The Gene Gun: Current Applications in Cutaneous Gene Therapy

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Introduction

Advances in genetics and molecular biology research have generated an increased interest in the field of gene therapy among clinical dermatologists. As researchers continue to close the gap between basic research and clinical science, several clinical applications of gene therapy have recently been attempted, and many others appear to be on the horizon. Originally, gene therapy was introduced as a mechanism to replace absent or defective genes in heritable disorders. In fact, many heritable cutaneous disorders have well-characterized genetic defects, making them favorable targets for gene therapy. (See Table 1 for list of genodermatoses with known gene defects). This field of research is currently being studied in detail, with many studies showing promising results both in animal models *in vivo* and in cell culture *in vitro*. However, the applications of gene therapy are not limited to the correction of genetic diseases. In fact, gene therapy has evolved to include genetic vaccination, suicide genes for cancer therapy, immunomodulation, and genetic pharmacology, among other applications (Figure 1).

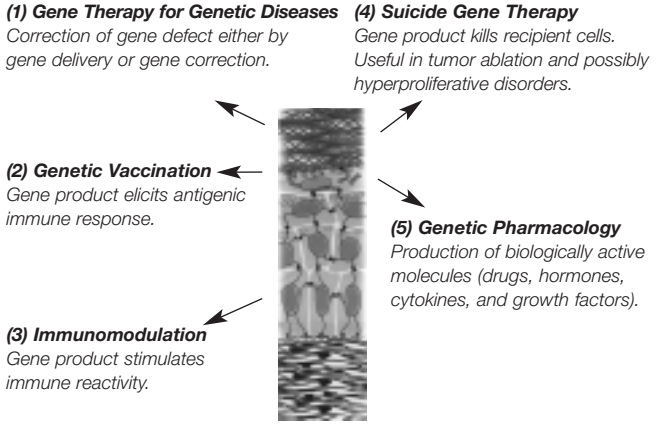

Fig. 1. Clinical applications of gene therapy. As indicated, (1) skin can be used as a target for correction of genetic defects in hereditary skin diseases. (2) Accessibility of skin makes it an ideal tissue to administer genetic vaccines. (3) Similarly, DNA encoding immunostimulatory molecules is introduced, causing an augmented immune response. (4) Genetically engineered cells, particularly tumor cells or hyperproliferative cells, can be targeted by a gene which converts a prodrug to a toxic compound causing the "suicide" effect. (5) Expression of pharmacological proteins in the skin can result in introduction of these molecules either systemically into the circulation or locally into the immediate environment.

Table 1. Genodermatoses with Known Gene Defects: Candidate Diseases for Gene Therapy

Disease	*Affected Gene*	*References*
Epidermal fragility disorders		
Dystrophic EB	COL7A1	74
Junctional EB	LAMA3, LAMB3, LAMC2	57
GABEB	COL17A1	56
EB-PA	ITGA6, ITGB4	56
EB-MD	PLEC1	75
EB-simplex	KRT5, KRT14	12
EDA/skin fragility	PKP1	49
Keratinization disorders		
Epidermolytic hyperkeratosis	KRT1, KRT10	12
Epidermolytic PPK	KRT9	12
Non-epidermolytic PPK	KRT16	12
Vohwinkel's syndrome	LOR	31
Ichthyosis bullosa Siemens	KRT2e	64
Pachonychia congenita type 1/2	KRT6a, 16, 17	64
X-linked ichthyosis	STS	7
Lamellar ichthyosis	TGM1	31
Palmoplantar keratoderma with deafness	GJB2	61
Erythrokeratodermia variabilis	GJB3	60
Darier's disease	ATP2A2	66
Striate palmoplantar keratoderma	DSP	2
Striate keratoderma	DSG1	62
Hair disorders		
Congenital atrichia	HR	1
Monilethrix	hHB1, hHB6	38, 83
Pigmentation disorders		
Waardenburg syndrome	PAX3	53
Albinism (different forms)	TYR, TYRP-1, OCA2, OA1	6
Tietz syndrome	MITF	53
Hermansky-Pudlak syndrome	HPS	6
Porphyrias		
Erythropoietic protoporphyria	FECH	51
Congenital erythropoietic porphyria	UROS	51
Familial porphyria cutanea tarda	URO-D	51
Variegate porphyria	PPO	51
Multisystem disorders		
Trichothiodystrophy	XPB, XPD	76
Nude	WHN	22
Fabry's disease	GLA	55
Ataxia telangiectasia	ATM	13
Hereditary hemorrhagic telangiectasia (HHT)	ENG, ALK-1	47
Cancer disorders		
Xeroderma pigmentosum	XPA, XPB, XPC, XPD, XPG, CSB	76
Basal cell nevus syndrome	PTC	4, 29
Peutz-Jeghers	STK11/LKB1	17, 65
Cowden syndrome	PTEN	19
Bannayan-Zonan syndrome	PTEN	48

