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Gene expression in an intact ex-vivo skin tissue model following percutaneous delivery of cationic liposome–plasmid DNA complexes

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Abstract

The skin represents an attractive site for the localised gene therapy of dermatological pathologies and as a potential antigen bioreactor following transdermal delivery. Potential also exists for the gene therapy of skin as a cosmetic intervention. The most exploited non-viral gene delivery system involves the complexation of cationic liposomes with plasmid DNA (pDNA) to form lipid:pDNA vectors that protect the DNA from nuclease-mediated degradation and improve transgene-cell interactions. Despite numerous studies examining the potential for these vectors in delivering genes to a variety of keratinocyte models, investigations into the topical application of such complexes to intact skin tissue is limited. This ex-vivo study, conducted with intact skin tissue derived from hairless mice, provides quantitative confirmation that topical administration of cationic lipid:pDNA complexes can mediate uptake and expression of reporter pDNA (33-fold higher compared with control) in viable epidermal tissue. The ex-vivo study design provides for intact skin tissue that has not been subjected to depilatory procedures of potential detriment to stratum corneum barrier function, and can be utilised for the quantitative and efficient examination of a potentially wide range of non-viral gene vectors designed for epidermal expression. © 2000 Published by Elsevier Science B.V. All rights reserved.

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The efficient delivery of gene therapy vectors to target cells, and indeed to appropriate subcellular compartments, remains relatively poor and the selective exploitation of gene therapy for local disease targets should provide for successful strategies. With increasing knowledge of skin pathobiology and characterisation of keratinocyte-specific gene promoters, the skin represents an attractive site for localised gene therapy. Delivery of therapeutic genes to viable epidermal tissue provides potential for local immune-modu-

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latory DNA-based treatment of dermatological pathologies such as hyper-proliferative dermatoses or cutaneous malignancies. Further, given the antigen presentation characteristics of skin tissue, the epidermis could serve as an antigen bioreactor for polynucleotide vaccine delivery. The recent elucidation of the molecular events of alopecia (Ahmed et al., 1998) also highlights the potential cosmetic interventions for gene therapy targetted to the hair follicle. While there is increasing work investigating the percutaneous delivery of oligonucleotides, including the use of electroporation (Regnier et al., 1998), iontophoresis (Brand et al., 1998), co-solvents (Nolen et al., 1994) and lipid-based systems (Ocheitree et al., 1996), the delivery of genes to the skin has received comparatively less attention (Alexander and Akhurst, 1995).

The interaction of liposomes with skin and their potential role in promoting the epidermal delivery of therapeutic molecules has long been investigated (Schaller and Korting, 1996; Schmidt and Korting, 1996). The most exploited non-viral gene vector involves the use of liposomes comprising cationic lipid species (Tomlinson and Rolland, 1996), with the cationic lipid interacting spontaneously with the negatively charged pDNA to form complexes which afford the properties of DNA condensation, protection against nuclease-mediated degradation and improved transgene-cell interactions. However, although numerous studies have examined gene delivery in a variety of keratinocyte models, investigations of the topical application of non-viral gene vectors to intact skin tissue is limited.

This current ex-vivo study, conducted with intact skin tissue that has not been subjected to depilatory procedures of potential detriment to stratum corneum (SC) barrier function, aimed to confirm that simple cationic lipid–plasmid DNA (pDNA) complexes can mediate transgene expression in viable epidermal tissue. The results also confirmed the utility of an ex-vivo skin model for the efficient examination of a potentially wide range of non-viral gene vectors designed for epidermal expression.

All reagents were of analytical grade and purchased from Fisher Scientific (Loughborough, UK) unless otherwise stated. The cationic lipid 1,2 - Dioleoyl - 3 - Trimethylammonium - Propane (DOTAP) was purchased from Avanti Polar Lipids (AL, USA). The 7.2 kb pCMVβ plasmid (Clontech, Palo Alto, USA), containing the β-galactosidase reporter gene expressed under the control of the immediate early promoter of human cytomegalovirus, was amplified in a transformed DH5α strain of *Eschericia coli* colonised onto an ampicillin selective LB agar, and purified using a Qiagen Plasmid Mega Kit (Qiagen, Crawley, UK).

