Topical Gene Transfer into Rat Skin Using Electroporation

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Purpose. To investigate whether electroporation can be used for topical gene delivery and for DNA expression in rat keratinocytes.
Methods. The localization of a fluorescent-labelled plasmid and the expression of a reporter gene (pEGFP-N1) coding for Green Fluorescent Protein (GFP) in stripped skin were assessed by Confocal Laser Scanning Microscopy (CLSM).
Results. The plasmid penetrated into the epidermis within minutes after electroporation and entered the keratinocyte cytoplasm within hours. A localized expression of GFP was observed for at least 7 days in the epidermis. Skin viability was not compromised by electroporation.
Conclusions. Electroporation enhances the delivery, and hence the expression, of topically applied plasmid DNA on the skin. It could be a promising alternative method to administer DNA, particularly for DNA vaccines, in the skin *in vivo.*

KEY WORDS: DNA; electroporation; skin; topical delivery.

INTRODUCTION

The skin is an attractive target organ for gene therapy because it is readily accessible and can be easily monitored. In addition to treating skin disorders, e.g., infectious, inflammatory, or cancerous diseases, or wound healing, the skin is also an attractive target for genetic vaccination (1,2). Hence, gene transfer to the skin has many potential applications but lacks a safe, efficient delivery method.

Electroporation involves the application of short duration (10 μs–100 ms) high intensity electric field pulses (50 V/cm–1500 V/cm) to cells and tissues. It results in the creation of transient aqueous pathways within lipid bilayers. Permeability for molecules and electrical conductance of the lipid bilayers are increased by several orders of magnitude. Moreover, the associated local electric field can contribute to transmembrane molecular transport by electrophoresis and/or electroporation (3). Consequently, electroporation is one of the most frequently used methods of introducing molecules, including macromolecules, into cells, and it has been widely used to introduce DNA into various types of cells *in vitro.*

Cell and tissue electropermeabilization can also be achieved *in vivo,* and different applications have been developed. Electrochemotherapy is a new therapeutic approach to transfer a nonpermeant anticancer agent like bleomycin into tumors by the application of high voltage pulses (for a review, see 4,5). Clinical trials of electrochemotherapy have shown impressive results for the treatment of melanomas and several cell carcinomas (5).

In vivo gene transfer by electroporation has been demonstrated to be efficient for introducing DNA (for a review, see 6) into mouse skin (7,8), chick embryo, mouse testis, rat liver (9), murine melanoma (10), rat brain, and mouse or rat muscle (11). Gene transfer into skin by electroporation is particularly attractive for several reasons. First, electroporation enhances the delivery of nucleic acids like oligodesoxynucleotides (ODNs) in the stratum corneum and the viable skin and permeabilizes both the stratum corneum and the keratinocytes (12,13); macromolecules (up to 40,000 Da) can be delivered into and through the skin by the application of high voltage pulses (14). Second, skin electroporation is well-tolerated (5,15,16). Third, the skin is particularly accessible. Fourth, gene therapy or DNA vaccination are possible applications. Fifth, the use of electroporation to deliver genes *in vivo* after local subcutaneous injection has also been demonstrated (7,8).

Different methods have already been evaluated to transfer genes into the skin. These include liposomes (17), direct injection of naked DNA (18), gene guns (19), microseeding or puncture (20), adenovirus (21), retrovirus (22), and electroporation after intradermal injection (7). Most of these studies on gene delivery in the skin or on electrically mediated gene transfer have used expression as evidence for successful delivery. The pathway of DNA transport, tissue viability, and/or DNA stability were usually not reported.

Based on the literature (12,13), our hypothesis was that electroporation could both enhance DNA transport into the skin and permeabilize the keratinocytes. The aim of this study was thus to investigate whether skin electroporation can be used for topical gene delivery and expression in keratinocytes. Hence, DNA was applied on the skin before skin electroporation. Firstly, to check if DNA can penetrate the keratinocytes and to gain insight into the mechanism of transport into the skin, the localization of fluorescence-labelled plasmid was studied as a function of time. Secondly, the expression of a reporter gene was investigated. Finally, the viability of the skin and the integrity of the plasmid following electroporation were also assessed.

MATERIAL AND METHODS

Animals and Chemicals

Hairless male rats aged 7–9 weeks old were housed in standard cages at room temperature (IOPS mutant from Iffa Credo, France). Standard laboratory food (A04, UAR-France) and water were given *ad libitum.* Salts for buffer preparation were of analytical grade (Vel, Belgium).