Abbreviations: EB, epidermolysis bullosa; GABEB, generalized atrophic benign EB; PA, pyloric atresia; MD, muscular dystrophy; EDA, ectodermal dysplasia; PPK, palmoplantar keratoderma.

The skin's easy accessibility and visualization makes it an attractive target for gene therapy.[25] The two major delivery strategies currently being tested in cutaneous gene therapy are *in vivo* and *ex vivo* delivery (Figure 2).[39] *In vivo* delivery involves the introduction of genetic material directly into the skin of the patient.[79] In contrast, *ex vivo* delivery involves removal of a skin sample from the patient, propagation of skin cells in tissue culture, introduction of genetic material into the cultured cells, and return of an epithelialized skin equivalent back to the patient (Figure 2).

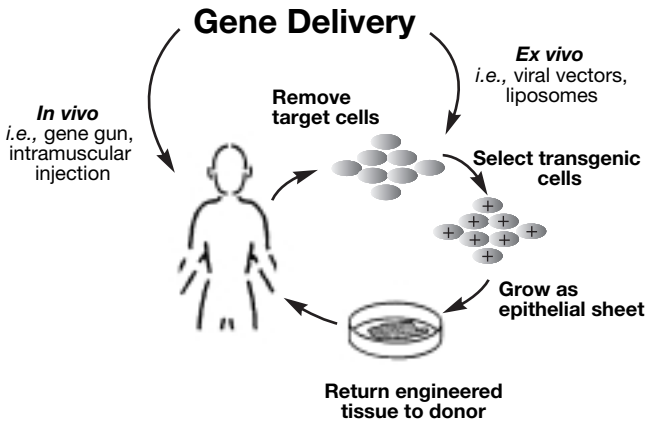

Fig. 2. Principles of the two primary strategies for cutaneous gene delivery. In the case of the in vivo *strategy, the genes are delivered directly into skin. In contrast, in* ex vivo *strategy, target cells are removed from the skin, transgenes are introduced into cultured cells, and the transduced cells are selected and grown into epithelial sheets that can be grafted back to the original donor.*

Currently, multiple modalities for gene delivery are being developed for both *in vivo* and *ex vivo* approaches. The most widely tested viral vectors are those derived from recombinant adenovirus or retrovirus. Newer viral delivery systems, including the adeno-associated virus, herpes simplex virus, and lentivirus offer possible alternative methods. Distinct advantages and disadvantages govern the applicability of each viral system in gene therapy. These restrictions are based upon the size of the insert, characteristics of the target cells (dividing vs. non-dividing), potential for long-term expression, immunogenicity, and the ability for genomic integration (Table 2).[32] In addition to viral systems, naked DNA can be delivered to the epidermis by gene gun, liposomes, and receptor-mediated endocytosis of DNA/ligand conjugates.

The Gene Gun Approach

Originally, particle-mediated gene transfer was developed by Sanford and colleagues in 1987 to deliver genes to plant cells using gunpowder acceleration.[34, 35] Eventually, helium replaced gunpowder as the particle propellant for most particle-mediated devices.[82] Currently, hand-held instruments have been developed to utilize the ballistic particle-mediated delivery system (the "gene gun" approach) to deliver genes into skin *in vivo*. Commercially, these devices are available, such as the Helios® gene gun (Bio-Rad, Hercules, California) (Figure 3A).

