Topical delivery of nucleic acids in the skin

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The skin is an attractive site for the delivery of nucleic acid-based drugs for the treatment of topical and/or systemic diseases as well as for DNA immunization. However, due to the barrier properties of the skin, the penetration of nucleic acids in or across the skin is limited. This review focuses on the chemical, biological or physical methods developed to enhance nucleic acid delivery into the skin. Oligonucleotides have been delivered across the skin using iontophoresis. Potentially therapeutic concentrations were reached in hair follicles using liposomes and in keratinocytes using electroporation. In vivo *transfer of genetic material to the skin has been achieved experimentally using dermal injection, topical application of naked DNA, DNA/lipids complexes or viral vectors as well as particle bombardment, puncturing or electroporation of plasmid DNA. Protein expression has been detected in the skin and/or the serum. The localization and duration of expression was affected by the delivery methods. The remaining challenges to address for optimal* in vivo *gene delivery in the skin are i) duration of expression (more than 1 to 4 weeks for the treatment of inherited skin and systemic diseases requesting protein supplementation), ii) delivery methods (if progress has been achieved, a safe, efficient, patient-friendly, non-invasive method for long-term expression at a high level in the target cells and extensive body area of a patient has not yet been developed), iii) targeted delivery.*

Key words: Transdermal delivery — Topical delivery — Skin — Oligonucleotides — DNA — Nucleic acid — Gene therapy — DNA immunization.

I. INTRODUCTION

Recent advances in biotechnology have brought new categories of therapeutic agents, protein-based drugs and DNA-based drugs to the forefront of therapeutic research. Due to their low oral bioavailability, they are usually administered by the parenteral route. New routes of administration have, however, been developed to avoid the drawbacks of injection. The scope of this review is to investigate the delivery of nucleic acid-based drugs, i.e., oligonucleotides (ODN) and DNA into the skin. The delivery methods designed to improve the transport of ODN and gene into or across the skin will be reviewed and discussed in terms of mechanisms and potential applications.

1. Skin structure and transdermal drug delivery

In order to understand the challenge associated with the topical delivery of nucleic acids, the structure of the skin and the mechanisms of transdermal drug permeation should be taken into account.

Mammalian skin is divided into two layers: the outermost epidermis and the inner dermis. Appendages, i.e. sweat glands and hair follicles, penetrate the epidermis. The dermis provides physiological support for the epidermis by supplying it with blood and nerve endings. The main cells are fibroblasts that supply the collageneous matrix. The epidermis (~ 100 mm) is a self-renewing stratified squamous keratinized epithelium. It consists of keratinocytes, which differentiate progressively and other cells including Langerhans cells and melanocytes. The outermost layer, the *stratum corneum*, is 10-25 mm thick and consists in multiple lipid bilayers surrounding dead keratin-filled corneocytes. The pathways of molecular transport across skin are the transcellular and paracellular route through the *stratum corneum* and the transappendageal pathways.

Transdermal or topical delivery is limited by the barrier properties of the skin, particularly the *stratum corneum*. Small potent lipophilic drugs can be delivered by passive diffusion without enhancement. They penetrate according to Fick's law of diffusion.

Experimental data and modeling of skin penetration by passive diffusion clearly demonstrate that molecular weight and log P are critical parameters. An increase in molecular weight above 500 to 1000 Da or a decreasing log P below 1 strongly decreases transdermal transport [1]. Hence, for more hydrophilic molecules and for macromolecules such as nucleic acids, methods had to be developed to expand the range of compounds delivered transdermally and to enhance their transport. Enhancement methods are based on two strategies: increasing skin permeability and/or providing a driving force acting on the drug. Based on these strategies, chemical methods (e.g., chemical enhancers, liposomes) or physical methods (phonophoresis, iontophoresis or electroporation) have been developed [1-3]. Chemical enhancers modify the skin barrier and enhance drug transport across the skin. They promote the transport of both hydrophilic and lipophilic drugs but significant enhancement of macromolecules transport has not yet been demonstrated. Several mechanisms seem to be involved including disruption of the lipid structure, increased drug solubility in the formulation, increased drug partitioning in the *stratum corneum* and/or interaction with the hydrophilic domain [2].

Encapsulation of drugs in vesicles such as liposomes, niosomes and transferosomes has been seen to enhance the topical and transdermal delivery of a variety of compounds. These vesicles promote transport by partitioning into the *stratum corneum* and acting as a drug reservoir and/or by increasing skin permeability. Cationic lipids have been used to deliver negatively charged nucleic acids. Some liposomes can target drug delivery to hair follicles [4, 5].

Sonophoresis or phonophoresis involves application of ultrasound techniques to enhance transdermal drug delivery. Therapeutic protein rates (up to 40 kDa) could be reached *in*

vitro with low frequency ultrasound by a mechanism of cavitation that disrupts the *stratum corneum* microstructure and provides pressure as a driving force [6]. Under controlled conditions, ultrasound is also an effective means of delivering plasmid DNA into cells. The subsequent expression of DNA in cells depends on a balance between transient cell damage and cell death [7]. Since no paper deals with nucleic acid delivery in the skin by ultrasound, this enhancing method will not be discussed further even though it could be potentially attractive.

Iontophoresis consists in applying a low electric field (< 0.5 mA/cm^2) for minutes or hours. Iontophoresis drives molecules across the skin by electrostatic repulsion and/or electro-osmosis and induces changes in the skin as a secondary phenomenon. It has attracted considerable interest for expanding the range of compounds delivered transdermally to hydrophilic, charged drugs of medium molecular weight (< 10 000 Da). It is now considered a safe procedure [8-10].

Electroporation consists in applying short high voltage pulses that create transient aqueous pathways across lipid bilayers and hence increase molecular transport across membranes [11]. Compounds ranging in size from small ions to large macromolecules can be introduced into cells *in vitro*. Gene transfection using electroporation has become a routinely used technique in molecular biology. *In vivo* electrically mediated gene delivery is a promising non-viral method for gene delivery *in vivo* in different tissues such as the liver or muscle [12, 13]. There is now considerable evidence that a high voltage rapidly causes a large increase in molecular transport across the skin both *in vitro* and *in vivo* [1, 2]. Even macromolecules can be delivered transdermally [14, 15]. Another potential advantage of high voltage pulsing is the permeabilization of the tissue exposed to the electric field.

