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A REVIEW OF GENE AND STEM CELL THERAPY IN CUTANEOUS WOUND HEALING

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Abstract

Different therapies that modulate wound repair have been proposed over the last few decades. This article reviews the two emerging fields of gene and stem cell therapy in wound healing. Gene therapy, initially developed for treatment of congenital defects, is a new option for enhancing wound repair. In order to accelerate wound closure, genes encoding for growth factors or cytokines have showed the most potential. The majority of gene delivery systems are based on viral transfection, naked DNA application, high pressure injection, or liposomal vectors. Embryonic and adult stem cells have a prolonged self renewal capacity with the ability to differentiate into various tissue types. A variety of sources, such as bone marrow, peripheral blood, umbilical cord blood, adipose tissue, skin and hair follicles, have been utilized to isolate stem cells to modulate the healing response of acute and chronic wounds. Recently, the combination of gene and stem cell therapy has emerged as a promising approach for future treatment of chronic and acute wounds.

Keywords

Liposomes; Non-viral Gene Therapy; Growth Factors; Umbilical Cord Derived Stem Cells; Cutaneous Stem Cells

INTRODUCTION

In the United States, over 2 million people suffer from cutaneous burns each year¹. These wounds lead to a loss of integrity of the skin and are a major cause of morbidity and mortality. The primary goals in treating burns are rapid closure, restoration of function, and aesthetically satisfactory scar development. In the past two decades, advances in cellular and molecular biology have expanded our understanding of wound repair and tissue regeneration. Wound healing encompasses a complex process that requires the integration of cell migration and proliferation as well as extracellular matrix deposition, angiogenesis, and remodeling^{2,3}. Transient gene therapy is of particular interest in this process since a transient increase in strategic growth factors is required until wound closure is achieved.

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Embryonic and adult stem cells, with their prolonged self renewal capacity and ability to differentiate into various tissues, may provide new strategies to treat acute and chronic cutaneous wounds. This article reviews several emerging techniques applications of gene transfer and stem cell therapy in cutaneous wound healing.

GENE THERAPY

Gene therapy, defined as the insertion of a gene into recipient cells, was initially considered only as a treatment option for patients with a congenital defect of a metabolic function or late-stage malignancy ⁴. More recently, skin has become an important target of gene therapy research. This research is made possible due to the ease of fibroblast and keratinocyte harvest and cultivation, thus allowing for gene transfer testing *in vitro* and the use of skin cells as vehicles in gene transfer ⁵. Skin is also easily accessible and the effects of therapy can be repeatedly monitored.

Two basic strategies for the introduction and expression of foreign DNA into host cells are 1) gene therapy, which is based on the permanent insertion of DNA, and 2) gene medicine, which is used for transient transformation and short-term expression of a gene product ⁵. Genes can be delivered by either *in vivo* or *ex vivo* approaches. *In vivo* techniques are based upon the direct introduction of genes to the target tissue. *Ex vivo* techniques rely upon the isolation and cultivation of selected cells with their transfection *in vitro* and a subsequent transplantation to a host. In both approaches, the selection of an appropriate vector for the introduction of genes is paramount for success ⁶.

Viral vectors

Gene transfer, using viral vectors, relies on the ability of viruses to carry and express their genes into host cells. Production of viral gene therapy vectors begins with genetic modification of the virus. Deletion of the original viral genes for replication or assembling is followed by insertion of the desired therapeutic gene ^{6,7}. The ability to reproduce recombinant viruses is restored by using specialized cells called “packaging cells” which are engineered to replace the function of the deleted viral gene ⁷. Gene therapy vectors are developed by the modification of different types of viruses. Retroviruses and lentiviruses are non-lytic replicators produced from the cellular membrane of an infected cell which leaves the host cell relatively intact. The lytic replication method involves the release of virions with the collapse of the host cell after infection. Human adenoviruses, adeno-associated viruses and herpes simplex viruses are examples of lytic replicators.

