Summary. The rapid advances associated with the Human Genome Project combined with the development of proteomics technology set the bases to face the challenge of human gene therapy. Different strategies must be evaluated based on the genetic defect to be corrected. Therefore, the re-expression of the normal counterpart should be sufficient to reverse phenotype in single-gene inherited disorders. A growing number of candidate diseases are being evaluated since the ADA deficiency was selected for the first approved human gene therapy trial (Blaese et al., 1995). To cite some of them: sickle cell anemia, hemophilia, inherited immune deficiencies, hyper-cholesterolemia and cystic fibrosis. The approach does not seem to be so straightforward when a polygenic disorder is going to be treated. Many human traits like diabetes, hypertension, inflammatory diseases and cancer, appear to be due to the combined action of several genes and environment. For instance, several wizard gene therapy strategies have recently been proposed for cancer treatment, including the stimulation of the immune system of the patient (Xue et al., 2005), the targeting of particular signalling pathways to selectively kill cancer cells (Westphal and Melchner, 2002) and the modulation of the interactions with the stroma and the vasculature (Liotta, 2001; Liotta and Kohn, 2001).

Key words: Skin, Gene therapy, Tissue engineering

Introduction

The skin constitutes a tempting target for gene transfer as it is the most accessible tissue of the body and it is possible to monitor the genetically modified area and replace the tissue in case of adverse side effects. Skin cells are easy to obtain and expand in vitro from a small skin biopsy. Moreover, extraordinary advances have recently been accomplished in the development of tissue-engineered skin equivalents for the finest and permanent skin regeneration in clinics (Pellegrini et al., 1999; Ronfard et al., 2000; Llamés et al., 2004). Finally, the epidermal stem cell compartment, the required target for any permanent gene therapy strategy in the skin, has been shown to be efficiently modified using different integrative vectors (Levy et al., 1998; Serrano et al., 2003; Del Rio et al., 2004). These approaches provide evidence for the rationale that corrective gene transfer is feasible and constitutes a starting point for further refinement and development of future in vivo and ex vivo genetic therapies at preclinical and clinical levels (Fig. 1). Thus, recent major progress has been made to correct genodermatoses. Cutaneous gene therapy is attractive not only for the correction of skin diseases but also because the epidermis can be used as a “bioreactor” to deliver a therapeutic protein to the systemic circulation. It has been proved that genetically modified human keratinocytes grafted to immunodeficient mice are able to act as a source of systemic proteins such as growth hormone (Teumer et al., 1990), factor IX (Gerrard et al., 1993) and leptin (Larcher et al., 2001). A major effort is now being directed to optimise critical issues such as long-term persistence of adequate therapeutic serum levels of the modified keratinocyte-derived proteins (Larcher unpublished data).

Two different gene delivery methods can be used to achieve the genetic modification of skin: ex vivo and in vivo. The ex vivo approach consists of the isolation and in vitro propagation of skin cells (either keratinocytes or fibroblasts or both). Then, cells are genetically modified before grafting them back to the patient as part of a tissue-engineered skin equivalent. In the in vivo setting, direct gene transfer is accomplished either through the delivery of plasmidic DNA (by direct injection, biolistic “gene gun” or electroporation) or using viral vectors (such as lentivirus, retrovirus and adenovirus).
Some critical issues must be considered for the adequate choice of the delivery system. Transient gene expression would be desirable for applications such as regenerative therapies. In this case, the use of non-integrative vectors such as replication-deficient recombinant adenoviruses appears to be the best option. They have been successfully used in in vivo gene transfer to skin by subcutaneous injection. The broad tropism of adenoviral vectors allows for keratinocytes, fibroblasts, smooth muscle cells, sebaceous cells and adipocytes to be modified using this strategy (Setoguchi et al., 1994). Adenovirus vectors are easy to produce in high titers, transduce both proliferating and quiescent cells and do not integrate in the genome of the transduced cells. Nonviral vectors avoid potential safety problems associated with viral vehicles, but they show extremely low gene delivery efficacy. Electroporation and gene-gun methodologies as well as synthetic vectors such as liposomal formulations, polymers and nanoparticles have been used to enhance the uptake of naked DNA in tissue.

Also, novel strategies mimicking viral mechanisms have been developed to overcome the major drawback of these systems when aimed at permanent correction of a given inherited disorder, which is their episomal nature that precludes long-term expression of the transgene. To achieve safe and stable integration, gene transfer based on the φC31 integrase methodology (Ortiz-Urda et al., 2002), on transposons (Ohlfest et al., 2005) and more recently on human artificial chromosomes (Mecklenbeck et al., 2002) are currently being explored at preclinical level.