2. Therapeutic use and delivery of ODN and DNA

2.1. ODN

The objective of ODN therapy is to inhibit gene expression in a highly sequence-specific and selective manner. Distinct strategies have been developed: i) the antigene strategy relies on a short ODN sequence to form triple helical structures with duplex DNA in order to interfere with gene expression at the level of transcription; ii) the antisense ODN are short ODNs designed to interfere with gene expression at the level of single stranded RNA to block RNA translation; iii) ribozymes are RNA-based ODNs which can catalyze the hydrolytic destruction of the target RNA in the RNA-ribozyme complex; iv) the aptamer approach aims at targeting molecules which bind to proteins involved in gene regulation and expression and inhibit their activity. The antisense strategy is the most developed approach. It relies on Watson-Crick base pairing to hybridize the target RNA and inhibit translation by several putative mechanisms including steric hindrance of ribosome reading and RNAse H activation to destroy the RNA component of the duplex [16-18].

The naturally occurring phosphodiester backbones of ODN are highly sensitive to enzymatic degradation. Chemical modifications of this backbone have been designed to bypass this problem and improve biological stability, hence making this a better candidate for *in vivo* therapeutic use. However, these chemical modifications affect both cellular uptake and the interaction with the target molecules (hybridization process, RNAse H cleavage, etc.) [18].

In theory, any disease with a gene expression component has the potential to be treated with nucleic acids. Among these numerous potential therapeutic applications, the antisense therapy has shown potential as a treatment for a variety of diseased states such as infection, inflammation or cancer. Viruses (e.g., HIV, HSV, HBV, etc.) are attractive therapeutic targets since their genetic sequence is unique with respect to the human host. Oncogenes (e.g., ras, abl, myc, myb, fos, etc.), are also potentially interesting targets for cancer [18]. Less obvious potential uses such as restenosis have also been investigated [16].

The therapeutic use of antisense ODN has been limited by i) the poor targeting to cells, ii) poor cellular delivery, e.g., uptake and sub-cellular trafficking, iii) low stability *in vivo* and iv) non-antisense toxic effects. Chemical modifications to reduce the susceptibility of ODNs to nuclease whilst retaining their ability to bind to their target site have been developed. A major challenge for antisense development is to overcome these barriers and strategies to improve antisense ODN delivery have been developed.

The mechanism for entry of ODN into cells is not yet completely understood. Both fluid phase endocytosis and receptor-mediated endocytosis have been reported. Most of the intracellular ODNs lie in the endosomal-lysosomal compartment. Since the site of action of ODN is the cytoplasm or the nucleus, the ODNs must enter these cellular compartments. Several methods have been developed to overcome the problem of poor cellular and cytosolic uptake of ODN: i) chemical modification such as introduction of a lipophilic group into the ODN structure, ii) entrapment in liposomes and/or complexation with cationic lipids [19], iii) water-soluble or nanoparticulate polymeric carrier [20] and iv) electroporation *ex vivo* [21]. These methods can also sometimes improve ODN stability. Liposomes and related carriers protect ODNs against degradation, modify their pharmacokinetics and biodistribution and potentially increase their uptake in cells. The poor efficiency of encapsulation of water- soluble ODNs in neutral liposomes can be overcome by increasing ODN lipophily or by using cationic lipids that bind spontaneously to nucleic acids.

Selective targeting to critical cells is another issue. Topical delivery is particularly attractive for the treatment of skin disease because it allows increasing ODN concentrations in the target tissue without any significant amount of ODN in the systemic circulation.

2.2. DNA

The potential use of DNA-based drugs could be: i) gene replacement to introduce a defective or missing gene, ii) gene therapeutic to deliver a gene expressing a protein with a specific pharmacological effect, iii) gene coding for a suicidal enzyme, iv) immunotherapy with DNA coding for cytokines involved in immune responses and v) DNA vaccine.

Gene therapy represents a new paradigm for therapeutic approaches. A gene under the control of an eukaryotic promoter is transferred into cells and is expressed in a protein that can

restore defective biological functions or reconstitute homeostatic mechanisms within cells. This is exemplified by the replacement of genetically defective genes in inherited disorders such as cystic fibrosis or inborn metabolic errors. Multifactorial acquired diseases could also be treated e.g., cell cycle control genes or suicidal enzymes for cancer, immune modifying cytokines for inflammatory or immune disorders [24].

DNA-based immunization represents a novel approach for vaccine development, particularly for therapeutic vaccines. It involves the administration of naked DNA that encodes antigens. The expression of the administered gene results in the induction of humoral and cellular immune responses against the antigen.

Effective gene therapy requires the administration of a gene encoding a therapeutic protein, delivery to target cells, migration to the cell nucleus and expression to a gene product. DNA delivery is limited: i) by DNA degradation by tissues or blood nucleases, ii) low diffusion, at the site of administration; iii) poor targeting to cells; iv) inability to cross membrane; v) low cellular uptake and vi) intracellular trafficking to the nucleus. Significant progress has been made to enhance the efficiency of gene delivery into tissue thanks to various strategies that have been developed. Methods to transfer genes *in vivo* include i) viral methods e.g., retrovirus and adenovirus, ii) chemically facilitated methods using vectors e.g., liposomes and iii) physical methods e.g., gene gun and electroporation. Each method has inherent advantages and limitations.

Viral vectors were the first routes explored to deliver genes into cells. The premise of virus- based gene therapy is that the viral vector carrying the therapeutic genes may exploit the natural virus pathways to achieve gene delivery and expression *in vivo* (or *in vitro*). It involves the genetic engineering of attenuated or defective viruses. Due to their ability to transfect cells, the most common viral vectors are retrovirus, adenovirus or adeno-associated virus (AAV) [23].

Non-viral methods i.e., chemical vectors and physical methods have been developed to overcome the problems (infection risks, potential mutagenicity, immunization, etc.) associated with viral vectors [24-27].