Plasmid

A 4.7-kb plasmid, pEGFP-N1 with CMV promoter (CLONTECH®, Germany) was used for the study of expression. The plasmid was transformed into $DH5_{\beta}$ *Escherichia coli* competent cells using standard procedures, and purified on a Gigaprep column (QIAGEN, The Netherlands).

A plasmid (pCMV-CAT) covalently linked with psor-

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alen–fluorescein pCAT (gift from Genemedicine, Texas, USA) was used for the localization study.

Topical Delivery of Plasmid

Animals were anesthetized during the experiment by diethyl ether (Vel, Belgium) inhalation. Since the target cells for gene expression were the epidermal cells, the stratum corneum—the main barrier to skin penetration—was partly removed. Hence, the abdominal skin was stripped 20 times with tape (Scotch Crystal, 3M™, France). A thin layer of stratum corneum remained in approximately 20% of the skin surface. A fold of skin was clamped into a custom-built clip. The clip was composed of two compartments each containing platinum electrode of 1 cm^2 (99.99% purity, Aldrich Chemie, Belgium). The electrodes were at the outer surface of each compartment (not in contact with the skin) and the distance between the two electrodes was 6 mm. The cathodic compartment (donor) was filled with 100 μl of plasmid solution (50 μg/100 μl) in a phosphate buffer of pH 7.4 (10 mM), while the other compartment contained 100 μl of the same buffer. No shift in pH was observed after pulsing. The electrodes were connected to the electroporation device Cytopulse PA-4000 (Cyto Pulse Sciences, Inc., Maryland, USA) for application of pulses. Cytopulse PA-4000 delivers square wave electric field pulses of variable voltage and duration. During a pulse, electrical behavior was measured with an oscilloscope (model 54602B, Hewlett-Packard, USA). The voltage and current were measured directly. Transdermal voltage, energy applied, and amount of charges transferred were calculated as previously described (15).

Different pulsing protocols were applied: 10 pulses of 1000V (applied voltage) of 100 μs duration 10× 1000V–100 μs, 10 × 335V–0.5 ms, and 10 × 335V-5ms (9–11). Pulsing caused slight muscle twitches in the rat. Plasmid was in contact with the skin 5 minutes before and after electroporation. A passive diffusion of 10 minutes was performed in the control animals.

Plasmid Localization in the Skin

A kinetic study was performed to localize the plasmid in the skin. Five minutes, 3 h, 8 h, and 24 h after topical delivery of the psoralen–fluorescein pCAT by electroporation, animals were sacrificed, skin was removed, cleaned with phosphate buffer of pH 7.4, and observed immediately by Confocal Laser Scanning Microscopy (CLSM). CLSM was chosen to localize the plasmid in the epidermal cells, as this technique allows localization of fluorescent molecules without distorting the living tissue. The skin samples were directly placed in a sample holder, covered with a coverslip, and placed into the CLSM system. The confocal microscope system used a BioRad MRC 1024 confocal unit equipped with an argon–krypton laser and mounted on a Zeiss Axiovert 135M-inverted microscope. The psoralen-fluorescein pCAT was detected using a BioRad filterblock, which selects the 488 nm laser line to illuminate the specimen, and transmits emitted light with a wavelength in the 522–535 nm range. Images were obtained using a Zeiss Plan-Neofluar 40× oil immersion objective (12).

Optical sectioning was performed parallel to the skin surface (xy planar optical section), at different focal planes (z), the z-axis being perpendicular to the plane of the skin surface. All images were the average of 3 scans, and were obtained with the same optical aperture, filterblock, lens, black level, scan speed, and laser intensities.

To investigate the autofluorescence properties of the skin, samples were investigated with the confocal microscope in the absence of psoralen–fuorescein pCAT. Some minor autofluorescence was found mainly in the cutaneous appendage (hair). No fluorescence was observed in the keratinocytes.