As mentioned above, permanent gene expression in the epidermis requires the efficient targeting of the stem cell population. Ex vivo gene transfer using oncoretroviral vectors (i.e. Moloney-based vectors) has been efficiently used in pre-clinical studies for this purpose (Del Rio et al., 2002). In this setting, proliferation of the target cells is a mandatory requisite. It has been hypothesized that under regular culture conditions a proportion of epidermal stem cells may not be induced to proliferate. Thus, a number of “dormant” stem cells may not be transduced using oncoretroviral vectors. Lentiviral vectors (HIV-based vectors) which are able to infect both proliferating and quiescent human keratinocytes may represent a promising strategy for genodermatoses such as epidermolysis in which the number of stem cells may be limited (Kuhn et al., 2002; Serrano et al., 2003). Retroviral and lentiviral vectors integrate randomly in the genome of their target cells, a characteristic that has recently generated significant safety concerns, particularly in applications involving genetic modification of stem cells (Cavazzana-Calvo et al., 2000). Major efforts are now being conducted to clarify whether the important side effects observed in an otherwise successful gene therapy trial using hematopoietic stem cells (Hacein-Bey-Abina et al., 2003) are specifically linked to that protocol or, alternatively, we are in the presence of a general phenomenon that may affect other tissue stem cells, other gene products and other transduction and grafting protocols. Last October, after a three year halt due to the side effects reported in the above mentioned hematopoietic gene therapy trial, a phase I-II trial of

![Fig. 1. Schematic diagram showing the applicability of human skin equivalents in tissue engineering and cutaneous gene therapy.](image-url)
cutaneous gene therapy for laminin 5-deficiency (JEB) based on autologous transplantation of epidermal stem cells transduced by an oncoretroviral vector was re-established in Italy (M De Luca and F Mavilio, personal communication). The results of this study will be critical to unravel some of these concerns.

Adeno-associated virus vectors (AAV) can efficiently transduce nondividing cells and do not elicit a significant inflammatory response. AAV vectors can ensure long lasting expression of the therapeutic gene with low risk of insertional oncogenesis. A new technology based on large-capacity DNA vectors capable of site-specific integration with lower risk for insertional oncogenesis has recently been described (Recchia et al., 2004). However, several technical improvements have to be accomplished before these vectors can be widely used in gene therapy protocols.

Vector design to achieve controlled transgene expression in the skin through inducible promoters points in that direction. For example, a growth factor inducible element (FiRE) has been shown to target transgene expression to cutaneous wound edge keratinocytes (Jaakkola et al., 2000). Pharmacologically regulated systems are of great interest for skin gene therapy, as modulation could be accomplished by simple topical application. Progress has been made in transcription systems induced by tetracycline, rapamycin, RU-486, steroid hormone and antibiotics (reviewed in Toniatti et al., 2004).

Genodermatoses

Among the inherited skin diseases, monogenic recessive disorders are the best candidate diseases to be treated by reintroducing a single normal copy of the gene into the keratinocyte. Some examples are recessive disorders such as Xeroderma Pigmentosum, X-linked Ichthyosis and Junctional and Dystrophic forms of Epidermolysis bullosa (Uitto and Pulkkinen, 2000; Spirito et al., 2001; Del Rio et al., 2004). An open question is how to circumvent a potential immune response against the foreign therapeutic protein in the host, in particular in patients carrying null-mutations. Large spontaneous immunocompetent animal models of some genodermatoses are now available and will be critical for accurate validation of cutaneous gene therapy strategies (Capt et al., 2005; Magnol et al., 2005; Spirito et al., 2006). Other skin disorders present a dominant negative mutation resulting in an aberrant protein that affects the function of its normal counterpart, such as Epidermolysis Bullosa Simplex (EBS) and the recessive form of Dystrophic Epidermolysis Bullosa (DEB) (Uitto and Richard, 2005). In this case, treatment should aim at suppressing the expression of the mutated gene or ameliorate the disease by controlled overexpression of the normal gene product. Most research efforts towards cutaneous gene therapy have been conducted to treat Epidermolysis bullosa (EB). The robust basic and preclinical data gathered during the past years have recently allowed the launching of a phase I-II clinical trial.

Epidermolysis bullosa

Epidermolysis bullosa (EB) is a group of inherited mechano-bullous skin diseases which results from defects affecting either keratin cytoskeleton or proteins involved in dermo-epidermal adhesion (Pulkkinen and...
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Uitto, 1999; Fine et al., 2000; Uitto and Richard, 2004; 2005) (Fig. 2). The clinical phenotype is associated with skin fragility, blisters, and erosion over trauma-prone parts of the body that may be aggravated by infection, usually during the first year of life. EB could also be associated with a significant extracutaneous multorgan involvement leading to impaired growth and anaemia. To date, medical care of EB patients is extremely limited and major research efforts towards keratinocytes-based gene therapy have been made for these disorders (Uitto and Pulkkinen, 2000; Spirito et al., 2001; Del Rio et al., 2004).

EB is classified into 3 major categories which differ in the affected gene and therefore in the level at which lesions split (Uitto and Richard, 2004, 2005).