Table 2. Frequently Used Viral Vectors in Gene Delivery

Type of Vector	*Integration to Genome*	*Advantages*	*Limitations*
Retrovirus			
Molony murine leukemia virus	Yes	Wide host range; high efficiency transduction of dividing cells; efficient expression of foreign gene product; stable integration; infects only once and does not replicate *in vivo*	Does not infect nondividing terminally differentiated cells; may be oncogenic; optimal insert size ~5 kb
Lentivirus (HIV)	Yes	Wide host range; stable transduction of dividing as well as nondividing terminally differentiated cells with long term expression; nonpathogenic; lack of expression of viral proteins	Difficult to produce high titer viruses
Adenovirus	No	Transduction of dividing and nondividing cells with high efficiency; wide host range; high viral titer and high expression; "gutless" vectors have insert sizes as large as 30 kb	Expression of viral proteins results in immune response and inflammation; short term expression; insert size only ~7 kb
Adeno-associated	Yes	Transduction of dividing and nondividing cells; all viral coding sequences can be deleted except those required for transduction; nonimmunogenic and nonpathogenic; long term expression of transgene; specific integration site in some cases	Limited transduction efficiency which depends on helper viral functions, although in newer systems helper virus is not needed; low efficiency of integration to genome; small insert size ~4 kb
Herpes simplex	No	Transduction of neurons and glial cells; wide host range; large insert size up to 30 kb; efficient infection; newly engineered vectors are avirulent in surrounding terminally differentiated cells	Short term expression; spreading of the infection to surrounding cell populations; immunogenic

The "gene gun" accelerates DNA-coated gold particles into target cells or tissues (Figure 3B). The gold particles are typically approximately 1 mm in diameter. Due to their small size the particles can penetrate through the cell membrane, carrying the bound DNA (typically 0.5–5.0 µg/particle) into the cell. At this point, the DNA disassociates from the gold particle and can be expressed. Since this method is cell receptor-independent, it can successfully deliver genes into different mammalian cell types; however, it is physically limited by the degree of penetration into the tissue. Previous experiments with the gene gun have shown high levels of local transgene expression,[82, 84] epidermal and dermal penetration in mouse skin,[44] and transient expression lasting at least 48 hr.[43] Some of the advantages of the gene gun over other *in vivo* delivery systems include: (1) freedom from use of complex biological systems (*i.e.,* viruses) and toxic chemicals (*i.e.,* liposomes/cationic agents/ligands), (2) cell receptor-independent delivery, (3) delivery of different sizes of DNA, including very large ones, (4) lack of extraneous DNA or protein introduced, and (5) possibility for repetitive treatments.

A. Helios Gene Gun

B. Gene Transfer with the Helios Gene Gun—The Process

Fig. 3. The gene gun technology for introduction of DNA into skin. A, Commercially available Helios gene gun manufactured by Bio-Rad. B, The gene gun introduces DNA into skin bound into small gold particles. For this purpose, DNA is precipitated onto gold microparticles, which are then loaded into a plastic tubing. Approximately one-half-inch pieces of the tubing are cut and loaded into the gene gun which uses pressurized helium to accelerate the particles into the skin. The velocity and the size of the gold particles determine the depth of penetration into skin layers. (Figure is courtesy of Bio-Rad Laboratories).

Current Applications of the Gene Gun

GENE REPLACEMENT

Since particle-mediated DNA delivery typically results in the short-term and inefficient expression of gene products *in vivo*, this approach has not been extensively investigated as yet as a means of gene replacement. Nevertheless, several studies have shown the successful introduction of reporter genes to a variety of skin and other organs.[10, 44, 82, 84] Furthermore, the introduction of dystrophin cDNA to skeletal muscle by gene gun in a mouse Duchenne muscular dystrophy model has resulted in detectable levels of dystrophin protein by immunocytochemistry for up to sixty days after bombardment.[85, 86] However, *in vivo* particle-mediated delivery results in a transient transfection, with no genomic integration. Therefore, the retrovirus and other related viruses capable of integrating genes into the host genome have shown more potential for long-term gene replacement in the skin. Long-term expression (>40 weeks) of a reporter gene, β-galactosidase, has been demonstrated in retrovirally transduced human keratinocytes that were grown in cell culture and grafted onto athymic mice.[37]