The most common materials used in non-viral preparations are purified supercoiled plasmids acting as gene expression systems and containing a gene encoding a therapeutic protein. Recombinant DNA techniques are used to clone DNA sequence encoding for proteins or antigens of choice into eukaryotic expression plasmids, which are readily and economically amplified in bacteria and then recovered with a high degree of purity and stability. However, the use of plasmid has been precluded by the inefficient uptake by cells and the low level and short-term expression compared to viral vectors. Hence, new methods to deliver plasmids have been developed.

Conventional liposomes have been tested as DNA delivery systems and were not very efficient. Improvements were achieved *in vitro* by using pH-sensitive or fusogenic liposomes [28]. Recently, there has been considerable interest in the development of cationic lipids for the delivery of nucleic acids into a wide variety of cells. Cationic lipids interact with the negatively charged phosphate backbone of DNA. The type of cationic lipids, the presence of other lipids, the DNA/lipid ratio and the positive/negative charge ratio influence the structure, size, charge and surface characteristics of the complexes. Cationic lipids promote the condensation of DNA, protect the DNA against degradation and modify its interaction with cell membranes and its intracellular trafficking [24, 29]. Transfection technology mainly uses liposomes composed of a binary mixture of cationic lipids and a neutral lipid such as DOPE (dioleyl-phosphatidylethanolamine) [28].

Formulation of plasmids with polymers has been explored as a means of enhancing plasmid stability and/or transfection efficiency. Both condensing cationic polymers, e.g., polyamido-amine cascade dendrimers or polyethyleneimine as well as non-condensing interactive polymers, e.g., polyvinylpyrrolidone have been successfully investigated [25,27].

Particle bombardment or biolistic technology provides a useful means for transferring foreign genes into a variety of cells in culture and tissues *in vivo*. It consists in accelerating and propelling DNA-coated particles using different kinds of so-called gene gun devices (based on voltage discharge, helium discharge or other techniques). The rationale of the gene gun, which bombards cells with DNA-coated microspheres, is to overcome the physical barrier of cell capture [30]. This method seems particularly attractive for nucleic acid immunization [31].

Electroporation has been reported to enhance by at least two orders of magnitude the expression of a naked plasmid injected in a tissue, leading to a protein expression higher than that induced by the injection of a plasmid complexed with or without cationic lipids [12, 13].

3. Delivery of nucleic acid-based drugs to the skin

The skin is an attractive target organ for the delivery of nucleic acid-based drug therapy because it is easily accessible and can be easily monitored. Delivery to a large target area could be feasible. Nucleic acid-based drug therapy can theoretically be used for treating inherited or acquired skin disorders such as infection, inflammation, cancer or wound healing as well as for the systemic delivery of proteins. Furthermore, promoter elements of tissue-specific genes, including keratin genes, have been identified. However attempts at therapeutic cutaneous gene delivery have been hindered by an inability to achieve efficient and long-lasting expression. Due to the continuous regeneration of the epidermis, the expression is generally brief, typically declining over a 2-7 day period.

The skin offers a unique potential as a target for DNA-based immunization. The skin contains numerous Langerhans cells and dermal dendritic cells that could acquire antigens either directly or through uptake and can present antigens to T cells in the lymphoid environment [32, 33].

In conclusion, transfer of nucleic acid-based drugs into the skin has many potential applications. Advances in the topical delivery of ODN or DNA have been achieved recently by the development of new delivery methods.

II. TOPICAL DELIVERY OF ODN

Due to their relatively large size and charges, ODNs do not penetrate through the *stratum corneum* by passive diffusion.

Penetration enhancement techniques have proved essential to improve the transdermal and/or topical penetration of ODN. *Table I* summarizes the different studies on the transdermal and/or topical delivery of ODN. It shows that both chemical and physical methods have been used to achieve significant percutaneous penetration.

1. Passive diffusion with or without chemical enhancers

Attempts to administer ODN across or into skin by passive diffusion have been reported. Either the ODNs were present in high concentrations in the donor compartment with chemical enhancer or the skin was stripped to remove the barrier to ODN penetration.

Nolen *et al.* [34] evaluated the percutaneous penetration and retention of methylphosphonate ODN. Ten- and 14-mer ODN applied in saturated aqueous or solvent solutions penetrated through mice skin *in vitro* and remained within the micromolar range in the skin. ODN penetration decreased with molecular weight or with the introduction of a negative charge. The *stratum corneum* was shown to be the main barrier to ODN penetration. S35-labelled phosphorothioate ODN applied under occlusion on the skin penetrated more readily across tape-stripped skin than normal rat skin. [35]

ODNs have been reported to penetrate in the skin *in vivo* by passive diffusion. "Superfusion" of c-fos antisense ODN inhibited the increase of c-fos induced by UV when 2 nmol ODN solution was applied four times in 3 h on shaved and stripped mice skin [36].

2. Liposomes

Several authors have reported that some liposomes can selectively target hair follicles to deliver both small and large molecules [4, 5]. Lieb *et al.* [37, 38] have shown that a liposomal-based formulation (lipofectine in alcohol-propylene glycol-surfactant) deposited about 2-6 times the quantity of ODN in the skin than a control formulation of ODN in buffer. About 1% of the dose applied was delivered to the hair follicles.

3. Iontophoresis

Iontophoresis is a potentially interesting and attractive method for the transdermal delivery of ODN to prevent risks and the pain associated with injection, to enhance the transdermal delivery of highly charged ODN of medium molecular weight by a mechanism of electrorepulsion and to achieve controlled ODN release. Additionally, iontophoresis could enhance the local concentration of ODN when a topical treatment is needed to treat infectious, inflammatory and/or cancerous diseases or to improve wound healing.

Vlassov *et al.* [39, 40] reported that ODN derivatives (P32 16-mer conjugated to benzylamine; 100 ml in 0.15M NaCl) applied to the skin of mice ear by iontophoresis resulted in an accumulation of radioactivity in animal tissues.