Stock solutions of DOTAP:pDNA complexes were prepared in deionised nuclease-free purified water by the addition of pCMVβ plasmid (1 mg/ml) to extruded small (120 nm Mass Median Aerodynamic diameter) unilamellar DOTAP liposomes (1 mg/ml) at a mass (w/w) ratio of 1.67:1 (DOTAP:pDNA) followed by mixing (five inversions) and incubation at room temperature for 20 min. For the skin expression studies the complexes were then diluted to the appropriate concentration with Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Paisley, UK) buffered with 25 mM HEPES and containing the antibiotics penicillin/gentamycin (100 U/ml and 50 μg/ml, respectively). Complexes were prepared immediately prior to experimentation and samples taken for analysis of diameter by photon correlation spectoscopy (PCS) at 25°C using a Coulter N4 Plus (Coulter Electronics, Luton, UK) with a 10 mW laser and a scattering angle of 90°.

Full thickness skin tissue of approximately 1 cm^2 area was harvested from the abdominal region of 7-week-old male hairless mice (University of Wales College of Medicine, Cardiff) and kept briefly on ice (not greater than 30 min) prior to placement between donor and receptor compartments of all-glass Franz-type diffusion cells. Throughout the experiment the basal surface of the skin sections were bathed, under stirring, by 3 ml DMEM, containing 10% Fetal Bovine Serum (FBS) and penicillin/gentamycin. For exposure of the apical skin surface to lipid:pDNA complex, 100 μl of DMEM (excluding FBS but including penicillin/gentamycin) containing 30 μg pCMVβ pre-complexed with 50 μg DOTAP was placed in the donor compartment of the diffusion cell for a period of 6 h; the 100 μl volume was just sufficient to cover the 1 cm^2 area of skin surface. Control treatments included addition to the donor compartment of 100 μl of DMEM (including penicillin/gentamycin) containing 50 μg DOTAP alone or 30 μg pCMVβ alone. Franz cells were incubated in a water bath to maintain the receptor fluid at 37°C and the donor fluid at temperatures associated with the surface of human skin (approximately 32°C). After the 6 h exposure of the skin tissue to pDNA, DOTAP or DOTAP:pDNA complexes the apical skin surface was surface-rinsed with DMEM to remove all treatments and then both donor (1 ml) and receptor compartments (3 ml) replenished with fresh DMEM/10% FBS and penicillin/gentamycin, and left to incubate at 37°C for a further 24 h to allow expression of β-galactosidase reporter protein.

At 30 h after initiation of the study, the skin tissue was harvested and homogenised in an Ultra-Turrax T25 homogeniser in the presence of immunoprecipitation buffer (1% Triton-X, 60 mM octylglucoside, 150 mM sodium chloride, 20 mM Tris, 2 mM ethylenediaminetetra-acetate, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 100 mM sodium orthovanadate, 1 mM phenyl methyl sulphonyl fluoride (PMSF) and 2 mg/ml leupeptin). The homogenate was centrifuged at $3000 \times g$ for 30 min and the clear supernatant removed for analysis. Following estimation of total protein (Lowry et al., 1951), the samples were loaded onto a 15% SDS/polyacrylamide gel and electrophoresed in running buffer (0.5 M Tris, 2% glycerol, 2% SDS) at 200V for 1 h. Gels were electro-blotted to polyvinyldene difluoride membrane (Biorad, UK) which was then blocked (5% non-fat dry milk; 10 mM Tris pH 7.5; 100 mM NaCl; 0.1% Tween) for 1 h at room temperature. Membranes were incubated with anti-β-galactosidase mouse monocolonal antibody (Promega, Southampton, UK) at 1:1000 dilution for 16 h at 4°C, followed by incubation (1 h at room temperature) with a HRP conjugated anti-mouse IgG (1:1500 dilution; Dako, Cambridge, UK). The generated chemiluminescent signal (Super Signal Ultra; Pierce, Chester, UK) was recorded onto Hyperfilm ECL (Amersham, Little Chalfont, Bucks, UK). Rainbow prestained molecular weight markers (Amersham, Little Chalfont, Bucks, UK) were concurrently electrophoresed. Image acquisition and band quantitation was undertaken on GS-700 densitometer with Molecular Analyst software (Biorad, Hemel Hempstead, UK). Statistical analysis of the band intensity data was undertaken by ANOVA with statistical signifance set at the 95% confidence level ($P < 0.05$).