Genetic Vaccination

Genetic vaccination is an intriguing application of gene gun technology. Within the broad classification of genetic vaccination, the two significant applications are the pathogen vaccines and the cancer vaccines. Immunization is achieved by the introduction of DNA[21] or mRNA[58] into the skin, leading to the expression of the foreign antigen and subsequently the elicitation of an immune reaction. DNA vaccines forego many of the potential safety concerns related to the biological hazards involved in live-attenuated, protein-purified, or killed vaccines.[40] Possible advantages of DNA vaccination over recombinant proteins for immunization include increased purity of antigens, higher effectiveness of immune elicitation, and lower cost.[40]

Intramuscular (IM) injection of DNA has been studied extensively for DNA vaccination; however, gene gun vaccination has also been shown to be effective in producing immunity. Cutaneous DNA vaccination takes advantage of the antigen presenting capabilities of Langerhans cells to elicit a T-cell mediated immune reaction.[36, 73] Several studies have suggested that different delivery modalities may affect the vaccine's effectiveness in eliciting either cell-mediated (largely Th1) or humoral immune responses (largely Th2).[54, 69] The injection of plasmid DNA in a saline solution into the skin or muscle results in a largely T helper 1-like (Th1) immune response in mice, while particle-bombardment to the skin results preferentially in a T helper 2-like (Th2) response. This bias may be related to the mode of delivery (DNA uptake vs. direct intracellular penetration) and/or quantity of DNA introduced. Dose-dependent non-specific immune responses can be triggered by plasmid DNA prepared from bacteria due to unmethylated DNA sequences.[63] Lower Th1 responses are observed in particle-mediated immunization since it requires one-hundredth as much plasmid DNA than direct injection.[73]

The first evidence of particle-mediated DNA immunization occurred when Johnston and colleagues noted that expression of human growth hormone via particle-mediated gene transfer resulted in the induction of antigen-specific antibody responses.[71] Since that time, several groups have demonstrated the successful immunization of laboratory animals against many viruses, bacteria, and parasites (reviewed by Donnelly *et al*,[18]). More importantly, the efficacy of DNA immunization has been demonstrated in primates,[8, 15] and human clinical trials with DNA vaccinations against hepatitis B, herpes simplex, HIV, influenza, and malaria have shown promising results.[81] Recently, particle-mediated DNA vaccination in mice has been successful in the immunization against other serious pathogens including hantavirus[27], Ebola virus[77], and rabies virus.[42]

The burgeoning field of cancer vaccination has opened many new avenues of investigation for clinical investigators and molecular biologists. In general, the goal of cancer vaccination is to prime the host immune system against tumor cells. Many distinct approaches are in development. Some of these approaches include the introduction of foreign class I or class II MHC antigen genes to tumor cells,[3, 52] the use of vectors encoding tumor-associated antigens such as gp100,

tyrosinase, carcinoembryonic antigen (CEA),[11, 30, 87] and the development of immunostimulatory techniques.[16] Human clinical trials are currently under way with cancer vaccines against prostate cancer, adenocarcinoma of the breast and colon, and several lymphomas.[81]

Suicide Gene Therapy

Many of the gene therapy applications currently in clinical trials involve the treatment or amelioration of various forms of cancers or infectious diseases. In fact, gene therapy has become a standard experimental approach for treating cancer patients that have failed conventional therapies. One of the most promising anti-tumor gene therapy techniques involves the concept of "suicide gene therapy."[23, 24] In this approach, a "suicide gene," such as herpes simplex virus thymidine kinase gene (HSV-TK), is introduced into tumor cells under the control of tumor-specific promoters. The use of tumor-specific promoters restricts expression of the transgene to tumor cells only. Tumor cells expressing the HSV-TK gene convert the prodrug, ganciclovir (GCV), by phosphorylation into a highly toxic form. This toxic metabolite disrupts DNA replication and results in the death of the cell. GCV by itself is virtually nontoxic to cells not expressing HSV-TK at therapeutic concentrations. GCV, therefore, is nontoxic to normal tissues. Although this technique has not been investigated extensively with particle-mediated delivery, many experiments using viral delivery and direct injection have been successful. Suicide gene therapy has been demonstrated to be effective in the treatment of neuroblastoma[14] and melanoma[78] in animal models, and many clinical trials using suicide genes in melanoma and other cancers are currently under way.[24, 33]