The iontophoretic transport of ODN *in vitro* was further studied by Oldenburg *et al.* [41] and Brand *et al.* [42-44]. Iontophoretic delivery of ODN resulted in substantial ODN flux. The cumulative amounts in the receptor compartment and/or the transdermal fluxes i) were directly proportional to the duration of iontophoresis ii) increased with the concentration of ODN iii) decreased with the length of the ODN iv) were affected by ODN conformation [41]. Controversial data on the effect of pH and ionic strength persist [41-42]. Factors other than size influence transport and their impact was greater at shorter lengths. Sequence and not base composition of equal-sized ODN affected fluxes across the skin [44].

Table I - ODN delivery in the skin.

Administration	ODN	Skin[a]	Transdermal fluxes	Accumulation in the skin	Ref.
Passive diffusion	PS 6-mer ≈ 40 μM	rat	< 0.04 nmol/cm^2.h	ND	[35]
Passive diffusion	PS 25-mer ≈ 40 μM	rat	< detection limit	ND	[35]
Passive diffusion	PS 25-mer 65 μM	hairless mice	< detection limit	ND	[42]
Passive diffusion	PS 15 and 24-mer 25.10^3 μM	human	< detection limit	ND	[45]
Penetration enhancer	MeP 14-mer supersaturated solution	hairless mice	0.05 nmol/cm^2.h	7.1 μM[e]	[34]
Penetration enhancer	MeP 14-mer supersaturated solution	hairless mice	1.8 nmol/cm^2.h	26.3 μM[f]	[34]
Liposomes[g]	PO 22-25-mer 450 μM	human[h]	ND	1% of dose[i]	[37, 38]
Iontophoresis (0.9 mA/cm^2-20 min)	PO 16-mer 10 μM	mice[j]	(≈ 2.10^{-4} nmol/g tissue)[j]	ND	[39]
Iontophoresis (0.9 mA/cm^2-50 min)	PO 22-mer 10 μM	mice	(≈ 5.10^{-3} nmol/g tissue)[j]	2.10^{-3} nmol/g tumor	[40]
Iontophoresis (0.5 mA/cm^2-12 h)	PO 6-mer ≈ 65 μM	hairless mice	≈ 0.16 nmol/cm^2.h	ND	[42]
Iontophoresis (0.3 mA/cm^2-2 h)	PO 20-mer ≈ 25 μM	hairless mice	≈ 0.03 nmol/cm^2.h	ND	[41]
Electroporation 720 x (80-139 V-1.1 ms)	PS 15-mer 25.10^3 μM	human	< 0.015 nmol/cm^2.h	ND	[45]
Electroporation 5 x (100-200 V-500 ms)	PS or 3' end derived PO 15-mer 3.3 μM	hairless rat	ND	1 μM	[46-49]

PS: phosphorothioate. PO: phosphodiester. MeP: methylphosphonate. ND: not determined.
[a] *In vitro* studies except [39, 40]. [b]Not studied isolated. [c]Epidermis. [d]EtOH/DMS 95/5. [e-f]Accumulation in the dermis [e]intact or [f]stripped skin. [g]Lipofectamine 25%, EtOH 15%, propylene glycol 22%, water 37.9%, Tween 80 0.1%. [h]Hair scalp. [i]Localisation in the hair follicles. [j]Ear lobe. [k]Mean concentration in the mice tissue (liver kidney blood, pancreas, guts and muscles), 30 and 90% ON degraded. [l]Administration to a subcutaneous tumor.

4. Electroporation

Since electroporation has been shown to enhance the transdermal transport of macromolecules, electroporation could be potentially attractive for transdermal delivery. Zewert *et al.* [45] showed that high voltage pulses enhance ODN transport through the human epidermis *in vitro* but the efficiency of transdermal permeation was low.

Regnier *et al.* [46-49] hypothesized that electroporation could be more interesting for the topical delivery of ODN based on the hypothesis that i) electroporation enhances the permeability of the *stratum corneum* and hence increases drug transport, ii) electroporation permeabilize underlying tissue as suggested by electrochemotherapy. It was demonstrated that electroporation increased the delivery of phosphorothioate and 3'derived phosphodiester ODN in viable skin by two orders of magnitude compared to passive diffusion. While ODN accumulated in the *stratum corneum*, micromolar concentrations were reached in the viable skin [46, 48]. Control of ODN delivery could be achieved by adjusting the pulsing conditions (number, voltage and duration of the pulses) and ODN concentration. The mechanisms of ODN delivery to viable skin were the creation of aqueous pathways and electrophoresis during pulsing [48]. On a more interesting note, the hypothesis that electroporation permeabilizes both the *stratum corneum* and keratinocytes was demonstrated. A rapid (within minutes) nuclear uptake of ODN in the keratinocytes was observed by confocal microscopy following electroporation but not iontophoresis [47].

Several methods have been investigated for the delivery of ODN across the skin. The topical delivery of ODN to hair follicles using liposomes or to keratinocytes using electroporation seems the most promising method. Even though transdermal delivery of ODN is enhanced by methods such as iontophoresis, relatively low transport efficacy could preclude the clinical development of an electrically controlled systemic delivery system.

III. TOPICAL DELIVERY OF DNA

Different methods have been investigated for the delivery of DNA into the skin. They are summarized in *Table II* and are based on the methods developed for gene transfection in cells and in tissues other than skin. Epidermal gene transfer has been achieved with *ex vivo* approaches where genes of interest have been stably introduced — mainly with viral vectors — in keratinocytes or fibroblasts and then grafted on nude mice. *In vivo* approaches, which are more patient-friendly and less invasive methods, seem more attractive. They include direct injection, topical delivery, liposomes, viral vectors or physical methods such as micropuncture, gene gun or electroporation.

1. Intradermal injection

The epidermis can take up and transiently express plasmid DNA after direct injection into animal skin.