Epidermolysis Bullosa Simplex (EBS), is an autosomal dominant disorder produced by mutations in the basal keratins K5 or K14 genes compromising the keratin cytoskeleton which is no longer providing the resistance stress needed to keep the mechanical stability of the epidermis. The genetic correction of this disease is complex due to the dominant-negative action of the mutant protein. Gene therapy approaches should aim at suppressing the expression of the mutated copy of the gene. However, an alternative approach has been proposed by Lane’s laboratory consisting of reinforcing the weakened intermediate filament cytoskeleton of EBS keratinocytes by providing an additional intermediate filament network of human desmin, which plays a similar role to the keratins in muscle cells (Magin et al., 2000; D’Alessandro et al., 2004). By retroviral gene transfer of human desmin into EBS keratinocytes, this group reported the formation of a well defined desmin filament network, independent of the endogenous keratin cytoskeleton which is not susceptible to the negative effect of the mutant keratin. These findings represent a promising result for the treatment of EBS disorders.

Junctional Epidermolysis Bullosa (JEB) is an autosomal recessive inherited disease produced by mutations in 6 different genes encoding 3 components of hemidesmosomes. The 3 chains that constitute the trimeric laminin 5 (LAMA3, LAMB3 or LAMC2), BPAG2/type XVII collagen (COL17A1) and α6/ß4 integrins (ITGA6 and ITGB4) could be affected. The hemidesmosomes are multiprotein complexes linking the epithelial intermediate filament network to the dermal anchoring fibrils, thus maintaining the integrity of integument in humans (McGrath et al., 1995; Vidal et al., 1995; Fine et al., 2000).

The initial efforts at genetic correction of EB were performed using keratinocytes of patients affected by the severe form of JEB associated with lack of expression of one of the 3 chains of laminin-5 heterotrimer. The restoration of laminin 5 expression has been achieved in primary JEB keratinocytes using either retroviral vectors (Dellambra et al., 1998; Vailly et al., 1998; Robbins et al., 2001) or non viral stable approaches mediated by the φC31 integrase (Ortiz-Urda et al., 2003a) or the transposon systems (Ortiz-Urda et al., 2003b). As mentioned before, the first trial for JEB using an oncoretroviral vector encoding laminin β3 chain was started in 2002 but was put on hold after the adverse effect observed in the X-linkend SCID trial and was restarted last October (DeLuca and Mavilio, personal communication).

Similarly, re-expression of type XVII collagen as well as integrin β4 has been accomplished by retroviral gene transfer to primary keratinocytes isolated from collagen XVII-null (Seitz et al., 1999) and β4-null (Dellambra et al., 2001) patients, respectively.

The recovered expression of laminin 5 subunits, type XVII collagen or integrin β4, in keratinocytes from JEB patients restored cell adhesion and colony forming ability without affecting their polarity and differentiation potential. JEB corrected cells were used to generate either skin equivalents in vitro or human skin in vivo after transplantation on immunodeficient mice. Genetically modified keratinocytes successfully assemble normal hemidesmosomal structures and correct the major hallmarks of the disease. Taken together these achievements have demonstrated the feasibility of phenotypic reversion of adhesion-defective disorders by keratinocyte-based gene transfer.

It is noteworthy, however, that although in vitro reconstructed skin and specially regenerated skin on immunodeficient mice are a valuable model to test gene therapy approaches, they are unable to recapitulate the host immune response. The identification of breeds of dogs with an inherited form of JEB provides the unique opportunity to verify the feasibility of the strategy in an immunocompetent environment. The genetic defect of dog keratinocytes has already been successfully corrected by retroviral gene transfer (laminin α3) (Spirito et al., 2006). Transplantation of autologous genetically modified dog skin equivalents are expected to provide information on the host immunoreaction and on persistence of the therapeutic transgene.

Dystrophic Epidermolysis Bullosa (DEB) is an autosomal dominant or recessive inherited disorder caused by genetic mutations in the gene (COL7A1) encoding collagen type VII, a large basement component which plays a critical role in epidermal-dermal adherence (Franzke et al., 2005). DEB is an untreatable condition characterized by unremitting blistering of the skin and mucosa, contractures and dystrophic scarring of the lesions. In the most severe form of the disorder (Hallopeau-Siemens recessive DEB), the lesions progress with time to invasive squamous cell carcinoma. Different groups have recently explored the feasibility of a permanent correction of RDEB primary human keratinocytes by ex vivo gene transfer. Promising results in animal models have been reported.

Ortiz-Urda and co-workers have used a φC31 integrase-based gene transfer system that stably integrated the COL7A1 cDNA into RDEB keratinocytes and fibroblasts isolated from patients (Ortiz-Urda et al., 2002). Chen and co-workers have achieved efficient
gene transfer using a self-inactivating lentiviral vector in immortalized RDEB keratinocytes (Chen et al., 2002). However, the exceedingly low transfer efficiency of non-viral vectors and the absence of suitable packaging cell lines for lentiviral vectors have hampered their use in clinical trials. Alternatively, the use of Murine Leukemia Virus (MolLV) based retroviral vectors are, at present, the most convenient and widespread tool for ex-vivo gene transfer of therapeutic genes in the context of clinical trials. Recently, Meneguzzi and co-workers have demonstrated that human primary RDEB keratinocytes, transduced with a retroviral vector expressing the human type VII collagen cDNA, were able to generate transplantable tissue-engineering skin equivalents (Gache et al., 2004).