Plasmid Expression in the Skin

Gene expression was assessed by the localization of Green Fluorescent Protein (GFP) in the epidermis. GFP is a fluorescent protein which can be excited at 488 nm, and the emitted fluorescence is monitored at >500 nm (23). At 2 days, 4 days, and 7 days after topical delivery of a reporter gene plasmid (pEGFP-N1) by electroporation, skin samples were removed from the rat, and cleaned with phosphate buffer of pH 7.4 and observed immediately by Confocal Laser Scanning Microscopy (CLSM), as described previously. The measures of the fluorescence intensities were performed with the BIORAD software (Lasersharp, BIORAD MRC-1024 Software) provided with the CLSM. Two independent investigators assessed the fluorescent areas.

In order to study the influence of the plasmid conformation on gene expression in the skin, the plasmid pEGFP-N1 was linearized by Hind III (a restriction enzyme) and delivered to the skin by electroporation at the same conditions as described above. An agarose gel electrophoresis (1%) was performed to check the plasmid form before electroporation.

Integrity of Plasmid Following Treatment

The plasmid remaining in the donor cell following treatment (passive diffusion, electroporation) was investigated by agarose gel electrophoresis (1% in Tris-Borate-EDTA [TBE] pH 8). The DNA bands were revealed by overnight immersion in ethidium bromide (1 μg/ml) to prevent changes in the migration of nucleic acids. DNA samples were treated with Hind III (Gibco BRL, Belgium), SDS 1%, or Proteinase K (Boerhinger Mannheim, Belgium) to investigate the structure of the DNA bands in the gel.

Skin Integrity: MTT Assay

The MTT assay (24) was performed to assess the viability of the skin after electroporation *in vivo*. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO, USA) is a yellow, water-soluble compound that is enzymatically reduced to dark purple and insoluble formazan by viable cells.

Biopsy samples of 4-mm were obtained from treated skin using a disposable biopsy punch (Stiefel, Germany) at time points up to 24 h post-electroporation, and rinsed with phosphate buffered saline (PBS). Excess solution was carefully removed and the biopsies were weighed and placed into individual wells of a six-well tissue culture plate (Greiner, Belgium). On the day of the assay, 2 mg/ml MTT was dissolved in freshly prepared PBS. Any undissolved crystal was removed by filtration through a 0.45 μm syringe filter. Two ml of MTT solution was added to each well, and the plate was incubated for 2 h at 37°C under stirring. After incubation, the

remaining MTT solution was removed by aspiration, and the tissue was rinsed twice with ml of PBS for 1 minute. The tissue was then shredded with surgical scissors, and the formazan precipitate was extracted into 4 ml (DMSO) (CarloErba, FARMITALIA, Italy), agitating with a tilted, rotating platform for 80 minutes. The group of enzymes responsible for the reduction of MTT is collectively referred to as tetrazolium reductase (TR), and data was expressed in absorbance units per mg tissue (TR index). Formazan absorbance was measured at 540 nm.

RESULTS

Plasmid Localization in the Skin

In order to determine whether plasmid penetrates into the keratinocytes, fluorescence-labelled plasmid was applied on the stripped skin for 10 minutes with or without skin electroporation. Five minutes and three hours after topical gene delivery by electroporation (10×-1000V-100 μs), the fluorescence-labelled plasmid was mainly localized around the keratinocytes. There was also some localization in the cytoplasm (Figs. 1a and 1b). A homogeneous cytoplasmic fluorescence was observed (instead of spotting fluorescence), which suggests the endosome–lysosome pathway was bypassed. Eight hours and twenty-four hours following the electroporation (10×-1000V-100 μs), all of the fluorescent-labelled plasmid was localized in the keratinocyte cytoplasm (Figs. 1c and 1d). Nuclear penetration could not be clearly seen, suggesting that transport in the nucleus remains a limiting step. The plasmid did not penetrate the dermis. There was no difference between the different electroporation protocols. After passive diffusion, some fluorescence was observed in the epidermis but to a lesser extent than after electroporation (Table I). These data demonstrate that a plasmid applied on stripped skin and subjected to electroporation penetrates into keratinocytes.

Plasmid Expression in the Skin

In order to investigate whether the plasmid topically delivered into skin keratinocytes by electroporation could induce gene expression, a plasmid containing a reporter gene (pEGFP-N1) coding for a fluorescent protein, the GFP was administered using identical procedures as for plasmid localization. Short high-voltage pulses (9) and long medium-voltage pulses (10,11) were utilized in order to investigate the influence of the electrical protocol on gene expression (25).