Following intradermal injection of pCMVβgal, expression was almost exclusively localized in the keratinocytes of the epidermis. β-galactosidase (βgal) staining persisted for three weeks in the epidermis whereas βgal mRNA and plasmid DNA levels declined and disappeared during the first week. In contrast to muscle, the plasmid was not stably maintained in an extrachromosomal state in the epidermal keratinocytes, and consequently, gene expression was not as long-lasting as in muscle [50]. When both pig skin or human skin (grafted or organ culture) were injected intradermally with naked DNA, the DNA was taken up and expressed in the epidermis whereas DNA injected into mouse skin was expressed in the epidermis, dermis and underlying tissue [51].

A jet injector was used to form a jet of 100-300 ml DNA solution. The introduced DNA was found in cells surrounding the jet path up to 2 cm away from the injection site. βgal staining was observed at the site of staining with a vital fluorescent dye tracking the injection path [52].

Intradermal injection of naked DNA can be used to induce cytokine expression. Expression in the epidermis of a biologically active cytokine, IL-8, was demonstrated [50]. The injection of an IL-10 expression vector in the hairless rat induced local, dose-dependent expression of IL-10 mRNA and protein as well as circulating IL-10. IL-10 released from the transduced keratinocytes could inhibit the effector phase of contact hypersensitivity at a remote area of the skin [53]. Overexpression of IL-6 after plasmid intradermal injection induced macroscopic erythema, keratinocyte proliferation and lymphocytic infiltration in the treated area [54].

Recently, it has been shown both in pigs and monkeys that intradermal injection of naked DNA together with a competitive nuclease inhibitor, aurintricarboxylic acid, triggers a 50 to 65-fold enhancement of the expression of a reporter gene (luciferase) compared with DNA injection. Virtually all transgene expression was observed in the epidermis [55].

Direct intradermal injection can also be used for DNA vaccination. When small amounts of naked DNA expressing the nucleoprotein of influenza were injected into mouse tail skin, long-lasting (> 70 weeks) protective cellular and humoral immune responses were induced [56]. Many studies confirm that intradermal injection provides an efficient means for immunization by inducing long-lasting cellular (Th1) and humoral (Th2) immune responses. Haensler *et al.* [77] reported that, even though local gene expression of the luciferase reporter gene was 10 to 100-fold greater when injected into the muscle compared with jet-injected skin, both methods for naked DNA vaccine administration were equally efficient in inducing specific CTL and IgG antibodies.

2. Topical delivery

Topical application of naked plasmid DNA to the skin is particularly attractive as a simple approach for delivering genes to large areas of skin. The low permeability of the skin limits the use of this approach but gene expression after topical delivery has been reported.

When naked plasmid DNA containing a reporter gene was topically applied to mouse skin, gene expression was detected in the skin samples as early as 4 h after DNA application, reached a plateau after 16 to 72 h post-application and decreased significantly by 7 days post-application [58]. This expression was confined to the superficial layers of the epidermis and to hair follicles. Topical application of DNA following shaving and brushing was as efficient as intradermal injection.

Table II - Non-immunological studies on gene delivery in the skin.

Administration	DNA	Test	Expression length	Model	Ref.
Naked DNA injection (ID)	- pCMVβ (20 μg) - pCMVIL8 (20 μg)	- Expression - Neutrophil recruitment	3 weeks	Pig	[49]
Naked DNA injection (ID)	pCMVβ (12.5-20 μg)	Expression	1 d investigated	- Pig - Human grafts - Balb/c mice	[50]
Naked DNA injection (ID)	phIL6 (max 8 μg)	- IL-6 expression in epidermal sheet - Erythema, lymphocytic infiltration	2 d investigated	Hairless rat	[51]
Naked DNA injection (ID)	phIL10 (max 8 μg)	- IL-10 expression in epidermis - IL-10 conc. in serum	1 d investigated Max. 72 h	Hairless rat	[52]
Naked DNA ± nuclease inhibitor	- PCMVLuc (100 μg) - pEGFP-C1 - pNGVL1-ntβgal	- Expression (luc) - Histology (GFP, βgal)	1 d investigated	- Rat - Pig - Macaque	[55]
Naked DNA injection (jet injector)	- pHCMVE1 CAT (100 μg-3X) - pHCMVE1-β-gal - pHCMVE1-Luc	Expression at site of injection	2 d investigated	Mice	[53]
Naked DNA injection (jet injector)	pVR-Luc+ (0.1-10 μg)	Expression	2 d investigated	Mice	[53]
Topical application	- pCMVCAT - pCMVLuc (20 μg)	Expression	16-72 h/7 days investigated	Mice	[58]
Liposomes (DOTAP)	- pIRV-Neo-K5 (10-50 μg)	Expression in epidermis and hair follicles	6 to 24-48 h (decrease —> 1 week)	Mice	[60]
NC-liposomes (DNA complexes)	pM-MuLV-SV-Lacz (83 μg?)	Expression in hair follicles	3 d investigated	Balb/c mice	[61]
Liposomes	(1.05 mg DNA in total twice daily) - pSG5IL-1ra - pSG5Lacz	Expression in hair follicles	8 d investigated	Hamsters	[62]
Retroviral vectors	BAG-βgal retrovirus	Expression in wounds	?	Living skin equivalent	[65]
Retroviral vectors	LZRS retrovirus for β-gal ($5 * 10^6$/ml)	Expression	4 weeks *in vivo* (post-grafting)	SCID mice + regenerated human epidermis	[66]
Retroviral vectors	- LTR GFP - LTR lacZ - SIN IP GFP - IN SIN IP GFP	Expression	12 weeks	- Keratinocytes - ID mice (regenerated human epidermis)	[67]
Retroviral vectors	Retroviral vector constructs with factor IX cDNA and keratinocyte-specific enhancer elements	Factor IX in plasma	Up to 40 d	Nude mice + transduced keratinocytes	[68]
Viral vectors	- AdRSVβgal - Adα1AT	- Expression of βgal in skin - α1AT in plasma	- 4 d investigated - 14 d α1AT in plasma	C54/6N mice	[70]
Viral vectors (SC injection)	Ad Luc	Expression in epidermis		Mice	[71]
Viral vectors (adenovirus and herpes virus)	- Ad.CMV.lacZ ($4*10^9$- $4*10^{10}$ particles) - Ad.RSV.TGFα ($4*10^{10}$ particles) - HSV-lacZ ($5*10^4$ particles)	- Expression of βgal - Hyperkeratosis and acanthosis (TGFα)	- βgal in skin up to 10 d - 4 d	CD-1 mice	[72]
Microseeding	- pWRG1630 (EGF) (15 μg) - pCMVβ (15 μg)	Expression in epidermis and dermis	4 d (max. 2 d)	Pig (skin or wound)	[74]

Table II - Non-immunological studies on gene delivery in the skin (continued).

