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Gene therapy in cutaneous wound healing

Tobias Hirsch, Malte Spielmann, Feng Yao, Elof Eriksson

Division of Plastic Surgery, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

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1. ABSTRACT

Medical treatment of complicated acute and chronic wounds represents a persistent and increasing medical and economic problem in our health-care system. In this review, we discuss the potentials and limitations of current and future gene therapy for the treatment of complicated, acute and chronic wounds. Chronic non-healing wounds result in significant morbidity, prolonged hospitalization, lost time from work and enormous health-care expenses. There are constant efforts to improve the therapeutic modalities to local treatment of wounds. One of them is gene-therapy where the delivery of peptides directly into the wound provides a relatively new and exciting possibility. The two groups of peptides of particular interest are growth-factors and anti-microbial peptides. Gene delivery of these peptides provides not only the possibility of more targeted local delivery but also larger concentrations. Many new techniques for gene delivery to wounds have been developed in recent years. The combination of mechanical and viral or chemical vectors appear to have the greatest yield. This review provides an update on gene delivery to cutaneous wounds.

2. INTRODUCTION

Medical treatment of complicated acute and chronic wounds presents a persistent and increasing medical and economic problem in our health-care system. Approximately five million patients in the United States suffer from chronic wounds (1). Chronic non-healing wounds result in prolonged hospitalization, significant morbidity, lost time from work, and enormous health-care expenses. According to the American Diabetes Association, approximately 82,000 non-traumatic limb amputations were performed in 2002 among people with diabetes (2). The Agency for Health Care Policy and Research reports that wound care for pressure ulcers is a $200 billion-a-year industry for hospitalization, durable medical goods, nursing home care, physicians, and transportation (3). Furthermore, infections play an increasingly large role in the high morbidity and mortality among patients with extensive post-traumatic defects, burns, and chronic wounds. Resistance of wound infections to conventional local antibiotic agents is increasing.

Table 1. Comparison of different gene transfer techniques in wound healing

Gene delivery	Capacity	Efficiency	Expression	Drawbacks
Viral				
Retrovirus	~ 7 kb	low	stable	insertional mutagenesis immunogenic
Lentivirus	~ 7 kb	high	Stable	insertional mutagenesis immunogenic
AAV	~ 5 kb	high	stable	immunogenic
Adenovirus	~36 kb	high	transient	immunogenic
Herpes Simplex	30-40 kb	high	transient	immunogenic
Chemical				
Ploykations		low	transient	low
Liposomes		low	transient	low
Physical				
DNA Injection		low	transient	low
Gene Gun		low	transient	low, foreign body reaction
Microseeding		low (plasmid). high (viral).	depends on vector used	depends on vector used
Electroporation		low	transient	low

Therefore, it is of paramount importance to develop new therapeutical strategies for wound treatment. The efficacy of promising pharmacologic agents such as growth factors and host-defense peptides (HDP). is strongly decreased by their short biological half-life within the wound (4). Thus, the limitations of local therapeutic regimens posed by resistance, short biological half-life, cost-intensive production, and the necessity for repeated administration (including wound manipulations and dressing changes) provide more than enough reasons to establish new and innovative therapeutic strategies (5).

One promising approach to overcoming these obstacles is gene therapy- defined as the insertion and expression of a foreign gene (transgene) in cells or tissue of a host organism (6). The goal is to express the transgene and synthesize therapeutic active proteins such as growth factors and host-defense peptides (7, 8). Proteins could be expressed directly at the desired location in target cells, and the patient would be responsible for the continuous delivery of therapeutic substances in the local wound (9). All in all, gene therapy could provide a promising alternative for the treatment of wounds (10).

Gene therapy has been most commonly applied to the treatment of genetic disorders, such as cystic fibrosis,(11) hemophilia,(12) and SCID (severe combined immunodeficiency) (13). The earliest cutaneous gene transfer was described by Morgan *et al*.,(14) who reported the first successful gene transfer to keratinocytes in 1987.