When RDEB-null keratinocytes were grafted onto the back of immunodeficient mice, the regenerated human skin exhibited fragile epidermal-dermal adherence and blistering, absence of type VII collagen protein, and no detectable anchoring fibrils. In contrast, and regardless of the viral vector used, all these hallmarks were recovered after grafting genetically corrected RDEB cells. Two facts are noteworthy in these studies. First, durable normalized col VII expression was achieved (Chen et al., 2002). Second, ex vivo correction of DEB was accomplished in a clinical setting by the use of a new skin substitute successfully applied in burn patients (Gache et al., 2004; Llames et al., 2004). Finally, retroviral vectors have also been demonstrated to mediate efficient transfer of dog collagen Type VII cDNA into primary RDEB keratinocytes (Baldeschii et al., 2003). The transduced cells fully reverted the RDEB phenotype in vitro, which set the basis for preclinical studies of RDEB gene therapy using a large immunocompetent animal model.

Keratinocytes account for the majority of the deposited collagen at the DEJ in human skin. However, it has been proved that type VII collagen secreted solely by normal (Ortiz-Urda et al., 2003c; Woodley et al., 2003) or ex vivo corrected dermal fibroblasts (Ortiz-Urda et al., 2003c) is enough to restore the DEJ after being grafted to immunodeficient mice. Moreover, the effect of direct intradermal injection of normal human fibroblasts or gene corrected RDEB fibroblasts (lentiviral vectors) into a RDEB human skin substitute on nude mice has also been tested. Remarkably, the type VII collagen synthesized and secreted by these exogenously injected cells (for up to three months), is precisely localized at the dermo-epidermal junction forming anchoring fibrils probably due to the great affinity of laminin-5 for this molecule.

In vivo gene transfer approaches have also been tested. Woodley et al. have been able to correct RDEB by a single intradermal injection of a lentiviral vector, encoding the type VII collagen transgene, into a human RDEB skin regenerated on immunodeficient mice by providing stable type VII collagen at the basal membrane for at least 3 months (Woodley et al., 2004a). Based on the stability of the type VII collagen, this group have proposed an even more simple strategy based on protein therapy, proving that intra-dermal injection of recombinant type VII collagen into a human RDEB skin regenerated on immunodeficient mice can correct the defect (Woodley et al., 2004b).

Xeroderma pigmentosum

Xeroderma pigmentosum is an autosomal recessive disease characterized by increased sensitivity to ultraviolet light that leads to cutaneous and ocular abnormalities. Patients are prone to develop cutaneous basal and squamous cell carcinomas and melanomas. About 30% of individuals also present neurological disorders although these may become apparent later than the cutaneous symptoms, which usually appear at one to two years of age.

Mutations in different genes (XP, XPB/ERCC3, XPC, XPD/ERCC2, XPE/UV-DDB/DDB2, XP/ERCC4, XPG/ERCC5 and XPV/POLH) define the seven complementation groups (XP group A to G) and the variant group XP-V to which patients are assigned. A defect in the nucleotide excision repair (NER) machinery is observed in cells from patients belonging to complementation groups A through G. Differently, the XP variant cells show normal excision repair but instead suffer from error-prone post-replication translesion synthesis. In addition, there is a range of symptom manifestations among individuals within any group.

Management of the disease is limited to strict avoidance of sunlight exposure. One innovative treatment for these patients is the topical application of active enzymes in liposome formulations (bacteriophage T4 endonuclease T4N5 and photolyase) (Kraemer and DiGiovanna, 2002). The activity of these enzymes results in the removal of lesions in the DNA. Treatment of 30 patients (T4 endonuclease V) for 1 year resulted in significant decrease in the onset of pre-cancerous lesions without apparent immune reactions (Yarosh et al., 2001).

As for other genodermatoses, long-term effective correction of XP skin can be approached through cutaneous gene therapy. Early experiments, using XP fibroblasts and integrative vectors encoding appropriate wild-type genes, have shown stable complementation of the cells and recovery of DNA repair (Carreau et al., 1995; Zeng et al., 1997). However, gene delivery efforts should be directed to the significant players of skin cancer in these patients, such as keratinocytes, the cells from which basal and squamous cell carcinoma originate. The first steps towards XP gene therapy have recently been achieved. Genetic correction of DNA repair-deficient XPC keratinocytes has been accomplished using a retroviral transduction strategy. In this setting, reexpression of the wild-type XPC protein resulted in restoration of normal DNA repair following UVB irradiation (Arnaudeau-Begard et al., 2003). In addition, an adenoviral vector carrying the XPA gene used for in vivo gene delivery to the skin of XPA-knockout mice led to the prevention of deleterious
effects in the skin, including development of squamous cell carcinoma (Marchetto et al., 2004). However, as for other inherited skin disorders, permanent gene delivery to the epidermal stem cell compartment through ex-vivo gene transfer strategies appears as the most realistic and clinically relevant approach. The latest in vitro development of human XP skin equivalents (Bernerd et al., 2001; Arnaudeau-Begard et al., 2003) is the basis for imminent preclinical trials. In vivo regeneration of XP skin onto immunodeficient mice is envisioned as a unique humanized model suitable to validate an adequate gene therapy approach to treat this disorder, as previously performed for other genodermatoses.

