes occurs in the anagen phase of the hair cycle and has defined optimal conditions for the transfection of human scalp follicles in vivo.

Extending these results, we achieved sufficient penetration of plasmid DNA into the interfollicular epidermis of various strains of mice including nude mice that lack anagen follicles and—for the first time—human interfollicular skin. Given the abundance of interfollicular keratinocytes beyond the scalp, axilla and groin, interfollicular uptake may even represent the principal route. In accordance to the liposomal formulations used in our study, Raghavachari and Fahl [32] have also reported that nonionic liposomes were the most efficient vehicles for transdermal DNA delivery.

Despite the low efficiency of keratinocyte transfection, the spray method may be particularly attractive since it is easy to apply and the formulation is very stable and simple. Despite these limitations, topical application represents a useful and cost-effective method for antibody production and potentially DNA vaccination.

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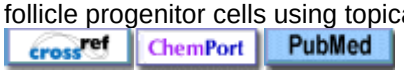

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