Immunomodulation

Immunomodulation is closely related to the fields of cancer vaccination and suicide gene therapy. In all three approaches, the desired effect is the destruction of unwanted tumor cells, mediated by either the stimulated immune system and/or gene product itself. One such approach involves the stimulation of the immune response to weakly immunogenic tumors, achieved by transfection with various cytokines (*e.g.,* granulocyte/macrophage colony-stimulating factor (GM-CSF).[46] Theoretically, the immune response will be augmented against the transfected tumor cells at the vaccination site, as well as at distant non-transfected tumors.[30]

Particle-mediated introduction of interferon α cDNA has been shown to produce local antitumor immunity in tumor establishment models.[72] IL-2, IL-6, interferon α, and interferon γ cDNA have also been shown to be effective in the reduction of tumor growth in mice.[70] More interestingly, gene gun delivery of IL-12 cDNA in murine models showed that local expression of IL-12 in epidermal cells near tumor cells resulted in regression of underlying tumors and inhibition of systemic metastasis, resulting in prolonged survival of test mice.[59] This study suggests that immune stimulation by gene gun techniques can result in local as well as systemic anti-cancer effects.

Genetic Pharmacology

A particularly promising application of gene therapy relates to genetic pharmacology.[25] With this approach, DNA encoding therapeutically beneficial genes is administered to tissues, and these gene products result in the pharmacological improvement of the patient. Current applications of genetic pharmacology being studied are the production of various clotting factors in hematologic disorders or use of erythropoetin-encoding DNA for enhanced red blood cell reproduction in patients with chronic anemias.[20, 36] Similarly, expression of various hormones, growth factors, etc., can modify pathological conditions. Methods of administration of such genes include, for example, intramuscular injections, introduction of the genes to the skin by gene gun delivery, or grafting of genetically modified keratinocytes into the skin.[39]

Just as the route of administration of a drug (*i.e.,* PO/IV/IM) may affect its therapeutic level, the mode of gene delivery may dramatically affect the therapeutic level of the transgene expression. For example, intramuscular injection of erythropoetin cDNA elicits a rise in hematocrit that persists for eight months.[82] However, the hematocrit can reach pathologic levels in the 75 to 90% range. In contrast, monthly gene gun applications of the erythropoetin cDNA to mouse skin resulted in a stable increase in hematocrit. With the gene gun, the level of erythrocyte production could be controlled by the adjustment of the dose and frequency of the transgene application.[82] Thus, the "gene gun" administration of plasmid DNA resulted in tighter regulation of secretion of the exogenous gene product.

The Gene Gun as a Research Tool: Promoter-Based Gene Expression

Promoter-based gene expression was initially developed in cancer research to restrict the expression of therapeutic genes to specific tumor cells. Since tumors often produce the same proteins as the original tissue of origin, the promoter sequences that control the expression of these proteins can be utilized to control the expression of the transgenes. For example, the α-fetoprotein promoter can be used to restrict expression to liver tumors,[28] the surfactant protein promoter to lung cancer cells,[68] and the tyrosinase promoter to melanoma cells.[26]

Promoter-based gene expression can be applied to cutaneous gene therapy to restrict the expression of introduced genes also to specific cell layers. Since many cell layers in the skin produce specific proteins, promoter sequences from the corresponding genes can be incorporated into the vectors to restrict the transgene expression to those layers. Layer-specific expression has been demonstrated in transgenic mice using the keratin 14 (K14)[80] and keratin 5 (K5) promoters.[9]

Using the gene gun, one can quickly identify the strength and specificity of different promoters in the skin. The use of particle-mediated gene delivery (*i.e.,* gene gun) to study promoter-based gene expression has only recently been developed. Cheng *et al.* compared various viral and cellular promoters in the epidermis and other tissue. Among ten promoters investigated, the CMV promoter was shown to be the strongest in the epidermis, followed by the mouse metallothionein gene promoter.[10]