The apparent structures formed between DOTAP liposomes and pDNA were visualised using an adaptation (Birchall et al., 1999) of an established negative stain electron microscopy technique (Zabner et al., 1995). Briefly, freshly prepared complexes were placed onto 100 mesh nickel grids and stained with freshly filtered and centrifuged 2% aqueous uranyl acetate for 30 s. The grids were washed twice with distilled water and imaged using a Philips 208 transmission electron microscope.

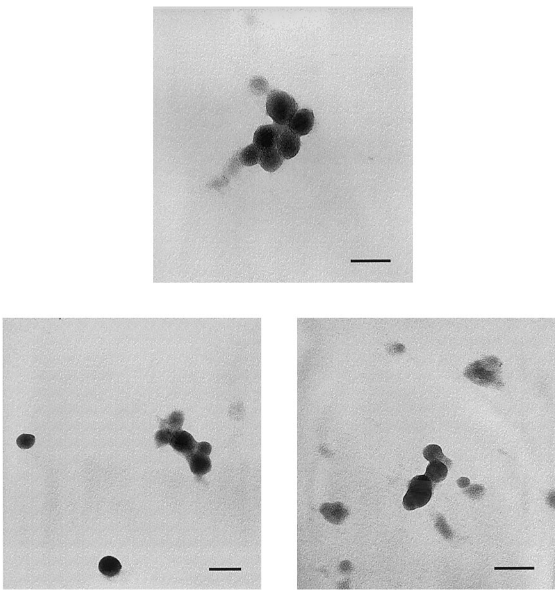

Fig. 1. Negative stain transmission electron micrographs of DOTAP:pDNA (1.67:1 w/w) complexes. Bar, 100 nm.

Fig. 1. shows a selection of TEM images of the DOTAP:pDNA complexes. The observed multilamellar aggregates, which are not apparent in the absence of pDNA, are thought to result from the interaction of lipid bilayers with pDNA (Tomlin-

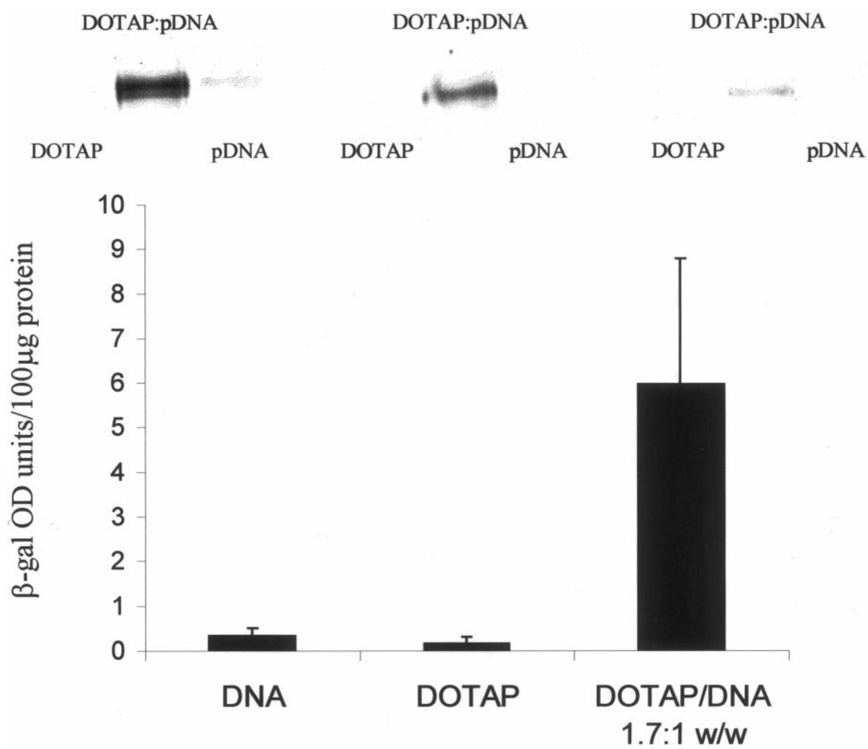

Fig. 2. Western blot analysis of β-galactosidase expression in full thickness harvested skin samples. The histogram represents mean chemiluminescent signal intensities.