Administration	DNA	Test	Expression length	Model	Ref.
Puncture	- pRc(CMV)(p53) 120 μg - pβactin Luc - pSV40 lacZ	Expression of bgal at site of injection	Up to 7 d (max. 4 d)	Mice	[75]
Gene gun	- pRSV-CAT (0.1 μg?) - pRSV-Luc (0.1 μg?) - pCMVβ (0.1 μg?)	Expression	1-2 d investigated	Mice	[30]
Gene gun	pHβ-Luc (2.5 μg)	10-20% cell mRNA	Up to 14 d	CD-1 mice	[77]
Gene gun (powder ject)	pKCR-lacZ (150-200 ng)	Expression	Begin 6 h, up to 72 h	c57bl/10 mice	[76]
Gene gun	- pCMVβ (15 μg) - CRPV-pLAII (5-10 μg)	Expression of CRPV (papillomas)	Up to 8 weeks	Rabbit	[78]
Electroporation (SC+EP) 2 x 400 to 600 V/cm for 100 to 300 μs	- pSV3neo (2-12.5 μg) - pHEB4 (10-12 μg)	Neomycin resistance	At least 30 generations	Newborn mice —> culture cells	[82]
Electroporation (6 x 1750 V/cm, 100 μs) ± nuclease inhibitor	- PCMVLuc (100 μg) - pEGFP-C1 - pNGVL1-ntβgal	- Expression (Luc) - Histology (GFP, βgal)	1 d investigated	Pig	[55]
Electroporation (topical)	pM-MuLV-SV-lacZ (40 μg)	Expression of βgal until the dermis	Investigated until 3 d	Hairless mice (SKIII)	[84]
Electroporation (topical)	- pCMVβgal (50 μg) - pEGFP-N1	- Expression of bgal in epidermis - Localisation of GFP in keratinocytes	- Up to 7 d - Investigated up to 1 d	Hairless rat	[85]

p: plasmid. βgal, lacZ: β-galactosidase. CMV: cytomegalovirus promoter (eukaryotic promoter). IL-8: interleukine 8. HCMVIE1: human cytomegalovirus immediate early gene 1 enhancer/promoter sequences. CAT: chloramphenicol acetyl transferase. Luc: Luciferase. pIRV-Neo-K5: 1.4 kb bovine KIII gene promoter (≈ human K5) to drive βgal expression, murine leukæmia promoter promotes neomycine gene resistance. pSG5: eukaryotic expression vector. pCAGLacZ: β-actin promoter. CMVIE enhancer, βgal gene and rabbit β-globin 3' flanking sequence. pMC1TK: herpes simplex virus thymidine kinase promoter. pUC19: plasmid control. pSV40-β: eukaryotic promoter sarcous virus (?)-β-galactosidase. BAG-βgal: BAG (replicative defective retrovirus) contains βgal gene and neomycin resistance. LZRS retrovirus: LZRS episomal plasmid in which the retroviral LTR drives the expression of βgal. LTR GFP: long-term repeat green fluorescent protein. SIN IP: self-inactivating internal promoter vector. IN SIN IP: insulated self-inactivating internal promoter vector. L: LTR, N: neomycin, C: CMV, IX: human clotting factor IX cDNA, P: keratinocyte dependent enhancer from HPV-16 (human papilloma virus 16), K14/5: transcriptional sequence from the 5' non-coding region of hK14/5 (human keratin 14/5) gene. K14R: reverse orientation. α1AT: α1 antitrypsin. TGFα: tumor growth factor α. HA: hemagglutinin. EGF: epidermal growth factor. NP: nucleoprotein. HEB4: E1A of adenovirus 2 which could immortalize primary rodent cells. Neo: neomycin.

Topical application of plasmid vectors expressing lacZ and hepatitis B surface antigen to intact skin induced antigen-specific immune responses that displayed Th2 features. It seems that topical gene transfer was dependent on the presence of normal hair follicles. In the case of HBV antigens, these immune responses approach the magnitude of those produced by intramuscular injection of the commercially available recombinant polypeptide vaccine [59].

Further studies are required to determine the clinical potential of this simple non-invasive method to transfect large areas of skin.

3. Liposomes

Several papers report that DNA complexed with cationic lipids can effectively pass through the epidermis into the skin. When DNA (pIRV-NeoK5-βgal) was complexed to DOTAP (1/1.6 w/w), the DNA rapidly penetrated mouse skin and was expressed in the epidermis, dermis and hair follicles. Expression was seen as early as 6 h post-application, persisted at high levels 24 and 48 h post- treatment but was markedly reduced by 7 days after application [60].

Selective detection of the lacZ DNA expression was observed by X gal staining in hair follicles of mice 3 days after topical administration of the gene lacZ entrapped in liposomes (phosphatidylcholine/cholesterol/dioleylphosphatidylethanol-amine 5/3/2), suggesting the feasibility of targeting the hair matrix and possibly the follicle stem cells [61]. Auricular hamster skin treated for 3 days with DNA interleukin-1-receptor antagonist protein (IL-1-ra) formulated with non-ionic/cationic liposomes (NC) had significant levels of transgenic IL-1-ra present for 5 days post-treatment. Expression of transgenic IL-1ra was specific to the areas of skin treated with NC liposomes [62].

Yu *et al.* [58] showed that when the DNA/lipid ratio (mg DNA/nmol lipid) was greater than 1/1, the expression levels observed after topical application of cationic lipids were comparable with those produced by application of DNA alone. Reporter gene expression decreased with increasing lipid concentrations. After topical application of liposomes containing the hemagglutinating virus of Japan, a 5 times lower transfer efficiency was reported than after naked DNA injection [63].