Although skin is an attractive target for gene transfer, it is difficult to deliver the transgene efficiently to the target cells and to elicit consistently high levels of gene expression in the host tissue (6, 15). Furthermore, the duration and kinetics of the transgene expressed must be limited or controlled to avoid persistent protein synthesis after wound healing is complete (6). Therefore, the development of the appropriate vector for gene delivery is of major interest.

3. VIRAL VECTORS

Over the past few years, intense efforts have been made to understand the molecular basis of the interaction of the host with viruses and viral vectors. Our findings allowed us to develop more efficient, specific, and safe vectors. Several viral and non-viral vectors to introduce the foreign gene into the host, each with specific advantages and side effects, are currently available, and more viruses are under development to serve as gene-therapy vectors (16-18). Five main classes of clinically applicable viral vectors, each with distinct properties are currently available: Adenovirus (Ad), adeno-associated virus (AAV), oncoretrovirus, lentivirus, and herpes simplex-1 virus (HSV-1).Viruses have evolved to gain efficient access to host cells and exploit their cellular machinery to replicate. Ideal virus-based vectors for most gene-therapy applications should harness the viral infection pathway but avoid expression of viral genes that lead to replication and toxicity.

3.1. Adenoviral vectors

Adenovirus were first described in 1953 (19). Since their first isolation from tonsils and adenoid tissue, 51 more human adenovirus serotypes have been identified and grouped into 6 species (A-F). that cause diseases of the airway epithelium, gastrointestinal tract, and eye (20-23). One of the most common is the serotype 5 adenovirus, widely used as a vector for gene therapy (24, 25). Most serotypes use the widely distributed CAR (coxsackie and adenovirus) receptor for primary attachment to the host cells (26, 27). After endocytotic uptake into the cell, the viral DNA migrates to the nucleus, where it remains episomal and begins to replicate (28). The genome consists of a linear double-stranded DNA 26-45 kb (Ad5 35kb) in length (24). The entire genomic sequence has been encrypted (GenBank Accession No. J01917).

The first-generation Ad5-based gene therapy vectors are E1-deleted with deletions of the E1a and E1b genes (early genes) responsible for the regulation of viral early and late gene expression,(29-33). often with all or part of the E3 region removed (23). Deletion of the E1 region is intended to render the virus replication-deficient and to eliminate potential oncogenicity. E3 genes are not essential for viral replication, and their deletion increases the capacity of the vector (25, 34, 35). Modification of these first-generation vectors led to a genome capacity of about 7.5 kb for transgene insertion (36). Transgene expression from first-generation vectors is limited and usually disappears after 2 to 3 weeks (37-39). The second-generation Ad vectors with additional deletions in the genome (E2A, E2B, E4) were developed to further reduce immunogenicity of the vector and to enhance the capacity for transgene insertion. Deletions have been made in the

early regions involved in DNA replication, including E2A, E2B and the E4 region (39-41). High-capacity, so-called gutless third-generation vectors, contain only the terminal *cis*-acting sequences of viral DNA (42). Consequently, they can encode up to 37 kb of transgene but require helper viruses for replication(35, 43) and are less efficient in expressing the transgene than are first-generation vectors (44).

Advantages of adenoviral vectors in cutaneous gene therapy are high-titer production,(45). the ability to infect both dividing and nondividing cells,(46, 47) and, because of episomal gene transcription, the transient expression of the introduced gene, which is of particular interest in gene therapy for wounds (48). Furthermore, no insertional mutagenesis has been detected (49).

The inflammatory response of the host is the main limitation on the potential of adenoviral vectors (50). Development of neutralizing antibodies and cytotoxic T-cells against the vector and expressed transgene product after adenoviral gene transfer *in vivo* has been described (51). Sylvester *et al.* detected an increase in immune responses in incisional cutaneous wounds after adenoviral gene transfer but no negative effect in wound healing (52). Repeated administration of adenoviral vectors elevated antibody titers but did not affect the transgene expression. We reported inflammatory reactions and altered wound healing after adenoviral gene transfer of high-dose vascular endothelial growth factor (VEGF) into porcine full-thickness wounds (53). Crystal *et al.* have presented a detailed analysis of the safety profile and toxicity of adenoviruses (54). Further, adenoviral constructs have been widely used in clinical trials and only rarely provoked serious clinical symptoms. In 10 years of research involving over 3,500 patients, only a few undesirable effects were found, and only in patients treated with unusually high systemic dosages (55, 56). Thus, on the basis of the literature and our findings, we consider that the cytotoxic potential of adenoviral vectors in topical application is low.