Similarly, using the gene gun and promoter-based expression, Shiraishi *et al.* delivered DNA to the cornea, conjunctiva, and skin. Using keratin 12 promoter sequences, restriction of reporter gene expression to the cornea was achieved.[67]

As an illustration of the applicability of the gene gun approach, we have bombarded mouse skin with a construct consisting of a *LacZ* reporter gene, encoding β-galactosidase driven by an involucrin promoter (Figure 4). Mice were sacrificed at 24, 48, and 158 hr. The *LacZ* expression was noted to have decreased by 48 hr and was undetectable by day 7. Although the construct, which was bound to the gold particles, was delivered to both the dermis and epidermis, gene expression was limited to the epidermis (Figure 4B). Therefore, a transgene regulated by an involucrin promoter was expressed only in the superficial epidermal layers, which coincides with the normal physiologic expression of involucrin (see Figure 4B).[45]

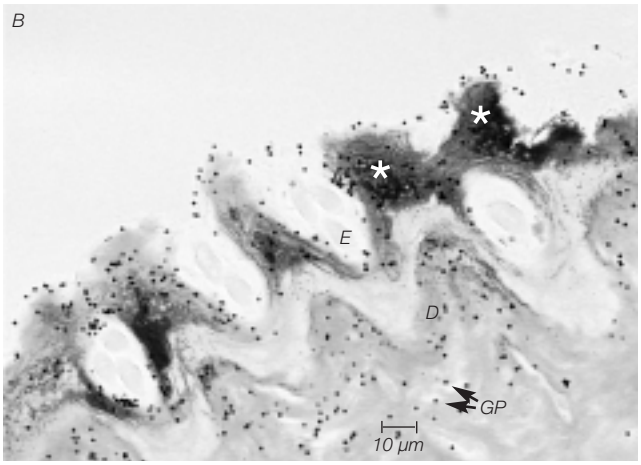

Fig. 4. Illustration of the use of the gene gun for introduction of a DNA construct into the skin of a mouse. A, Targeting of the ventral skin of a mouse with the gene gun. B, Expression of the DNA construct in the skin of the mouse. The construct, which consisted of LacZ *encoding the β-galactosidase reporter gene under control of the involucrin promoter, is expressed exclusively in the upper layers of the epidermis, as indicated by the β-galactosidase stain (darker areas indicated by asterisks). Note the presence of gold particles (GP) both in the epidermis (E) and the dermis (D), yet there is no β-galactosidase expression on the dermal side, attesting to the specificity of the promoter.*

Of particular interest is the prospect of targeting epidermal stem cells by promoter-based techniques. The epidermis is a rapidly proliferating and differentiating tissue. Therefore, a dilemma in cutaneous gene therapy relates to the difficulty in maintaining gene expression due to the rapid epidermal turnover. Since keratinocyte stem cells are the progenitors of all other keratinocytes in the epidermis, gene delivery to these key cells remains an important long-term goal to maintain sustained gene expression.[5] Ultimately, if a unique promoter can be found to be specific for stem cells, such a promoter can be utilized for identification and enrichment of stem cell populations. Evidence that keratin 19 may be a marker for stem cells[50] has prompted investigation into the use of the keratin 19 promoter for stem cell identification.

Conclusions

As the field of gene therapy progresses, no one delivery system will likely be superior in all situations. Each delivery system is distinctive. For example, certain delivery systems allow for long-term expression (*i.e.,* retroviruses), while others result in short-term expression only (*i.e.,* gene gun). Also, in genetic vaccination, differences in the patterns of immunostimulation elicited by intramuscular and cutaneous gene gun delivery may lead to more effective vaccination by one modality than another based upon the specific application. Finally, certain delivery systems are more amenable to systemic delivery (*i.e.,* viral), while others are effective in local delivery only.

The gene gun has proven to be useful in gene therapy research and has many potential clinical applications. Although the gene gun has limited applicability in "conventional gene therapy" (*i.e.,* gene replacement), the prospects of using the gene gun in genetic vaccines, genetic pharmacology, and cancer therapy are particularly exciting. The gene gun's ability to deliver genes to a variety of cell types without viruses or toxic chemicals offers distinctive advantages over other delivery systems. Therefore, the gene gun will likely remain a standard gene delivery technique in future gene therapy applications.

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