**Gene therapy to improve wound healing**

Non-healing acute and chronic wounds are a diverse group of diseases of different aetiology and manifestation. The most common cause of acute wounds is thermal injury. Chronic wounds include arterial, diabetic, pressure and venous ulcers and cause significant morbidity and impaired quality of life. The comprehension of the tissue repair process and its failure has been essential for the development of new therapeutic approaches. The analysis of genetically modified mouse models has been particularly important to understand the role of individual genes (Scheid et al., 2000). The first event in the normal wound repair process is the immediate activation of the coagulation cascade followed by an acute inflammatory response. Leucocytes clear the wound and release growth factors that induce the formation of a temporary granulation tissue. The provisional matrix provides a scaffold that allows extracellular matrix remodelling, angiogenesis and re-epithelialization (Clark, 1996; Martin, 1997; Singer and Clark, 1999). Growth factors and cytokines orchestrate all these events to perform tissue repair (Werner and Grose, 2003).

Clinicians realize the difficult task involved in the management of impaired wound healing associated with chronic and acute wounds. A conventional treatment consists of transplantation of epidermal sheets (Gallico et al., 1984; De Luca et al., 1989). An improved strategy is based on the grafting of skin substitutes, resembling native human skin as closely as possible, and whose clinical efficacy has already been reported by different groups, including ours (Hansbrough et al., 1989; Falanga, 1998; Chang et al., 2000; Llamés et al., 2004, 2006). Different growth factors and their receptors such as PDGF (Reuterdahl et al., 1993), VEGF (Frank et al., 1995; Nissen et al., 1998), FGF (Vogt et al., 1998), and KGF (Marchese et al., 1995) appear to play a critical role in wound repair. Since defects in their expression may impair wound healing, many groups have been interested in testing them as candidate genes for gene therapy. The use of growth factors such as recombinant proteins in clinical assays to improve wound healing has been discouraging, probably due to their rapid degradation in the wound environment (Falanga, 1993; Meyer-Ingold, 1993; Lauer et al., 2000; Werner and Grose, 2003). After a first report showing that the expression of human epidermal growth factor mediated by gene gun transfer accelerated wound repair in porcine and mouse models (Andree et al., 1994), the idea that gene therapy may also contribute to the therapeutics has become popular.

Two applications can be easily distinguished for cutaneous gene therapy aimed at wound handling. One is the ex-vivo gene transfer of healing promoting genes to keratinocytes combined with either autologous or allogenic skin equivalent transplantation. The other is the in vivo gene transfer of these factors directly to the wound site using vectors capable of achieving high levels of protein expression and transducing both proliferating and quiescent cells (Sylvester et al., 2000; Gruss et al., 2003).

**Growth factors**

Platelet derived growth factor (PDGF) is produced locally by most of the cells at the site of injury promoting the granulation process (Heldin and Westermark, 1999; Werner and Grose, 2003). Reduced expression of PDGF and its receptors has been associated with impaired cutaneous wound healing in mice (Beer et al., 1997; Gao et al., 2005) and in vivo gene transfer of both PDGF A and B cDNA by direct particle bombardment of wounds promotes wound repair in rats (Eming et al., 1999). PDGF-A overexpression mediated by retroviral gene transfer improves graft performance during the first critical week after transplantation due to the acceleration of graft invasion by fibrovascular cells and the deposition of collagen (Eming et al., 1995, 1998). In addition, the effect of PDGF-B is dramatically enhanced when it is produced from an adenoviral vector (PDGF-B/Ad5) in three different models: diabetic mice, ischemic rabbit ear (Liecghy et al., 1999a, 1999b) and human skin substitute regenerated on immunodeficient mice (Sylvester et al., 2000). In addition, topical administration of recombinant PDGF–BB protein, which was the first growth factor approved by FDA (becaplermin gel; Regranex®, Janssen-Cilag Ltd), has already been shown to be safe and effective in treating human chronic wounds (Margolis et al., 2005). These encouraging results have promoted the first gene therapy clinical trial for the treatment of chronic ulcers using an adenoviral vector encoding human PDGF (PDGF/Ad5) (NIAMS-044; N01 AR-9-2238; Margolis et al., 2000, 2004).