Human adenoviruses are double-stranded DNA viruses that are the most common and best described vectors used in viral gene therapy. These viruses have been shown to exert a 95% efficiency for infecting skin cells *in vitro* ⁸ and are safe in regards to infection rates in a skin-transfection model ⁹. It is important to note that integration of a viral genome into the host genome does not occur, therefore, the expression of the genes remains transient ^{10,11}. Liechty et al. ¹² transferred platelet-derived growth factor-B (PDGF-B) via the adenoviral vector to chronic wounds in the ischemic rabbit ear model and found that these ischemic wounds, treated with only one administration of viral gene, re-epithelialized more quickly than non-ischemic control wounds. As an adverse reaction to the adenovirus, an acute inflammatory response occurred probably due to the capsid protein of the virus ¹³. The authors concluded that adenoviral application alone may lead to a marked inflammatory response and impaired wound healing ¹². This same group recently reported positive effects of lentiviral-mediated transfection of stromal-derived growth factor-1 alpha (SDGF-1α) in diabetic mice showing notable improvements in wound healing with complete epithelialization after 2 weeks ¹⁴.

Adeno-associated viruses are single-stranded, non-enveloped DNA viruses that are mostly non-pathogenic to humans. Deodato et al.¹⁵ and Galeano et al.¹⁶ developed a model for external gene delivery of vascular endothelial growth factor-A (VEGF-A) by the serotype 2 of Adeno-associated viruses. Wound healing was significantly accelerated 6 to 10 days after surgery (as measured by wound area reduction), with well-structured granulation and increased vascularization after 18 days¹⁵. These findings were confirmed in a rat burn model using the same gene and vector to demonstrate an increase in epithelial regeneration and neo-angiogenesis¹⁶. New approaches with adeno-associated viral vectors indicate that different serotypes might influence the targeting abilities and transduction rates of endothelial cells to improve gene expression¹⁷. Adenoviral delivery of antimicrobial peptide genes from keratinocytes, under inflammatory conditions, which has been shown in human keratinocytes *in vitro*¹⁸.

Retroviral vectors are single stranded RNA viruses that replicate through a DNA intermediate and can be designed as highly tissue specific and safe from recombination after transfection¹⁹. They are, however, limited by low transfection efficiency, relative particle instability, and the inability to transduce non-dividing cells. The latter limitation is not of great concern in wound healing models, since the most of the target cells are in the proliferating phase, and has been partly overcome by using lentiviral vector parts, which have surface glycoproteins replaced by genes from another viral genome, thus making non-dividing cells a target for retroviral vectors²⁰. Morgan et al.²¹ used retrovirus-mediated gene transfer in athymic mice by constructing recombinant human growth hormone grafted on an epithelial sheet. This study showed that cultured keratinocytes reconstituted an epidermis which was similar in appearance to that resulting from normal cells, but from which human growth hormone could be extracted. Eming et al. showed the feasibility of retroviral gene transfer in introducing genes encoding for PDGF or insulin-like growth factor-I (IGF-I) into cultured human keratinocytes^{22–25}. This *in vitro* approach was supported by animal experiments with gene-modified composite grafts which showed an increase in fibrovascular ingrowth in the dermal template²³.

To summarize, viral vectors are the original and most established technology for gene delivery. A wide range of applications have been developed and many virus-mediated gene transfer models are successful. The production of viral vectors, however, is time and cost consuming, transfection efficacy is variable, and the risk of local or systemic infections, leading to fatal outcomes, remains a concern.

Non-viral vectors

In 1995, Hengge et al. first described the direct injection of DNA coding for interleukin-8 genes. By injecting naked genes into the skin, they found a significant recruitment of dermal neutrophils²⁶. However, the injection of naked DNA into the skin has been proven to have a low transfection efficacy and a high rate of initial degradation even before the injectate reaches the cytosol. Naked DNA constructs are not likely to penetrate the cells due to their fragility in the extracellular environment, large size, and electrical charge²⁷.