Adenovirus was the vector of choice in 287 clinical trials to date or 25.1% of all clinical trials in gene therapy (57). Adenoviruses are one of the most frequent vectors for *in vitro* and *in vivo* gene delivery and investigations of gene therapy for cutaneous wounds. Adenovirus-mediated gene transfer of the human host defense peptide LL37 to second-degree burns infected with *Pseudomonas aeruginosa* inhibited bacterial growth up to 10,000-fold in a rat burn model (58, 59). We showed that adenovirus mediated transgene expression in porcine full-thickness wounds and partial thickness burn wounds *in vivo* is feasible and that significant quantities of therapeutic proteins can be expressed in the wounds (53). Adenoviral gene transfer of endothelial nitric oxide applied in impaired wounds in type 1 diabetic mice produced significant improvements in wound healing and regeneration (60). Other studies reported that adenoviral gene transfer of platelet-derived growth factor-BB (PDGF-BB) elicited detectable expression for up to 2 weeks and enhanced wound reepithelialization in an *in vivo* ischemic wound model (61). Finally, adenoviral vectors are utilized in clinical trials investigating the therapeutic effect of adenovirus-derived gene transfer of vascular endothelial growth factor (Ad-VEGF). in patients with chronic lower limb ischemia and PDGF-BB in the treatment of chronic venous stasis ulcers and chronic diabetic ulcers (7, 8, 62).

3.2. Adeno- associated vectors

Although eight identified serotypes of adeno-associated virus (AAV). have been described, the most promising is the apathogenic AAV type 2 (AAV-2), a member of the parvovirus family discovered as a co-infecting agent during an outbreak of adenovirus infection (63). AAV-2 itself is a single-stranded, replication-deficient DNA virus with a genome of about 4.7 kb that integrates specifically into chromosome 19 within 19q13,4 (64). The generation of recombinant adeno-associated vectors (rAAV-2) as vector systems for safe long-term transgene expression is especially interesting. These “gutless” vectors lose the ability to integrate specifically and remain episomal, since they are missing the Rep protein, which regulates replication and integration into the host genome (65). AAV-vectors transduce both dividing and non-dividing cells. The advantage of this vector is its low immunogenicity due to the stability of the viral capsid, which shows almost no signs of a cytotoxic T-cell response (66). These advantages enable the AAV-vectors to achieve gene expression *in vivo* for up to several months (67). The reproduction and packaging of AAV-2 vectors require helper-virus functions normally provided by adenovirus or herpesvirus. New investigations show that the helper virus can be substituted by an adenoviral helper plasmid, which avoids helper virus contamination (68).

Transduction rates by rAAV in human keratinocytes *in vitro* were up to 70% (69). In 2000, *in vivo* gene transfer into porcine skin was successfully achieved after intradermal injection of rAAV particles, which led to transgene expression in epidermal keratinocytes for more than 6 weeks (70). Deodato *et al*. showed significant decrease in healing time of excisional wounds in a rat model with rAAV-vector encoding human vascular endothelial growth factor (hVEGF) (71). In 2003, the same group demonstrated that AAV vector–mediated hVEGF gene transfer stimulated angiogenesis and wound healing in diabetic mice (72). An important barrier to efficient transduction of rAAV-2 vectors is the requirement of conversion from single- to double-stranded DNA for successful expression in the target cells. Recently, this obstacle was surmounted through the development of double-stranded vectors that exploit a hairpin intermediate of the AAV replication cycle, thereby mediating 10- to 100-fold higher levels of transgenic expression *in vitro* and *in vivo* (73). The major drawback of AAV-based gene therapy is the limited size of the vector (4.5 kb). However, new developments have overcome this limitation through viral DNA heterodimerization, in which a large gene and its endogenous promoter is split into two individual rAAV vectors(74). These innovations may make the AAV vector an attractive tool for *in vivo* gene transfer for the treatment of cutaneous wounds and injuries.