Topical application of DAN encapsulated in liposomes can

induce short-term gene expression. The formulation of the lipid-based complex is a critical factor that needs to be optimized.

4. Virus

Both retrovirus and adenovirus have been used to transfect DNA in skin.

Transgene can be introduced into fibroblasts or keratinocytes *ex vivo* and lead to the expression of gene products with local or systemic effects. The keratinocyte is an attractive target for the purpose of *ex vivo* gene therapy. The epidermis can be biopsied to provide the source of keratinocytes, which can be expanded in culture. *Ex vivo* retroviral and adenoviral methods have been used to transfect these cells *ex vivo* before re-implantation *in vivo*. Most studies using retroviruses have been performed *ex vivo*.

The theoretical advantages of *ex vivo* therapy, relatively easy delivery and stable integration of the gene, are outbalanced by the expensive and long-lasting procedure and the risk associated with the procedure. However, this approach is an interesting tool for assessing the feasibility and understanding the mechanisms of gene delivery in skin.

4.1. Retrovirus

Partial and full-thickness wounds made *in vitro* in a human living skin equivalent were placed in contact with a transduced cell line producing a replication defective retrovirus containing the βgal gene. Expression of bgal was uniformly present at the wound edge and along the base of the entire partial thickness wound [64].

Human keratinocytes transduced with a retroviral vector for bgal with 99% efficiency were then grafted onto immuno-deficient mice to generate a human epidermis. Although integrated vector sequences persisted unchanged in epidermis at 10 weeks post-grafting, retroviral LTR-driven βgal expression ceased *in vivo* after approximately 4 weeks [65]. Whilst expected in non-integrating viral vectors such as adenovirus, in the case of retrovirus, this loss of gene expression occurred despite the retention of vector sequences for several turnover periods. In contrast, insulated and uninsulated LTR defective internal promoter vectors displayed consistently strong levels of sustained marker protein expressions for up to 10 to 12 weeks [66].

Keratinocytes transduced by a retroviral vector have been shown to express the human clotting factor IX but a low human factor level was detected for less than a week in the plasma of mice grafted with these cells [67]. Factor IX in plasma was two to three times greater with HPV16 and hK5 elements as promoters than with vector containing the CMV promoter alone [68]. Kolodka *et al*. [69] also showed long-term engraftment and persistence of transgene expression in retrovirus-transduced keratinocytes that could be keratinocyte stem cells.

4.2. Adenovirus and other viruses

Gene transfer to the skin using adenovirus has also been demonstrated both *ex vivo* and *in vivo*.

When murine keratinocytes infected with replication deficient adenovirus (Ad) coding for human α1 antitrypsin (α1AT) were transplanted in mice, hα1AT was detected in the serum for at least 14 days. When Ad RSVβgal or Adα1AT were administered subcutaneously to mice, expression of βgal was detected after 4 days in the epidermis and dermis and human α1AT was detectable in the serum for at least 14 days [70].

Lu *et al.* [71, 72] showed that the subcutaneous administration of an adenoviral vector containing the luciferase reporter gene induced a strong expression of the transgene in dermal cells but only a small portion of epidermal cells were transduced.

Two other viral vectors (adenoviral vector (ad.CMV.lacZ) and the herpes simplex virus (HSV) amplicon vector were tested to transduce murine epidermis through topical application. After topical application of ad.CMV.lacZ, the entire surface of the treated skin exhibited bgal staining which persisted for 7 days, with little or no expression at 10 days. Skin treated with HSVlac showed high bgal activity initially but was essentially negative after 7 days. Significant necrosis and toxicity were observed in HSV-treated skin. Quantitative analysis showed that the viral vector-mediated gene transfers were superior to gene gun delivery of plasmid DNA. Epidermal gene transfer by either gene gun delivery or viral vectors was transient and likely to be due to the episomal localization of adenoviral vectors as well as terminal differentiation and elimination. Four days after topical application of an adenoviral vector containing a human TGFα expression unit, the murine epidermis developed hyperkeratosis and acanthosis [72].

Implantation in nude mice of HeLa keratinocytes transduced by the adeno-associated virus harbouring the erythropoetin cDNA induces a high level and long-term (> 1 month) increase in hæmatocrit [73].

These data demonstrate that the retroviral and adenoviral vectors can be used to achieve local or systemic delivery of DNA-encoded protein.

5. Microseeding and puncture

Physical perforation of the skin, e.g., microseeding [74] and puncture [75], can also be used to deliver DNA into skin.

In microseeding, DNA is delivered directly to target cells by multiple perforations with oscillating solid microneedles. Expression of pCMVβgal or hEpidermal growth factor in microseeded skin peaked 2 days after transfection and was higher than after gene transfer by intradermal injection or gene gun. βgal-expressing cells were detected in the epidermis and dermis. Pigs microseeded with hæmagglutin expression plasmid were protected from infection by influenza virus [74].

High frequency puncturing of the skin with fine short needles used for tattooing human skin allowed the transfer of reporter genes as well as the expression of a transgene leading to the induction of cytolytic T lymphocytes. The bgal activity corresponded to the localization of the charcoal marker deposits in the epidermis and subepidermal tissue. Expression lasted for at least 7 days [75].

These data suggest that microneedles could be useful for DNA vaccination or for somatic gene therapy of large body areas.

6. Gene gun

The gene gun has been shown to induce gene expression in different tissues including skin.

Yang *et al.* [30] demonstrated a transient expression of RSV-CAT or CMV-βgal in mouse skin after bombardment with DNA-coated gold microparticles. About 20% of skin epidermal cells expressed bgal activity. βgal expression could be detected as early as 6 h after the administration of DNA-coated particles and persisted for up to 72 h [76]. When the skin was bombarded with 1-5 mm tungsten or gold particles coated with a bactin-promoter-luciferase reporter plasmid, 10-20% of the cells in the epidermis expressed mRNA. Expression of luciferase in the mice ear was detectable at high levels (4000-fold over background) and persisted for up to 10 days. Microprojectiles that penetrated the skin retained the DNA in the tissue and did not induce extensive cell damage or inflammation [77].