GFP expression in keratinocytes was observed in the epidermis two days post-electroporation (Fig. 2a and Fig. 2b), with expression being localized in distinct areas in the electroporated zone. The GFP expression persisted in some animals for at least seven days, but decreased in intensity and the number of expressing areas with time (Table II). The GFP fluorescence was homogeneously localized in the cytoplasm. The type of electroporation protocol did not significantly influence GFP expression in keratinocytes.

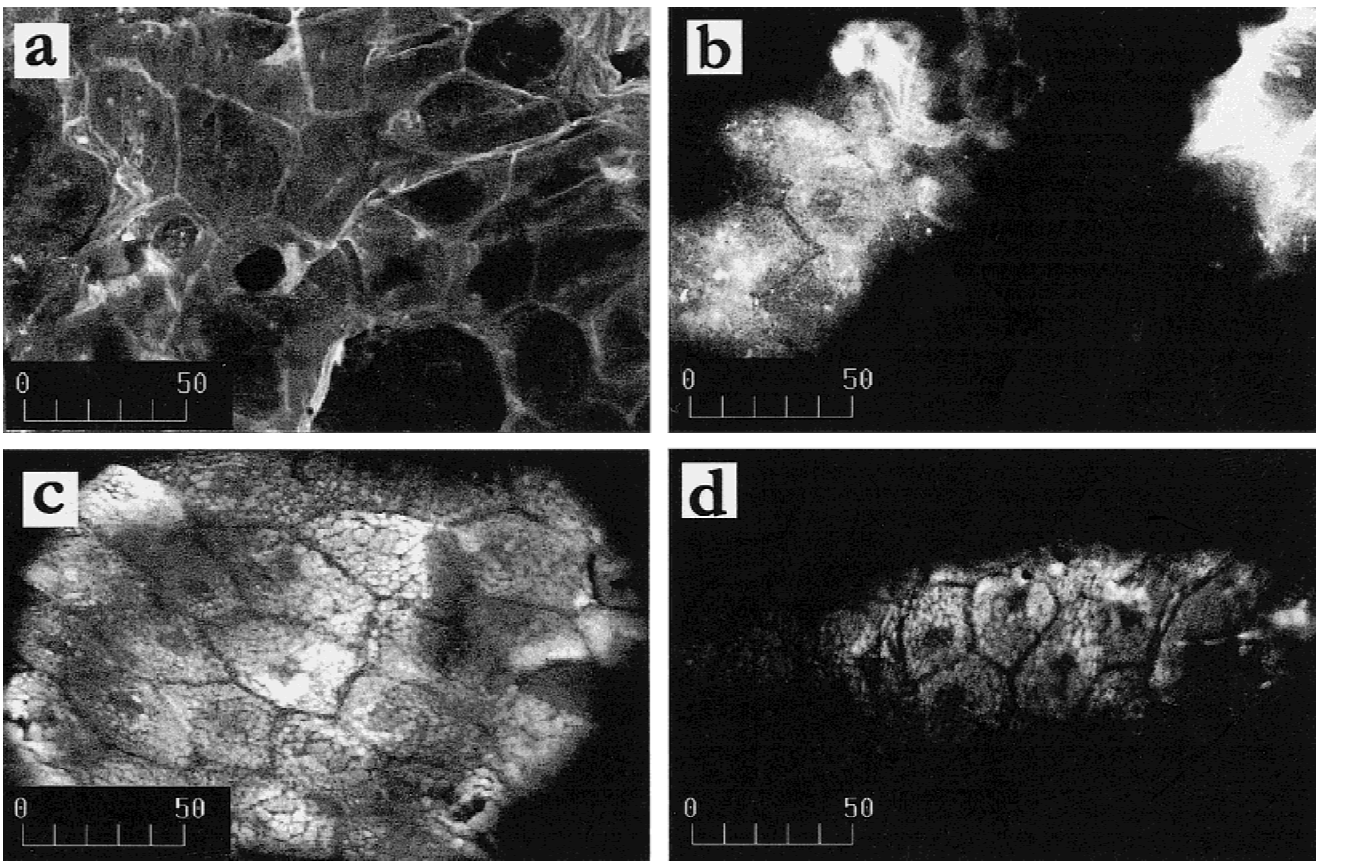

Fig. 1. XY-planar CLSM sections showing the localization of the fluorescence labelled plasmid in the epidermis 5 min (a), 3 h (b), 8 h (c), or 24 h (d) following skin electroporation (10×-1000V-100 μs). The image was acquired at depth 12 μm (a), 18 μm (b), 12 μm (c), and 10 μm (d) below the surface of stripped skin. Scale bar: 50 μm.