3.3. Retro- and Lentiviral vectors

Oncoretroviral vectors were the first class of viral vectors to be developed and, now in 2006, are still one of the most commonly used vectors in ongoing clinical trials (24%; n= 276) (57). Retrovirus is an enveloped virus containing a single-stranded RNA molecule of approximately 8 kb. After infection, the viral genome is reverse-transcribed into double-stranded DNA, which integrates into the host genome (75).

While retroviral vectors offer a means to correct genetic diseases permanently by stably expressing transgenes, all current integrating gene transfer vectors carry a finite risk of insertional mutagenesis(76). As reported by Fischer and coworkers, the development of leukemia in 3 of 11 children with X-linked SCID following *ex vivo* gene transfer and successive cell transplantation shows the limiting factor for the use of this promising vector for gene therapy (76). However, new methods for evaluating the sites of vector integration are being developed, and long-term follow-up (1–7 years) of retroviral gene transfer to hematopoietic stem cells in dogs and non-human primates showed no evidence for clonal expansion or insertional mutagenesis (77). The major drawback to the use of retroviral vectors is the difficulty in attaining high titers and their short half-life *in vivo* (2–9 hours) (78). The use of specially designed vector-producing cell lines (VCL) might overcome this obstacle by expressing all vector components (transfer vector, packaging, and envelope) (79). However, the generation of replication-competent retroviruses, as evidenced by several outbreaks of wild-type virus from recombinant virus, and potential insertional mutagenesis present a persistent risk (80, 81).

Lentiviruses are unique among members of the retrovirus family for their ability to infect quiescent non-dividing cells (82). Lentiviral vectors have been derived from human immunodeficiency virus (HIV) and non-human lentiviruses. Vectors that are based on HIV retain <5% of the parental genome, and <25% of the genome is incorporated into packaging constructs, which minimizes the potential of the generation of a replication-competent HIV (83). Biosafety has been further increased by the development of self-inactivating vectors that contain deletions of the regulatory elements in the downstream long terminal-repeat sequence, eliminating the transcription of the packaging signal that is required for vector mobilization (84). Recent studies showed that lentiviral vectors were 10 times more efficient than retroviral vectors at transducing growth-arrested cells (85). Furthermore, lentiviral vectors were considerably more resistant to *in vivo* silencing than were retroviral vectors (85). Unlike adenoviral vectors, which also do not require active cell division for successful infection, lentiviral vectors do not seem to stimulate a significant immune reaction (86).

Several studies have investigated the potential of retroviral and lentiviral vectors for cutaneous gene transfer: In 1999, Ghazizadeh *et al.* reported the first successful *in vivo* gene transfer resulting in sustained transgene expression in the epidermis (87). The aim of this study was to develop an effective strategy for treating cutaneous disorders through retrovirus-mediated *in vivo* gene transfer. They established a method for *in vivo* transduction but detected only low rates of transgene expression.

Lentiviral gene therapy for cutaneous wounds has been focused on the transfer of genes encoding wound-healing growth factors (88), (89). Escanez *et al.* investigated an in vivo model focused on the regeneration of human skin on the back of nude mice. Human keratinocytes in the epidermal compartment were genetically modified with a retroviral vector encoding keratinocyte growth factor (KGF). After a small circular full-thickness wound was created on the mature human skin graft, the reepithelialization was significantly accelerated by the gene therapy during the first 3 days post-wounding (88). In 2005, lentivirus-delivered PDGF was expressed successfully in the regenerated dermis in full-thickness wounds in a diabetic db/db mouse model. Lentiviral PDGF transfection into the diabetic wound enhanced PDGF concentrations, improving vascularization and collagen organization. However, reepithelialization and granulation tissue of the affected area were not significantly different from that of controls (89).