Eriksson et al. modified the direct injection technique, termed “micro-seeding”, which delivers naked DNA directly into target cells via solid needles mounted on a modified tattooing machine²⁸. Elevated levels of transferred DNA could be maintained for one to two weeks²⁸, but transfection was only observed in the superficial layers of skin with minimal penetration into deeper tissue. Another technique used to penetrate the cellular membrane employs the “gene gun”. In this approach, 1–5 µm gold or tungsten-coated particles carrying DNA plasmids are propelled into skin cells²⁵. Gene transfer is mostly transient and reaches its highest expression between the first and the third day after injection^{23,24}. *In vivo* transfection with epithelial growth factor (EGF) cDNA in porcine partial-thickness wounds

has demonstrated an increase in the rate of wound healing and re-epithelialization²⁹. Recent studies in the rat model indicate that gene gun particle mediated transfection of different PDGF isomers significantly improved wound healing by increasing its tensile strength²⁵. Differing results have been reported on the depth of transfection, with one study showing the gene gun technique primarily delivering particles no deeper than the epidermis with transfection rates of up to 10%³⁰. Dileo et al. had higher levels of gene expression in the epidermis and dermis by using a modified gene gun which delivered plasmids at higher discharge speed. Gene expression in skin and muscle reached its peak 24 hours after application and remained detectable for at least 1 week with little tissue damage³¹.

The technique of electroporation has been successfully used to accelerate the closure of diabetic and chronic wounds^{32,33}. Lee et al. describe the synergistic use of electroporation, where an electric field is applied to tissue, in combination with tissue growth factor- β 1 (TGF- β 1) cDNA in a diabetic mouse model³⁴. In the group which received electroporation and gene transfer the wound bed showed an increased rate of re-epithelialization, angiogenesis, and collagen synthesis. Apart from a transitional effect between the second and fourth day after wounding, the wound healing process itself was not significantly accelerated by the combined use of electric stimulation and gene transfer³⁴. Marti et al. showed that electroporation and simultaneous administration of keratinocytes growth factor (KGF) plasmid DNA increased wound healing when compared to controls receiving no treatment (92% vs. 40% of the area healed). No significant improvement in comparison to administration of KGF plasmid DNA alone was observed³⁵. Taking into account these inconclusive results, the benefit of this concept remains in question.

Another method receiving particular attention as a reliable and highly efficient therapy is the cutaneous gene delivery with cationic liposomes. Cationic liposomes (CL) are synthetically prepared vesicles with positively charged surfaces that form loose complexes with negatively charged DNA to protect it from degradation in the wound environment. The net positive charge of the complex binds readily to negatively charged cell surfaces to facilitate uptake via endocytosis^{36,37}. Genes encapsulated in CL can be applied either topically or by direct injection^{36,38}. Alexander et al. used topical application of CL constructs containing the Lac Z-gene to induce transfection and expression in the epidermis, dermis, and hair follicles in shaved 4-week-old mice. Expression was observed as early as 6 hours after topical application, which persisted at high levels for 48 hours, and was detectable for seven days³⁹. Several studies have been performed to determine CL gene transfer for growth factors^{25,40,41}. Sun et al.⁴¹ administered fibroblast growth factor-1 (FGF-1) by topical application and subcutaneous injection to the injured skin of diabetic mice. Transfection with FGF was found to increase tensile strength. In a preliminary study of IGF-I cDNA constructs applied to thermally injured rat skin, Jeschke et al. detected a transfection rate of 70–90% in myofibroblasts, endothelial cells and macrophages, including multinucleate giant cells⁴². In an *in vivo* approach, thermally injured rats treated with liposomal IGF-I cDNA significantly improved body weight and increased muscle protein when compared to burn controls. An accelerated rate of re-epithelialization of nearly 15% was observed when compared to naked IGF-I protein and IGF-I protein encapsulated in liposomes³⁷. Furthermore, no evidence was found that the dermal injection of IGF-I cDNA-complexes led to transfection or increase in β -galactosidase or IGF-I expression in blood, liver, spleen or kidney, thus gene transfection and production of growth factors remains localized³⁷. Animals transfected with IGF-I cDNA increased their basal skin cell proliferation, suggesting that myofibroblasts, endothelial cells and macrophages, identified as transfected, produce biologically active IGF-I⁴².

As a positive attribute, non-viral gene therapy is performed without a viral vector, which eliminates the risk of infection and cost of vector production²⁷. The transient nature of gene

expression is also a benefit in wound healing applications. The use of CL appears to be efficacious and clinically safe. On the negative side, some non-viral gene transfer methods tend to be non-specific and highly variable within the levels of gene expression reported.