Vascular endothelial growth factor (VEGF) has been explored as a candidate to induce angiogenesis since its expression is enhanced in keratinocytes at the wound site and induces potent proliferation and migration of endothelial cells (Nissen et al., 1998, 2003). The impaired wound healing in the diabetic mouse model has been related to a reduction of VEGF expression (Frank et al., 1995; Lauer et al., 2000; Altavilla et al., 2001). We and others have also found that VEGF overexpression in
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the skin of transgenic mice increases vascularization (Detmar et al., 1998; Larcher et al., 1998). Thus, our lab undertook grafting of ex vivo gene transferred pig primary keratinocytes overexpressing VEGF (through lipid mediated cDNA transfection) to nude mice as part of a transplantable skin substitute. The result of this strategy was a dramatic increase in the number of blood vessels in the host stroma (Del Rio et al., 1999).

Similarly, Supp and co-workers using human keratinocytes overexpressing VEGF through retroviral gene transfer, have also found an improvement in graft performance (Supp et al., 2000a,b; Supp and Boyce, 2002). On the other hand, in vivo VEGF delivered either by means of an adenoviral vector or an adeno-associated viral vector has shown improved angiogenic response and has subsequently enhanced the overall wound healing process in different animal models of impaired wound repair (Deodato et al., 2002; Romano Di Peppe et al., 2002; Galeano et al., 2003a,b). Preliminary results from our lab indicate that the use of VEGF-producing autologous keratinocytes, as a part of a transplantable skin substitute, improves stable engraftment and tissue regeneration quality in a porcine immunocompetent model (García et al., 2003). Together, these results warrant additional studies to evaluate the clinical usefulness of VEGF gene transfer to improve skin wound healing.

The potential value in the acceleration of re-epithelialization of keratinocyte growth factor (KGF), a well known growth factor playing an important role during cutaneous injury, has been explored (Werner, 1998; Werner and Gröse, 2003). Expression of KGF by fibroblasts is stimulated during normal wound healing, and its expression is significantly reduced and retarded in diabetic and glucocorticoid treated mice (Werner et al., 1992, 1994a; Brauchle et al., 1995). In addition, dominant negative KGF receptor transgenic mice are characterized by a severe delay in wound re-epithelialization (Werner et al., 1994b). Preclinical data from animal models demonstrated that KGF stimulates both epithelialization and granulation tissue (Statano-Coico et al., 1993; Pierce et al., 1994; Andreadi et al., 2001). Interestingly, KGF also stimulates capillary endothelial cells together with endothelial barrier stabilization (Gillis et al., 1999). Additionally, KGF gene transfer demonstrated a beneficial effect on wound healing in different animal models. In a pre-clinical wound healing model based on the transplantation of ex vivo modified skin equivalents to assess graft take, as a wound healing end point (7-15 days post-transplantation), KGF overexpressing human keratinocytes were able to demonstrate beneficial effects on early graft performance in immunodeficient mice (Erdag et al., 2004). On the other hand, enhanced wound healing has also been reported after in vivo gene transfer of KGF to excisional wounds of diabetic mouse (naked DNA injection with subsequent electroporation) (Marti et al., 2004) and to thermally injured rats (liposomal injection) (Jeschke et al., 2002). Finally, protein therapy using recombinant KGF was found to reduce the duration and severity of oral mucositis in a phase III clinical trial (Spielberger et al., 2004). Collectively, these results support KGF as a candidate growth factor for gene therapy.

Hepatocyte growth factor (HGF) is a potent mitogen for hepatocytes but it has also been revealed as a factor with interesting activities in cutaneous tissue repair, having an effect on epithelial cell proliferation, migration and morphogenesis (Brinkmann et al., 1995). Recently, the overexpression of HGF gene by human keratinocytes (retroviral-mediated gene transfer) after transplantation on nude mice regenerated an epidermis showing a transitory hyperproliferation that subsides by 2 weeks (Hamoen and Morgan, 2002). Therefore, activated skin substitutes containing cells that express and locally deliver HGF whose time frame action coincides with the graft take process may be useful in cutaneous tissue repair and wound healing. Recently, a phase I clinical trial using HGF has demonstrated that intramuscular injection of naked HGF plasmid achieves clinically significant improvement of ischemic ulcers (Morishita et al., 2004). These findings are preliminary and do not establish the long-term safety of HGF but encourage further studies.

Recently, we have described a novel wound healing model based on the injury of skin-humanized mice (Escamez et al., 2004). The main advantage of our system is that full thickness wounds are performed in a mature, “quiescent” regenerated human skin (9-12 weeks after grafting). This model offers the possibility to test both in vivo and ex vivo gene transfer approaches by using normal-control skin or stable genetically modified human skin, respectively (Fig. 3). So far, we have applied exogenously recombinant human KGF protein by intradermal injection to wounds and it recapitulated the accelerated wound closure previously reported in other animal models. This skin-humanized mouse model represents a useful platform to study candidate genes to improve wound healing.