Retroviral and lentiviral gene therapy of wounds thus shows promise. However, the development of replication-competent mutants during the gene therapy process and the possibility of insertional mutagenesis (76, 90) indicate the hurdles that need to be overcome before its use as a standard therapeutic tool.

3.4. Herpes viral vectors

Herpes simplex virus (HSV) type 1 is a human neurotropic virus that has linear double-stranded DNA with a genome of about 152 kb. This virus replicates in epithelial cells and establishes life-long infection in neuronal cells within the ganglia.

As a vector for gene transfer, HSV is able to infect a number of mammalian cells and tissue types (91-93) but is used mainly for gene transfer to the nervous system (94). Regarding the safety of the vector, our laboratory created a novel anti-HSV-1 recombinant virus capable of inhibiting its own replication and that of parental wild-type virus (95). These encouraging results make HSV an attractive candidate for gene delivery to skin and wounds because of the large size of its genome and its capacity to accommodate up to 50 kb of foreign transgenes (93). Glorioso *et al*. showed that herpesvirus-delivered VEGF and nerve growth factor to dorsal root ganglia alters the development of diabetic neuropathy in mice (96, 97). Despite the usefulness of HSV for delivering gene products to a spectrum of tissues, a problem common to these vector systems is the delivery of high levels of the protein of choice to the proper tissue in a sensitively regulated manner (98). We recently generated a novel replication-defective HSV-1 vector that encodes the tet-On gene switch T-REx (Invitrogen, CA). We showed that infection of cells with this T-REx-containing replication-defective HSV vector can induce up to a 1000-fold higher regulated gene expression by tetracycline, which compares favorably

among the single tetracycline-inducible viral vector systems developed to date (91) and is at least 20-fold more effective than currently existing regulatable replication-defective HSV vectors and HSV-based amplicon vectors.

4. CHEMICAL GENE TRANSFER

Numerous chemical methods for gene delivery have previously been investigated, but only liposomal gene transfer remains a promising technique at present (98).

Cationic liposomes are positively charged lipids that form vesicular structures (99). They are able to form complexes with negatively charged DNA molecules, after which they interact with the cell membrane and allow DNA to be introduced into cells via endocytosis (98). The efficacy of liposome-mediated gene transfer *in vitro* and *in vivo* varies greatly, depending on the liposome as well as the cell type (100).

Plasmid/liposome-complex (lipoplex) derived gene transfer of fibroblast growth factor (FGF) to excisional wounds in diabetic mice accelerated wound closure in comparison to that of topically applied recombinant FGF (101). Jeschke *et al*. reported that multiple injections of plasmid/liposome complexes encoding for IGF-1 are feasible and accelerate wound healing in rat burns (102). They further showed that delivery of KGF via lipofection to acute wounds in rats improved epidermal regeneration (103). We found that lipoplex-mediated *ex vivo* gene transfer resulted in high expression of growth factor over 12 days in porcine keratinocytes. Transplanting these cells into porcine full-thickness wounds in diabetic animals led to the expression of 450 ng/ml IGF-1 in the wound (unpublished data). Compared to non-diabetic animals, diabetic pigs have significantly lower concentrations of IGF in the wound fluid from full-thickness wounds (0.5–3 ng IGF/ml wound fluid versus 10–40 ng IGF/ml of wound fluid). In this study we showed that lipoplex-mediated *ex vivo* gene transfer to wounds overcomes the gap between healthy and diabetic animals. Another promising approach to the use of liposomal complexes is their combination with viral vectors (104, 105). It has been shown that liposomes enfold the viral vector, thus increasing the efficiency of transfection and possibly diminishing the immune response of the host, (106) which is one of the major problems in the application of adenoviral vectors (107).

Jacobsen *et al.* showed that the combination of adenoviral vectors and cationic liposomes leads to a significantly higher transduction efficiency and higher levels of transgene expression in both primary human keratinocytes and a rat burn model (104).

Advantages of liposomal gene transfer is its low immunogenicity in the host environment (108), the option of repetitive administration *in vivo* (109), transient expression of the transgene without stable integration into the host genome(110-112), the ability of the liposomal gene to carry large amounts of DNA(113), and the commercial availability of the liposomes (104).