New directions

A potential problem of single growth factor gene therapy is that simply increasing the concentration may not promote all phases of wound healing. A single growth factor cannot counteract all the deficiencies of a burn wound, nor control the complexities of chronic wound healing. Lynch et al.⁴³ demonstrated in a partial thickness wound healing model that the combination of PDGF and IGF-I was more effective than either growth factor alone, while Sprugel et al.⁶ found that a combination of PDGF and FGF-2 increased the DNA content of wounds in the rat better than any single growth factor. Our group subsequently investigated the efficacy of KGF cDNA in combination with IGF-I cDNA compared to the same genes individually⁴⁴. It was noted that this combination accelerated re-epithelization, increased proliferation, and decreased skin cell apoptosis compared to the single construct alone. The re-epithelialization in the burn model was over twice that of the untreated control with a significant improvement in cell survival⁴⁴. Transfection of multiple growth factor genes at strategic time points of wound healing (sequential growth factor therapy) is therefore the next logical step in augmenting wound healing. Work is in progress in our lab on using liposomal gene therapy transfer in an established porcine wound-healing model (Figure 1). Other labs have also conducted work on porcine models using retroviral technology⁴⁵.

Other delivery routes including biomaterials⁴⁶, calcium phosphate transfection⁴⁷, diethylaminoethyl-dextran⁴⁸, and microbubble-enhanced ultrasound⁴⁹ have been investigated. Slow-release matrices⁵⁰ and gene-delivering gel matrices⁵¹ are used for prolonged transgenic expression. The concept of a genetic switch is another exciting development, where transgenic expression in target cells can be switched 'on' or 'off', depending on the presence of or absence of a stimulator such as tetracycline⁵². Biotechnological refinements, such as wound chamber technique⁵³, may also improve the efficacy of gene delivery to wounds. These new techniques need further studies to define their efficacy and clinical applicability. More studies are also needed to define growth factor levels in different phases of wound healing and to elucidate the precise timing of gene expression or down-regulation required to better augment wound healing and control of scar formation.

STEM CELLS AND WOUND HEALING

Cutaneous wound healing requires a well-orchestrated integration of the complex biological and molecular events of cell migration and proliferation, as well as extracellular matrix deposition, angiogenesis, and remodeling³. Creating a viable skin substitute by assembling individual components *in vitro* has not been successful⁵⁴. Stem cells, due to their ability to differentiate into various tissue types by asymmetric replication, may create such a complex structure. A variety of sources, such as bone marrow, peripheral blood, umbilical cord blood, adipose tissue and skin/hair follicles, have been utilized to isolate stem cells to modulate the healing response of acute and chronic wounds.

The embryo with its developmental plasticity and high proliferative capacity has traditionally been the preferred source for the isolation of pluripotent stem cells. Stem cells, defined based on the findings of Ernest A. McCulloch and James E. Till^{55,56}, are characterized by 1) a prolonged self renewal capacity and 2) the ability to differentiate into mature stages and different tissue types by asymmetric replication^{57,58}. These properties have led to the expectation that human embryonic stem cells can be useful in understanding

disease mechanisms and the development of effective drugs for treating patients with various diseases and injuries^{59,60}. Despite their unique potential, embryonic stem cell use remains controversial. Ethical concerns regarding the use of human embryos for cell harvesting have been raised by both the scientific community and public media. Even though there are new studies suggesting that embryonic stem cells may be obtained without destroying the embryo⁶¹, it remains in question whether this approach would eliminate ethical concerns. To circumvent these issues, Yamanaka et al.⁶² produced pluripotent somatic cells by direct reprogramming to generate pluripotent stem cells from adult human dermal fibroblasts and other human somatic cells which were comparable to the differentiation potential of human embryonic stem cells⁶³. In addition, differentiated adult tissues have been shown to harbor pluripotent stem cells with unexpected plasticity⁵⁹. Thus, stem cells derived from adult sources may have the same clinical and experimental potential as embryonic stem cells.