Antimicrobial peptides

Infection is the major cause of skin graft failure in burn patients and sepsis increases mortality rates. Immediately after injury, gram-positive organisms usually invade the burn wound surface. Subsequently, more virulent gram-negative organisms may replace them, proliferate and disseminate to underlying viable tissues and reach the circulation. The increasing problem of multi-drug resistant bacteria limits the use of topical and systemic antibiotics to treat infection and results in secondary opportunistic infections with fungal pathogens. The most common cause of nosocomial infection in severely burned patients is Methicillin-resistant S. aureus (MRSA), multiple resistant P. aeruginosa and vancomycin-resistant Enterococci infection (Steintraesser et al., 2004). Recent studies focus on the use of novel compounds to treat infection
such as naturally occurring antimicrobial peptides. They are major components of the innate defence system from plants to vertebrates and display a broad spectrum of microbicidal activity. Antimicrobial peptides are attractive candidates for clinical development because bacteria may not easily develop resistance to them. Some of them are induced in human keratinocytes in inflammatory conditions, such as for example β-defensins 2 and 3 (Harder et al., 2001; Nomura et al., 2003) and the cathelicidin LL-37 (Frohm et al., 1997). A defect in HBD-2 expression in full-thickness burn wounds and in burn blister fluid has been previously demonstrated. This fact might influence microbial growth (Ortega et al., 2000).

Several biotechnological companies have been interested in the therapeutic application of recombinant peptides to treat infection. Some examples of the developed products are: MBI-226 for the treatment of central venous catheter related infections (Migenix Inc., Vancouver), Immuccept for the treatment of nosocomial pneumonia (Iminex Pharmaceutical Inc., Vancouver) and P-113D for cystic fibrosis (Demegen Inc., Pittsburgh). The high cost of scaling up a human quality recombinant protein makes the antimicrobial peptide gene therapy approach a promising therapeutic strategy for the treatment of infected wounds. An adenoviral vector for the delivery of LL-37 was previously employed to reverse the bacterial killing defect observed in cystic fibrosis by using a human bronchial xenograft model (Bals et al., 1999). We have also demonstrated the efficacy of adenoviral-mediated overexpression of different antimicrobial peptides in preventing bacterial growth in a human skin equivalent setting (Carretero et al., 2004). It has also been reported that transient adenoviral delivery of LL-37 is effective in reducing bacterial growth in an infected rat burn model (Jacobsen et al., 2005).

In addition to the activity of antimicrobial peptides as natural antibiotics, other actions have been described on eukaryotic cells that could be beneficial for wound repair. LL-37 is upregulated in the re-epithelializing front of human skin wounds. However, no protein expression is detected in non-healing chronic ulcers. In addition, inhibition studies using antibodies against LL-37 showed a dose-dependent impaired re-epithelialization in ex vivo wounds (Heilborn et al., 2003). LL-37 has also been shown to induce proliferation and migration of airway epithelial cells (Shaykhiev et al., 2005). Growth factors, such as IGF-I and TGF-α, as well as proinflammatory cytokines may act as inducers of antimicrobial peptide expression in wounded tissue (Sorensen et al., 2003). Interestingly, mice deficient for the murine homologue CRAMP presented a decreased vascularization during wound healing studies in skin-humanized mice. The experiments are performed in regenerated skin (12 weeks post-grafting). At day 0 excisional wounds were performed by using 2mm biopsy punches. The healing response is analyzed at day 3 post-wounding (pw). Two different protocols are used for gene transfer to human skin. Upper panel: The ex vivo approach consists of the in vitro genetic modification of human keratinocytes using retroviral vectors before grafting them as part of a skin equivalent onto immunodeficient mice. Stable GFP expression is observed in the entire graft. Lower panel: The in vivo approach consists in the direct subcutaneous injection of adenoviral vectors. Using this approach only local expression of GFP is observed around the wound consistent with the injection site. B. Immunohistochemical analyses of the reepithelialization process at different days post-wounding (pw) using an antibody directed against human involucrin. The involucrin positive labelling indicates that the closure of the wound is performed by human keratinocytes.
repair, suggesting an important role of this peptide in cutaneous wound neovascularization. LL-37 has been shown to induce angiogenesis through formyl peptide receptor-like 1 expressed on endothelial cells (Koczulla et al., 2003). Other activities of this peptide are related to its anti-endotoxic properties (Scott et al., 2002) and the involvement in the enhancement of adaptive immunity either by chemotaxing different immune cells (De et al., 2000) or modulating dendritic cell differentiation (Davidson et al., 2004). Altogether, these data support the use of an antimicrobial peptide gene therapy for wound repair, as it might control the spread of infection as well as contribute positively to tissue regeneration by acting at different stages of the wound healing process.

**Antitumoral gene therapy to treat melanoma**

Cancer is commonly defined as an illness where multiple genes and environmental factors are interacting in a complex way to give rise to the disease phenotype. Moreover, tumor generation is a multistep process, in which successive genetic changes, each conferring a growth advantage, leads to the progressive transformation of normal cells into highly malignant derivatives. These changes range from deregulated proliferation and limitless replicative potential to tissue invasion and metastasis (Hanahan and Weinberg, 2000).