The current drawbacks of liposomes is the possibility of high toxicity to cells and tissues and low efficiency(100, 114), both crucial for successful gene therapy in wounds. The studies of the combination of viral vectors and liposomes, the combination of lipoplexes and electroporation, and the *ex vivo* approach all show encouraging possibilities as optimized combined gene therapy in the near future (104, 115).

5. PHYSICAL METHODS FOR GENE DELIVERY

There are several physical methods for gene delivery to skin. Because of its accessibility, skin is a relatively good target for physical gene transfer technique.

5.1. Ballistic gene delivery

Ballistic gene delivery with a so-called gene gun delivers transgenes on DNA-coated gold particles (1–5 μm in diameter) that are driven by physical force into tissue to introduce foreign DNA into the cells (116, 117). We have successfully transferred the gene for human epidermal growth factor into partial thickness wounds with this technique in large-animal studies (118). Steinstraesser *et al*. reported that delivery of the reporter gene LacZ with a gene gun is feasible in first- and second-degree burn wounds in rats (5). However, the same group found that adenovirus-mediated gene transfer of this particular transgene elicits higher expression in the same *in vivo* model (104). Thus, the major limitation of this technique is low *expression in vivo*, but an advantage is that it elicits little to no immunoresponse and mutagenesis in the host.

5.2. Microseeding

Microseeding is a technique developed in our laboratory for *in vivo* gene transfer whereby the plasmid DNA solution of choice is delivered directly to the target cells of the skin by a set of oscillating solid microneedles driven by a modified tattooing device (53). We demonstrated that levels of EGF expression in microseeded skin and wound sites were two- to three-fold higher than in particle-bombarded skin and wound sites and approximately four- to seven-fold higher than that produced by single injection. The efficiency of this gene-transfer technique can be further enhanced by applying the plasmid as a liposome-DNA complex (119).

The advantage of this technique is that, unlike ballistic-derived gene transfer, it does not deposit any foreign material in the tissue. Furthermore, this technique opens new avenues for the delivery of viral genes to wounds and skin. We successfully delivered microseeded adenoviral vectors encoding the Ad-VEGF at doses ranging from 1×10^7 to 2.7×10^{11} particles per wound to full-thickness wounds in a large-animal model and found that VEGF expression in wound fluid followed a dose-response pattern (53). Thus, microseeding provides a effective strategy for administering gene therapy to skin and wounds.

5.3. Electroporation

In vivo electroporation is used for enhancement of gene uptake into cells after injection of naked DNA by the application of controlled electric fields to facilitate cell permeabilization (120). There are currently many choices in electrode design, and good comparative studies are clearly necessary. Zhang *et al.* examined skin electroporation using different electrodes to improve gene transfer. A meander electrode appeared to be a more patient-friendly design, as it avoids the need to pinch the skin and achieves the same results as calliper electrodes (121). The pattern of electrical pulses also varies considerably between studies, ranging from moderate voltage (e.g., 200 V/cm) pulses of tens of milliseconds to high-voltage microsecond pulses (122).

Malone and coworkers demonstrated that the administration of electrical field pulses after intradermal injection of naked DNA enhances transfection activity in the skin by about 115-fold (123). In 2004, the same group investigated the use of electroporation in full-thickness wounds in a mouse model (124). Their results suggested that electroporation significantly improves the efficiency of plasmid transfection in cutaneous wound tissue. The application of high-voltage, short-duration square-wave electrical field pulses to wounded tissue enhanced gene expression more than 10-fold and therefore shows potential as a method to reduce the dose of plasmid or increase transgene expression without negatively affecting wound healing (124).

In general, electroporation appears to be an attractive tool for enhancing gene expression in wounds, but additional *in vivo* studies are required to determine whether this will improve gene therapy and wound healing in humans.