Bone marrow derived stem cells

Human mesenchymal stem cells derived from bone marrow are relatively easy to obtain and have received considerable attention. They are self-renewing and capable of differentiating into various tissues and cells, such as liver epithelium, lung, gastrointestinal tract, and skin cells^{64,65}. Bone marrow-derived cells, used in chronic wounds, have been of particular interest. Fathke et al.⁶⁶ showed, in the chimeric mouse model, that distant bone marrow-derived cells contribute to the reconstitution of the dermal fibroblast population in cutaneous wounds. Bone marrow stroma cells were further found to synthesize higher amounts of collagen, FGF, and VEGF, when compared to native dermal fibroblasts, indicating a potential use in accelerating wound healing⁶⁷. The effects of bone marrow impregnated collagen on wound healing were studied in a microcirculatory mouse model, showing significant increases in angiogenesis⁶⁸. Patients with chronic leg ulcers demonstrated successful wound closure after a treatment with these impregnated collagen matrices. Falaga et al.⁶⁹ utilized a fibrin polymer spray to apply cultured autologous mesenchymal stem cells obtained from bone marrow aspirates to accelerate the rate of healing of acute and non-healing cutaneous wounds in both humans and mice. This approach may represent a feasible method for introducing cells into wounds.

There are several difficulties related to these techniques. Stem cells must be cultured in sufficient numbers for topical application and grow in the wound for a therapeutic response. A limitation in severe burn trauma is that bone marrow suppression has been observed either as a result of silver sulphadiazine toxicity⁷⁰ or sepsis^{71,72}, and ultimately, bone marrow mesenchymal stem cells significantly decrease with age⁷³.

Umbilical cord derived stem cells

Human umbilical cord blood is a rich source of hemopoietic stem cells for clinical application^{74,75}. It has been proposed that umbilical cord blood could become a source of stem cells with naive immune status⁷⁶. Currently, the presence of mesenchymal stem cells in umbilical cord blood is not known. Studies by Erices et al.⁷⁷ showed that mesenchymal stem cells together with hematopoietic precursors are circulating in the blood of preterm fetuses, however, Mareschi et al.⁷⁸ failed to isolate these cells in preterm umbilical cord blood. In contrast, Romanov et al.⁷⁹ found that cord vasculature contains a large amount of mesenchymal stem cell-like elements. These elements form colonies of fibroblastoid cells that were successfully expanded in culture, suggesting that umbilical cord stroma could be utilized as an alternative source of mesenchymal stem cells. It was also suggested that stem cells from umbilical cord blood are able to differentiate into epithelial cells under *in vitro* conditions and could therefore be used as a starting material for isolation and expansion of cells in large skin defects⁸⁰. It should be pointed out that umbilical cord blood-derived stem

cells are usually obtained from allogenic sources, potentially leading to immunological rejection upon transplant or transfusion.

Phan et al. recently found the amniotic membrane of the umbilical cord to be an extremely rich source of stem cells for burns resurfacing (unpublished data). The isolated cells, termed “Cord Lining Stem Cells”, can be divided into subpopulations of epithelial cells (Cord Lining Epithelial Cells) and mesenchymal cells (Cord Lining Mesenchymal Cells) (Figure 2). They express stem cell markers, such as Oct-4 and Nanog, and form healthy colonies in culture. Both have been utilized to treat partial thickness and full thickness burns as well as chronic diabetic wounds (ongoing clinical trials). Growing these cells on specific scaffolds creates an accessible way of applying them to the wound. Immunological rejection has not been observed in these ongoing clinical trials.

Adipose-derived stem cells

Adipose-derived stem cells may represent another available source of pluripotent cells with characteristics similar to bone marrow-derived mesenchymal stem cells⁸¹. Human liposuction aspirates have been used to culture adipo-derived mesodermal stem cells and differentiate them into chondrogenic cells⁸². Recent studies indicate that adipose-derived stem cells promoted human dermal fibroblast proliferation by direct cell-to-cell contact and by secretory induced paracrine activation which significantly accelerated the re-epithelialization of cutaneous wounds⁸¹. Beanes et al.⁸³ further observed that human connective tissue stem cells from fat lose their stem cell potential with age.