Table I. Localization of Fluorescent-Labelled Plasmid Delivered Topically by Electroporation or Passive Diffusion

Localization	Electroporation	Passive Diffusion
5 min	Extracellular Weakly cytoplasmic	Weak and fuzzy images
3h	More cytoplasmic	Few fluorescent areas
8h	Only cytoplasmic	Very weak
24h	Plasmid still present in the cytoplasm	Experiment not carried out

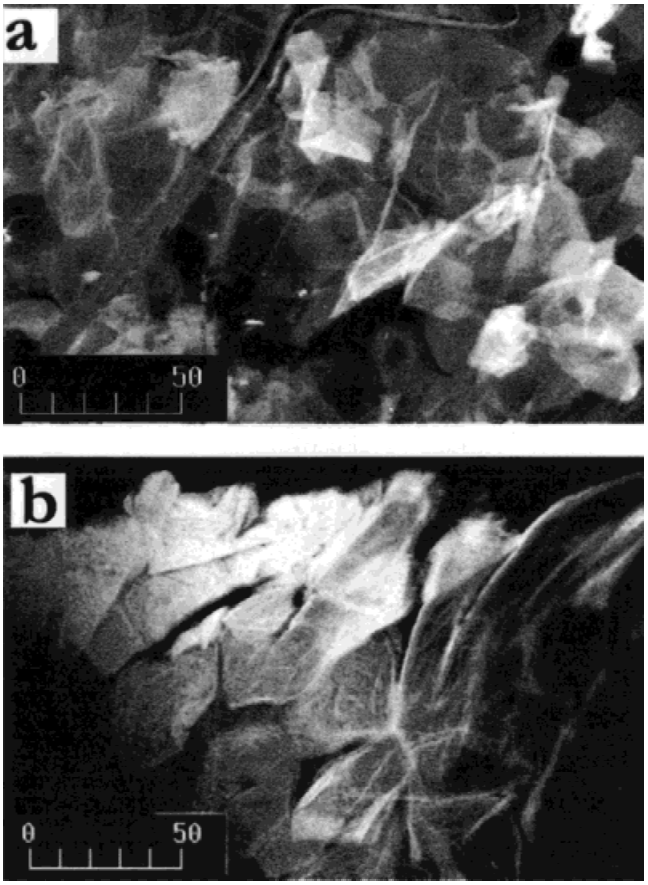

Fig. 2. XY-planar CLSM sections showing the expression of pEGFP-N1 in the epidermis at 2 days (10×-1000V-10 μs) (a) or 4 days (10×-335V-5 ms) (b) after skin electroporation. The image was acquired at depth 4 μm (a) or 2 μm (b) below the surface of stripped skin. Scale bar: 50 μm.

Compared to electroporation, passive diffusion resulted in a weaker and more diffuse expression, which was only detected for two days after passive diffusion of the plasmid (Table II). When the plasmid was linearized, the expression of GFP following electroporation was decreased as compared to nonlinearized plasmid (data not shown).

Plasmid Integrity Following Treatment

In order to check whether the plasmid remained stable after 10 minutes of contact with the skin with or without electroporation, agarose gel electrophoresis of the plasmid remaining in the buffer was performed.

Figure 3 shows that the structural integrity of the plasmid

Table II. Expression of pEGFP-N1 in the Skin: Frequency, Depth, and Fluorescence Intensities

Expression	Electroporation	Passive diffusion
2 days	++[a] >50 μm[b] 122 ± 27[c]	+[a] 32 ± 9[c]
4 days	+[a] <50 μm[b] 44.5 ± 14[c]	0[a] 8 ± 1[c]
7 days	+/–[a] <50 μm[b] 36 ± 1[c]	0[a] 10 ± 1.4[c]

[a] visual appreciation of the number of areas expressing GFP.
[b] depth under the surface of the skin.
[c] mean intensity ± SEM in the GFP positive area for 10×-1000V-100 μs (arbitrary unit).