6. GENE REGULATION

One of the most important issues in gene therapy is controlling expression of the foreign gene once it has been introduced into the host tissue. Especially in the treatment of wounds, only transient (time-limited) expression of the gene is desired once the wound has healed completely, and further gene expression is not necessary. Plasmids, lipoplex, and adenoviral vectors are known for their transient but varying expression in skin and wounds,(13, 125) whereas retro- and lentiviruses integrate stably into the host genome and thereby achieve persistent gene expression (126). Thus, we must develop tools to enable precise control of gene expression in the host. In recent years, significant progress has been made in developing genetic switches for control of transgene expression. Four major systems for gene regulation are presently available(127): ecdysone(128), RU486/antiprogestin-mifoprestone (129), and tetracycline-dependent systems (130).

Our laboratory developed a new tetracycline-regulatable switch (91, 93, 131) (T-Rex, Invitrogen, Carlsbad, CA, USA). Unlike the tetracycline gene-switch systems described above (129), which rely on the hCMV minimal promoter and the hybrid transactivator, tTA or rtTA; the T-REx system uses tetR itself and the tetO-bearing full-length wild-type hCMV major immediate-early enhancer promoter. Because no mammalian cell transcriptional regulatory proteins are used, the pleiotropic effects on the expression of cellular genes resulting from transcription squelching by tTA and/or rtTA(132-134) is eliminated in the T-REx system. Regulated gene expression can be increased by more than 1000-fold by T-REx. *Ex vivo* gene transfer to porcine full-thickness wounds confirmed the *in vitro* gene regulation of several 1000-fold in localized wound microenvironment (unpublished data). With the cloning of a wound healing-promoting gene under the control of this tetracycline-inducible switch, timing and levels of its expression could be adjusted during the healing process. Precise control of transgene expression would be another milestone on the road to a standard therapy protocol for clinical application in humans. To date, none of other gene switch systems have been tested in achieving regulated gene expression in the context of wound healing research

7. GENE SILENCING AND TRANSCRIPTIONAL FACTORS

The field of gene silencing with siRNA(135-137) and the introduction of transgenes on an mRNA- and ribozyme level presents another possible future approach to understanding and treating wounds on a molecular level. Schwarz *et al.* showed that simultaneous balllistic delivery of epidermal growth factor mRNA with the translational factor eukaryotic initiation factor 4E (eIF4E) mRNA, which upregulates growth-related proteins, significantly increased wound-breaking strength in abdominal wounds in rats (138). Other studies showed that expression of transforming growth factor-beta (TGF-beta) can be successfully knocked down by cell transfection with small-interfering RNA (siRNA) (139). Wang and coworkers showed that siRNA induced silencing of connective tissue growth factor (CTGF) in porcine skin fibroblasts significantly decreased the levels of mRNA of several matrix molecules, growth factors, and proteinase inhibitors, highlighting the effect of the CTGF-dependent pathway and providing a basis to a better understanding of the function of this growth factor in wound repair (140). These findings offer a promising perspective for studying the role and interaction of growth factors and cytokines in wound healing and wound inflammation at the molecular level.

8. CLINICAL TRIALS

Several small clinical trials investigating gene therapy in wound healing have been done in recent years or are ongoing. Isner and Walsh reported a phase I clinical trial in nine patients with nonhealing ischemic ulcers and/or pain during rest due to peripheral arterial disease. Gene transfer was performed by injecting naked plasmid DNA encoding VEGF directly into the muscles of the ischemic limb. Patients who had undergone this treatment showed significant improvement in the ankle brachial index, with newly visible collateral blood vessels seen by contrast angiography in the limbs. Qualitative evidence of improved

distal flow could be detected by magnetic resonance angiography in eight limbs. Ischemic ulcers healed or markedly improved in four limbs, including successful limb salvage in three patients initially recommended for below-knee amputation. Complications were limited to transient lower-extremity edema in six patients and a transient increase in serum levels of VEGF (141).
These findings were confirmed by Kim *et al*.(142), who published the results of their gene therapy trial of plasmid-derived VEGF gene transfer by intramuscular injection of the limb of nine male patients with severe peripheral arterial disease. In this trial, only three patients showed edema due to the VEGF enhancement of vascular permeability as a negative side effect. Ischemic pain of the affected limb was relieved or improved markedly in six of seven patients. Ischemic ulcers healed or improved in four of six subjects. The mean ankle brachial index improved significantly, and six of nine patients showed an increase in collateral vessels around the injection sites.