Melanoma is a very severe form of skin cancer that begins in melanocytes. Although melanoma accounts for only about 4% of all skin cancer cases, it causes most skin cancer-related deaths (Hanahan and Weinberg, 2000). Current treatment of malignant melanoma using chemotherapy or high-dose of interferon and IL-2 is ineffective and associated with a high cost and toxicity (Meric et al., 2003).

The recent advances in our understanding of the functional genomics and molecular abnormalities underlying the progression of malignant melanoma, the identification of melanoma specific tumor antigens and the easy accessibility to tumor lesions have brought to the clinical trial arena the use of gene therapy as a promising strategy against this disease (Rosenberg, 1999; Hengge, 2001).

The canonical ways to targeted gene therapy aimed at melanoma cells themselves were the introduction of suicide genes, the expression of tumor suppressor genes, the inactivation of oncogenic signalling pathways and the introduction of genes encoding immunologically relevant molecules. So far, limited positive results have been reported in previous clinical trials. However, these studies have shown the feasibility and safety of this approach (Sotomayor et al., 2002).

Although these strategies directed to kill cancer cells themselves or inhibit their proliferation have been the focus of oncological treatments, recent advances have proved that the tumor environment is actively involved in the development of cancer. Tumor progression results from an imbalanced interaction of tumor cells with host cells and the extracellular matrix (ECM), in which neoplastic cells recruit vasculature and stroma through production and secretion of stimulatory growth factors and cytokines, while host-activated cells and ECM modify the proliferative and invasive behavior of tumor cells (Liotta, 2001; Liotta and Kohn, 2001). Pleiotropic molecules capable of affecting more than one component of the tumor-stroma system will be potential targets of gene therapy for the treatment of melanoma.

An interesting example of this is the glycoprotein SPARC (Secreted Protein Acidic and Rich in Cysteine). This secreted molecule is expressed during embryogenesis and tissues undergoing remodeling (Lane and Sage, 1994). SPARC interacts with several ECM components, binds and modulates the activity of specific growth factors, and regulates matrix metalloproteinase expression and activity (Bradshaw and Sage, 2001). Moreover, SPARC expression is associated with tissue remodelling processes, like wound healing and angiogenesis, both of which include physiological steps of invasive phenotypes activity (Bradshaw and Sage, 2001). Several reports associated SPARC expression with the invasive and metastasic capacity of different human cancers (Ledda et al., 1997a; Jacob et al., 1999; Briggs et al., 2002; Schultz et al., 2002; Rich et al., 2003). In addition, we have demonstrated that suppression of SPARC expression in human melanoma cells abrogated their tumorigenic capacity (Ledda et al., 1997b). SPARC is expressed not only by malignant cells but also by surrounding fibroblasts, endothelial cells and certain inflammatory cells. Interestingly, its production by stroma cells has also been associated with the neoplastic progression of tumors in which SPARC is hardly detected in the malignant cells themselves (Podhajcer et al., 1996; Brown et al., 1999; Lussier et al., 2001; Koukourakis et al., 2003). Suppression of SPARC, using adenoviral vectors carrying the antisense-RNA technology, in three different human melanoma cell lines, promoted the in vivo PMN recruitment and rejection of tumor cells in nude mice. Suppression of SPARC expression also promoted the rejection of bystander non-engineered melanoma cells in vivo and triggered the in vitro anti-tumor cytotoxic capacity of human PMN. Overall, these results suggest that SPARC produced by malignant cells might be involved in the escape of tumor cells from primary anti-tumor immune surveillance (Alvarez et al., 2005).

Another attractive case of a pleiotropic protein that may be used for improving melanoma treatment is the molecule called pigment epithelium-derived factor (PEDF). Initially identified as a neuronal differentiation factor produced by culture human retinal pigment epithelial cells (Tombran-Tink and Barnstable, 2003), PEDF has recently proved to be a potent inhibitor of angiogenesis in the eye by inducing apoptosis in actively dividing endothelial cells (Stellmach et al., 2001). The potential role of PEDF as an antiangiogenic agent in the context of solid tumors has recently begun to be explored, pointing to a more widespread angioinhibitory
role for this multifunctional factor (Crawford et al., 2001; Abramson et al., 2003; Wang et al., 2003). Studies in PEDF-knockout mice showed increased vessel density in several organs, combined with marked hyperplasia of the pancreas and prostate epithelium. Interestingly, highly tumorigenic prostate cell lines showed reduced PEDF expression compared with less tumorigenic ones. Thus, it is likely that PEDF has an inhibitory effect on prostate tumor development (Doll et al., 2003). We have recently reported that retroviral mediated overexpression of PEDF produced a dramatic direct growth inhibition of primary melanoma and metastases, which contributes to the canonical antiangiogenic effect of PEDF (Garcia et al., 2004). Our results emphasize that PEDF is targeting both the tumor cells and vasculature, leading to a more efficient blockade of tumor growth than that achieved by using purely antiangiogenic compounds.

Thus, the search for multifunctional molecules as new putative targets that may affect several tumorigenic pathways could be one of the main foci of scientific investment in oncotherapy.

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