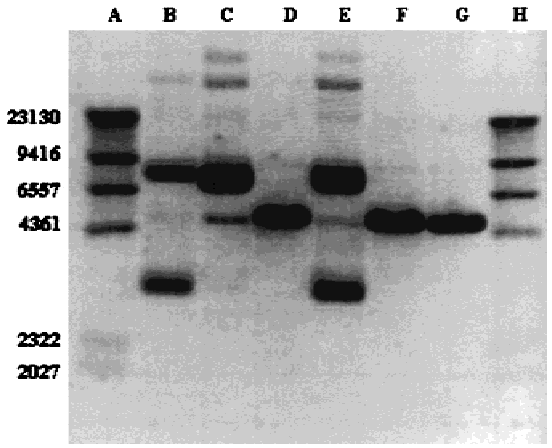

Fig. 3. Agarose gel electrophoresis (1%) of pEGFP-N1. A, H: molecular weight marker; B: pEGFP-N1 before skin contact; C, E: pEGFP-N1 after skin contact; D, F: pEGFP-N1 after skin contact + Hind III; G: pEGFP-N1 before skin contact + Hind III.

was unaffected by electroporation, before contact with the skin. After 10 minutes of contact with the skin, neither linearization nor a smear (band at 4.7-kb) was observed, indicating that the plasmid was not extensively degraded, the open circular form appeared more intensive, and additional bands at (9 kb–23 kb) were repeatedly observed when the native plasmid was electrophoresced. When the electroporated plasmid (in contact with the skin) was linearized with Hind III and electrophoresced, no such high molecular weight bands were seen. Incubation with proteinase K showed that these high molecular weight bands were not due to protein binding, whether covalent or not. Antibiotic (streptomycin sulfate and penicillin G, 100 U/ml, Gibco-BRL, Belgium) in the buffer did not change the profile of DNA bands. However, these high molecular weight bands decreased when Ethylene Diamene Tetracetic Acid (EDTA) (10 mM) was added in the buffer.

Skin Integrity

In vitro, a balance between transfection efficiency and cell viability has to be reached (26). Therefore, we investigated the effect of electroporation on the skin viability by measuring the TR activity with the MTT test. The TR index was unaffected; none of the electroporation protocols

changed the TR index after 5 min or 3 h, as compared to control values ($p > 0.05$). (Fig. 4)

DISCUSSION

This study was based on the hypothesis that electroporation can permeabilize the keratinocytes and can enhance the transport of macromolecules into skin. We assessed whether electroporation is an efficient method for delivering topically-applied naked plasmid DNA into keratinocytes.

The first key issue addressed was to determine whether DNA plasmid delivered across partially stripped skin penetrates keratinocytes. None of the *in vivo* studies investigated the transport kinetics of DNA using electroporation. CLSM showed that plasmid penetrates into the keratinocyte cytoplasm after skin electroporation *in vivo.* Cytoplasmic penetration did not, however, occur immediately. In contrast to oligonucleotides (11) which reached the nucleus within minutes after skin electroporation, the plasmid was first associated with the keratinocyte membrane before intracellular penetration. Literature on DNA transfer in isolated cells also reports that DNA transfer by electroporation involves different steps. The interpretation is that the high voltage pulse allows the electrotransfer of DNA to the cell membrane, and the insertion of DNA in the aqueous pathways of the lipid bilayer. After pulsing, the long DNA molecule penetrates slowly in the cytoplasm through the "pores." The diffusion coefficient for the translocation of DNA through electroporated membranes is lower than the diffusion coefficient of small molecules (27,28). It is likely that a similar phenomenon occurs in the skin. The roles of high voltage pulses would be to drive the DNA into the skin by electrophoresis, and to act on the cell permeabilization.

The endosome–lysosome pathway was avoided, as evidenced by a homogeneous staining of the cytoplasm. Interestingly, the plasmid permeation was localized in distinct areas in the electroporated zone. The transport of small molecules has also been reported to be associated with localised transport regions (LTRs) (29). In contrast to fluoresceine isothiocyanate (FITC) (14) or oligonucleotide penetration (12), plasmid penetration was limited to the superficial layers