Cutaneous stem cells

Recent advances in isolating cutaneous stem cells have lead to a greater understanding of their potential use. Hair follicles, with the ability for self-renewal, represent a promising source of multipotent stem cells⁸⁴. Transplantation of cells from the murine bulge region showed that these cells could repopulate the epidermis, sebaceous glands, and the epithelial layers of the hair follicle⁸⁵, while Roh et al.⁸⁴ showed that stem cells extracted from the human bulge region exhibit hair follicle differentiation and form epidermal and sebaceous cells *in vitro*. Most authors agree that these stem cells, based on their accessibility and the easy access to skin, will become an important source for therapeutic applications.

Stem cells and gene transfection

An *ex vivo* technique for stem cell transfection has been described by Mathor et al.⁸⁶ using clonogenic keratinocytes with the characteristics of cutaneous stem cells. The authors were able to induce interleukin-6 (IL-6) cDNA using a retroviral construct to achieve a stable transgene expression. Epidermal sheets from these transfected keratinocytes grafted on to athymic animals showed IL-6 production for up to seven days after grafting. *In vivo* approaches include a recent study by Hachiya et al.⁸⁷ in which different lentiviral vectors were used to transfect human skin grafted onto severe combined immunodeficient mice. These mice showed an effective targeting of epidermal stem cells which underwent terminal differentiation resulting in transgene expression. Stem cells of the follicular outer root sheath and matrix can also be transfected using by non-viral method. Our group has shown that cationic liposomes carrying genes encoding for β -galactosidase were able to transfect hair follicle cells at the edge of the wound⁸⁸. This was confirmed by positive immunohistochemical staining in epithelial stem cells both on the outer root sheath and the matrix of hair follicles (Figure 3). Taking into account the main qualities of the therapeutic options discussed, the transfection of cutaneous stem cells with an optimized gene therapy vehicle could provide a future approach.

SUMMARY

Gene therapy and stem cell research have become areas of great importance. Gene therapy has evolved from a purely experimental scientific endeavor to a clinically pertinent treatment for many organ systems. In wound healing, there still remain challenges in the selection of optimal target cells, development of sequential therapeutic methods, and identification of factors which may be detrimental to the introduction of genes. Stem cell research has made a significant contribution to the study of basic mechanisms of cell proliferation and differentiation and has proven essential in the development of cellular therapy. It is evident that the plasticity of the different types of stem cells, both *in vitro* and *in vivo*, will have clinical applicability in the future, however, further research is needed on the intrinsic molecular mechanisms that keep stem cells pluripotent or direct them along particular differentiation pathways.

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Figure 1.

Levels of β -Galactosidase expression in porcine dermal and epidermal tissue as measure of gene transfection efficacy using multichannel confocal fluorescence imaging. (A–D) Control tissue. (E–H) Tissue transfected with Lac-Z cDNA delivered via liposomal vectors, Day 5 post-transfection. (A) Nuclei stained with 4',6-diamidino-2-phenylindole (DAPI). (B) Fluorescein (FITC) β -Galactosidase expression. (C) Laser scanning brightfield image. (D) Overlay. (E) Nuclei stained with DAPI. (F) FITC β -Galactosidase Expression. (G) Laser scanning brightfield image. (H) Overlay – Composite image of DAPI, FITC, and brightfield to illustrate gross orientation of Lac-Z expression.

Figure 2. Photomicrographs of (A) Morphology of Cord Lining Epithelial Cells and (B) Cord Lining Mesenchymal Cells. Cells were isolated from the outer membrane of the umbilical cord and differentiated into both epithelial and mesenchymal cells using specific culture conditions. Magnification 200X.

Figure 3.

Sections of rat skin samples **(A)** stained for β -galactosidase (blue) and **(B)** dual stained with anti-cyokeratin-19 antibodies using peroxidase immunohistochemical techniques (magnification 1000X). Alkaline Phosphatase was used as substrate, hence bright pink stained cells are CK-19 positive. Sections were lightly counterstained with hematoxylin (5 seconds) to identify nuclei. Section shows colocalization of CK-19 and Blue-Gal stain in dermal sheath cells (marked DSC) along with hair follicle cells (ESC).