Hepatocyte growth factor (HGF) has been shown to be a potent angiogenic growth factor in several animal models (143, 144). A clinical trial of HGF gene transfer via plasmid DNA into ischemic limbs of six patients with critical limb ischemia (arteriosclerosis obliterans, n=3; Buerger disease, n=3; Fontaine III or IV) was published in 2004. Five of six patients had a significant reduction in pain (<1 cm visual analog pain scale), and five patients had an increase in the ankle pressure index. The long diameter of 11 ischemic ulcers in four patients was reduced >25%, and no severe negative side effects occurred during the therapy (145). Another approach is being pursued by Margolis and Cross(8, 62): They are investigating the potential of adenovirus-mediated gene therapy of PDGF-BB to diabetic foot ulcers and venous leg ulcers in an ongoing clinical trial with an estimated 27 patients that is expected to be completed in February 2007.

Although all these clinical trials deal with a small number of patients, the average results are encouraging, and additional and larger clinical trials should be initiated soon.

9. PERSPECTIVE

In this review, we discussed the potentials and limitations of current gene therapy for the treatment of wounds. The recent development of new and improved vectors for gene therapy has been encouraging. Less-immunogenic vectors have been developed, and the efficiency of *in vivo* transgene expression has been improved.

Although many approaches and different techniques for gene delivery exist, none is ideal, and more work is necessary to further improve gene-therapy vectors. Preclinical studies in large-animal models are required to evaluate vector performance and efficiency in situations close to those in clinical therapy.

Future gene therapy vectors will certainly be different from those used today. Vectors will be customized for each therapeutic application, or even for each patient. We might be able to perfect the combination of non-viral delivery technology with viral-vector properties. Future vectors in gene therapy for wounds need to target cells specifically, to be less immunogenic and mutagenic and safer, and involve a gene-regulation system capable of controlling transgene expression efficiently and for an appropriate and predetermined duration. Overall, the vector must be able to express the transgene at therapeutic levels.
We have overcome many hurdles but must continue to improve the vectors used for gene therapy. By maintaining a strong focus on these issues, we will significantly improve the outcome of chronic and acute wound healing treatment with gene therapy. We hypothesize that, in the near future, improved delivery systems will be available to treat and heal wounds and cutaneous pathologies effectively, safely, reliably, and efficiently.

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Abbreviations: Ad: Adenovirus, Ad5: Adenovirus serotype 5, AAV: Adeno-associated virus, HSV-1: Herpes simplex virus serotype 1, CAR: Coxsackie-adenovirus-receptor, VEGF: Vascular-endothlial growth-factor, PDGF-BB: Plastelet-derived growth-factor- BB, KGF: Keratinocyte growth-factor, FGF: Fibroblast growth-factor, IGF-1: Insulin-like growth-factor, EGF: epidermal growth-factor, TGF-beta: Transforming growth-factor beta, siRNA: small-interfernce RNA, CTGF: connective-tissue growth-factor, HGF: Hepatocyte growth-factor

Key Words: Review, Gene Therapy, Gene Transfer, Wounds, Wound Healing, Cutaneous Wounds, Skin Injury, Growth Factors, Antimicrobial Peptides, Host Defense Peptides, IGF-1, EGF, VEGF, KGF, PDGF-BB, FGF, siRNA, TGF-beta, CTGF, HGF, Gene Vector, Viral Vector, Adenoviral Vector, Retrovirus, Retroviral Vector, Lentiviral Vector, Herpes Simplex Viral Vector, HSV, Microseeding, Electroporation, Gene Gun, Ballistic Gene Transfer, Liposomes, Lipoplex

Send correspondence to: Elof Eriksson, MD, PhD, Division of Plastic Surgery, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, Tel: 617-732-7409, Fax: 617-732-6387, E-mail: eeriksson@partners.org

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