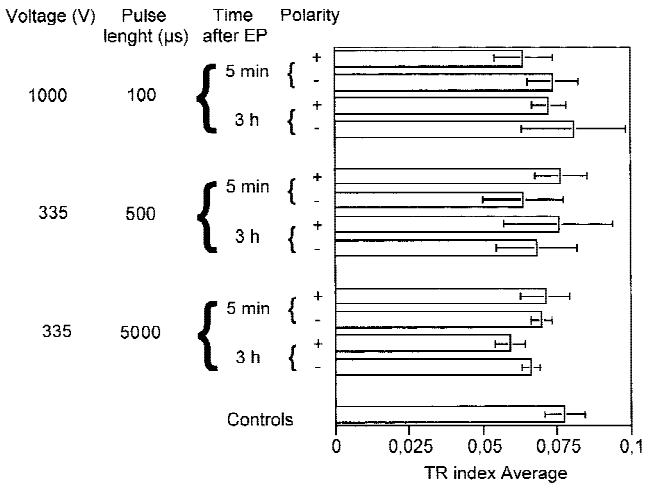

Fig. 4. TR index (MTT assay) 5 min or 3 h after skin electroporation. The results were compared with control skin (Student's t-test, $p > 0.05$).

of the skin, probably due to their larger size (4.7 kb ≡ 3.10^6 Da).

A small amount of plasmid penetration by passive diffusion is probably due to the partial stripping of the skin and the 10 minutes of contact. Such DNA expression following passive diffusion concurs with reports by Yu *et al.* (30) who demonstrated that topical application of DNA to mouse skin following shaving and mild abrasions induces a rapid gene expression. Our results show that electroporation strongly enhances DNA transfer into keratinocytes compared with passive diffusion. These data prove that electroporation enhances DNA transport in viable skin probably via an electrophoretic mechanism and permeabilizes keratinocytes.

The stratum corneum is the main limiting barrier for transdermal drug delivery. Due to the high molecular weight of plasmids, the skin was stripped to check if DNA could be transferred into viable epidermis by electroporation. Further experiments will investigate DNA delivery through intact skin. Approximately 80% of the skin surface was devoid of stratum corneum following the stripping procedure. Interestingly, the DNA was localized in the keratinocytes of these areas, in agreement with electroporation literature. Actually, the current follows the least resistant pathway (here, the stripped areas).

Once we had shown that DNA plasmid could be transferred to keratinocytes by skin electroporation, the next step was to determine whether a gene expression could be induced by the topical delivery of a reporter gene using high voltage pulses. The expressed GFP was observed for up to 7 days in the keratinocytes. Expression decreased in intensity with time, probably due to the skin turnover. Compared to passive diffusion, electroporation strongly increased the expression duration and the number of transfected sites.

It has already been established that electroporation allows *in vivo* gene transfer of injected DNA in tissues (7,8). In our experiments, no injection was needed. The DNA crosses the stratum corneum remaining after stripping, permeating into the epidermis and the cell membrane of the keratinocytes.

Other methods that have been used to deliver DNA in the skin have resulted in expression for different durations of time. For example, viral methods which use adenoviruses or retroviruses allow a more prolonged expression (several weeks), but have the disadvantages of viruses (e.g., immunogenicity, cell division); naked DNA injection (18) and gene gun (19) which induce expression proteins for up to 3 weeks and 8 weeks, respectively, but require injection; liposomes (17) and puncture (20) which induce a peak expression at 48 h which persists for up to 10 days (similar duration to our results).

In the literature, two types of protocols of DNA transfer *in vivo* by electroporation are reported, namely short high-voltage pulses (9) and long medium-voltage pulses (10,11). In our studies on the localization and expression of plasmid DNA, no obvious difference was observed between the two types of protocols.

Due to the presence of nucleases in the skin, the DNA integrity was checked. Importantly, neither a linearization of the plasmid, nor an extensive degradation underlined by a smear, was detected. However, the plasmid showed more open circular form and some higher molecular weight bands after contact with the skin, probably due to a cationic enzy-

matic-dependent process. In contrast to linearization, which decreased GFP expression in the skin, an expression of GFP was observed, suggesting that the plasmid was still active.

Electroporation may be a promising alternative method to deliver genes into skin, particularly for genetic vaccination. The use of plasmid DNA simplifies gene preparation relative to the use of viruses. The expression is limited to cells electroporated in the presence of plasmids. Targeting could be further extended by the use of cell-specific promoters on the delivered plasmid. Since the expression was not uniform and limited to a week, application in gene therapy would be limited. However, the transient expression in the epidermis could be sufficient to induce an immune response. Skin electroporation may thus be an alternative strategy to deliver genetic vaccines. Further studies are ongoing.

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