son and Rolland, 1996; Birchall et al., 1999). The size and structure of these particulates is heterogenous with unimodal analysis by PCS confirming a mean particle diameter of 584.9 ± 114.3 nm for such complexes. The DOTAP:pDNA ratio used in this preliminary study (1.67:1 w/w) was selected on the basis of observations from previous skin studies (Alexander and Akhurst, 1995). The use of more cationic complexes, however, may provide for more complete complexation of the pDNA and result in smaller and more homogenous systems. Fig. 2 shows a Western blot demonsrating β-galactosidase expression in the full thickness harvested skin samples at 30 h after initiation of study (i.e. 24 h after removal of the pDNA from the skin surface). All tissue incubations and protein analyses were run concurrently to ensure valid within-experiment comparisons. The histogram represents the averaged Western blot band intensities for the treatments and while there is significant variation for the DOTAP:pDNA data (coefficient of variation ≈ 45–50%) this lipid: pDNA complex can be seen to clearly mediate a greater (16-fold; $P < 0.05$) level of β-galactosidase expression in the skin compared with pDNA alone, and compared with DOTAP alone (33-fold greater; $P < 0.05$). No significant difference between pDNA alone and DOTAP alone was evident, or indeed between these control treatments and untreated skin, either freshly isolated or incubated for 30 h under matched conditions. Previous investigations have confirmed that isolated mouse skin treated with DMEM under conditions adopted in this experiment maintain tissue viability and barrier function for at least a 24 h period (Collier et al., 1989). It should be noted that within this study design the opportunity for penetration of pDNA across stratum corneum was limited to the first 6 h period, and any subsequent detriment in tissue viability and barrier function after this time would serve only to compromise the level of reporter gene expression attained.

In this ex-vivo experiment with skin tissue from hairless mice devoid of an active hair growth cycle but possessing pilosebaceous units, we show significant quantitative reporter gene expression following topical application of pDNA–cationic liposome complexes. It may be expected that an intact stratum corneum would represent an inpenetratable barrier for such particulate structures, although the work of Ceve et al. (1996) provides some evidence for a transporter role for certain lipid-based vesicles (termed transfersomes) in carrying drugs across the stratum corneum (SC). Nevertheless, there is increasing data supporting the efficacy of liposomes to deliver drugs to the pilosebaceous unit (Li et al., 1993; Li and Hoffman, 1995; Lauer et al., 1995, 1996; Weiner, 1998). At the junction of the SC with the hair follicle there is a lipophilic matrix of triglycerides, waxy esters and cholesterol, which collectively form sebum. This pocket of oily substance forming the entrance to the pilosebaceous unit is approximately 70 μm in diameter. It can be envisaged that the physico-chemical properties of lipid–pDNA complexes allow for partition into sebum with access directly to cells of the pilosebaceous unit. With this in mind the immunohistochemistry data of Alexander and Akhurst (1995) is intriguing to note: these investigators reported that the topical application of a cationic liposome–pDNA vector to in-vivo mouse skin in the anagen or active phase of the hair growth cycle resulted in more efficient reporter gene expression than when applied in the telogen phase of the hair growth cycle. This would implicate a significant role in pDNA uptake and expression for cells of the follicular pathway. Indeed, in-vivo experiments from the laboratory of Weiner (Niemiec et al., 1997) show that topical application of pDNA complexed with novel mixtures of non-ionic/cationic lipids exhibit transgene expression localised to perifollicular cells. With limited tissue diffusion of such large complexes, observations of significant expression in dermal fibroblasts following topical application of a cationic liposome–pDNA vectors (Alexander and Akhurst, 1995) may reflect disruption of SC barrier function as a result of chemical and physical techniques used to remove animal hair prior to application of gene vectors.

In summary, using an ex-vivo model with skin tissue derived from hairless mice we provide quantitative confirmation of uptake and expression of reporter pDNA following topical application of pDNA–cationic liposome complexes. The route(s) of diffusion of these complexes to viable epidermal tissue may well involve follicular and/or trans-stratum-corneal pathways. Further research is warranted into the route of diffusion of these vectors and whether smaller lipid:pDNA (Birchall et al., 1999) and lipid:polycation:pDNA (Gao and Huang, 1996; Li and Huang, 1997; Sorgi et al., 1997) systems that provide for more complete pDNA condensation and protection can mediate greater expression of reporter plasmid. The ex-vivo study design as adapted in this investigation provides for intact skin tissue that has not been subjected to depilatory procedures of potential detriment to SC barrier function, and can be utilised for the quantitative and efficient examination of a potentially wide range of non-viral gene vectors designed for epidermal expression.

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