A high efficient long-term expression of cotton tail rabbit papilloma virus (CRPV) DNA in rabbit skin was detected following particle-mediated DNA transfer. The delivery force and hence penetration of gold particles coated with βgal plasmid was optimized. Rabbit inoculated with gold particles (1-3 mm) coated with CRPV-DNA formed papillomas at all of the 45 sites for at least 8 weeks [78].

The major application of particle bombardment for gene transfer is DNA immunization. Both humoral and cellular immune responses are elicited via gene gun-mediated nucleic acid immunization. Gene gun vaccination offers the advantages of minimal amounts of DNA (0.5-1 mg) and provides a simple means of delivering DNA intracellularly to the epidermis [31].

Fynan *et al.* [79] demonstrated highly efficient immunization against the influenza virus with 2 to 3 times less DNA than injection in saline. This could be due to the combination of efficient transfection with efficient antigen presentation and recognition. Tang *et al.* [80] detected antibody responses to the human growth hormone (hGH) after genetic inoculation with microprojectiles coated with a plasmid hGH gene. These initial studies have been extended for immunization against various diseases e.g., influenza, hepatitis B or HIV. Clinical trials on genetic vaccination using gene gun are continuing.

7. Electroporation

Electroporation has been widely used to introduce DNA into various types of cells *in vitro* and is a promising method to enhance gene transfer in various tissues. The recent literature suggests that the combination of local administration by injection and pulsed electrical field is a very efficient, clinically compatible method to achieve DNA transfection. It has emerged as a leading technology for developing non-viral gene therapy and nucleic vaccines [12, 81].

The earliest published work that utilized *in vivo* electroporation for gene delivery was conducted by Titomirov *et al.* [82]. The plasmid DNA coding for the Neo-R gene was introduced subcutaneously into newborn mice followed by high-voltage pulses applied to the skin. After electroporation, the skin was harvested and skin cells were placed into selective culture medium. Plasmid DNA was shown to persist in the cells for at least 30 generations without selection. Rols *et al.* [83] have injected a plasmid coding for βgal in murine tumors and then applied square wave pulses. βgal expression in tumor cells harvested 3 days after electric field treatment was detected and the maximum percentage of βgal positive cells was 4%.

It has recently been shown that intradermal DNA injection combined with electroporation enhanced transgene expression 20- to 40- fold over free injection of the luciferase reporter gene. By co-injecting the nuclease inhibitor arintricarboxylic acid with DNA before applying electric pulses, transfection expression was increased 115-fold over free DNA. In contrast to DNA injection, transfected cells were concentrated in the dermal and subdermal layers [55].

lacZ plasmid was applied topically to the skin prior to electric field application. Pressure (from the electrodes) was maintained for either 1- or 10-minute intervals after pulsing. The expression was only investigated for up to 3 days and appeared localized as far as the dermis (370 μm deep) [84].

Dujardin and Préat [85] have shown that a fluorolabelled plasmid penetrated the epidermis immediately after skin electroporation and entered the cytoplasm within hours. Gene expression was induced for up to 7 days in localized areas of the epidermis.

In conclusion, direct DNA injection allows transfection of skin tissues. To overcome the low efficiency of transfection, a gene gun can be used alone or in conjunction with electroporation. Topical delivery to the skin of naked DNA, DNA/lipid complexes or viruses has been developed in order to render DNA delivery non-invasive and more patient-friendly.

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The skin represents an attractive site for gene-based drug delivery. The skin is the most accessible site for systemic delivery and for targeted topical delivery. However, ODN and DNA penetration is limited by the barrier properties of the skin. Different strategies, discussed above, have been developed to overcome this limiting barrier and to deliver ODN or DNA into or across skin.

ODN can be delivered at potentially therapeutic concentrations (micromolar range) into the skin. Methods such as liposomes, iontophoresis or electroporation could be interesting to deliver antisense ODN acting on specific viral, malignant or inflammatory diseases of the skin.

Enhancement of transdermal transport of ODN by chemical and physical methods has been reported. However, the bioavailability of transdermally administered ODN has not yet been investigated and might be too low for the development of a commercial product.

Cutaneous gene delivery could be potentially attractive for the treatment of hereditary or acquired skin diseases, the treatment of systemic disease or genetic immunization.

Ex vivo and *in vivo* approaches for the delivery of viral or plasmid DNA have been developed. The *ex vivo* approach involves extensive cell culture and grafting. On this occasion, time-consuming, invasive methods are not likely to be used routinely in patients. *In vivo* transfer of genetic material to the skin has been achieved experimentally using dermal injection, topical application of naked DNA, DNA/lipids complexes or viral vectors as well as particle bombardment, puncturing or electroporation of plasmid DNA. Protein expression has been

detected in the skin and/or the serum. The localization and duration of expression is affected by the delivery methods. Efficiency, safety, the therapeutic aim and patient compliance will be the critical factors in determining the optimal method of DNA transfer.

The remaining challenges to address for optimal *in vivo* gene delivery to the skin are:

- duration of expression: due to the epidermis turnover, expression remains transient (i.e. less than 1 to 4 weeks except when retroviruses were used *ex vivo*). If short-term expression is sufficient for immunization or treatment of acquired skin disorders (e.g., wounds, viral disease, etc.), long-term expression will be required for the treatment of inherited skin and systemic diseases requesting protein supplementation;
- delivery methods: many methods have been shown to enhance the topical delivery of DNA and its expression in the skin. Although progress have been achieved, a safe, efficient, patient-friendly, non-invasive method allowing long-term expression at high levels in the target cells and large body area of a patient has not yet been developed;
- targeted delivery: the problem of tissue targeting is by-passed using topical delivery. Nevertheless, selective targeting to specific types of cells could be desirable for some applications. Keratin promoters to target keratinocytes or liposomes to target hair follicles could be a promising approach.

In conclusion, even though its barrier function impedes gene penetration and its immunological function can induce an immune response against encoded protein, the skin remains an attractive tissue for ODN and DNA administration for the treatment of topical and systemic diseases as well as for immunization.

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