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The skin as a vehicle for gene therapy: hemophilia B, an application model

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Abstract

Artificial skin offers important advantages in gene therapy for its biosafety and simple monitoring. An easy access of keratinocytes through small biopsies and their in vitro expansion enriched with epithelial stem cells, make them an ideal target for long-term therapeutic transgene expression. Corrective cutaneous gene therapy has been recently applied in clinical trials on dermatological genetic diseases. In systemic monogenic diseases such as hemophilia B, the graft of genetically modified skin in murine experimental models has achieved a modest increase of clotting factor IX in plasma that may attenuate severe symptoms of the disease. (Gac Med Mex. 2015;151:249-52)

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Keratinocytes as a therapeutic resource

One of the essential organs in our body is the skin, which is responsible for protection against external agents and microorganisms, as well as for keeping bodily water concentration and temperature. The predominant cells that make up the skin are keratinocytes, which have been very attractive as a target for *ex vivo* gene therapy, since they are obtainable with minimally invasive methods, their replication capability is continuous due to the presence of stem cells both *in vivo* and *in vitro,* they are easily transplantable with already well-established techniques, their gene expression is regulated, there are culture techniques by means of which they acquire their complete features by mimicking the epithelium under *in vivo* conditions (dermoepidermal equivalents), they can be secreted at the systemic level and, since the graft is easily monitorable and removable if needed, their biosafety characteristics are good.

Artificial skin, based on keratinocytes bound to different dermal bases, has been widely used for the treatment of patients with burns and ulcers, from which the development of these plates has focused on other therapeutic purposes. The use of these "full-thickness artificial skins" for transplantation warrants graft success by decreasing its fragility and improving its healing. With the development of different protocols for culture optimization, artificial skin and its genetic modification with therapeutic viral vectors offer very interesting alternatives for the treatment of genetic conditions, even non-dermatological, such as hemophilia.

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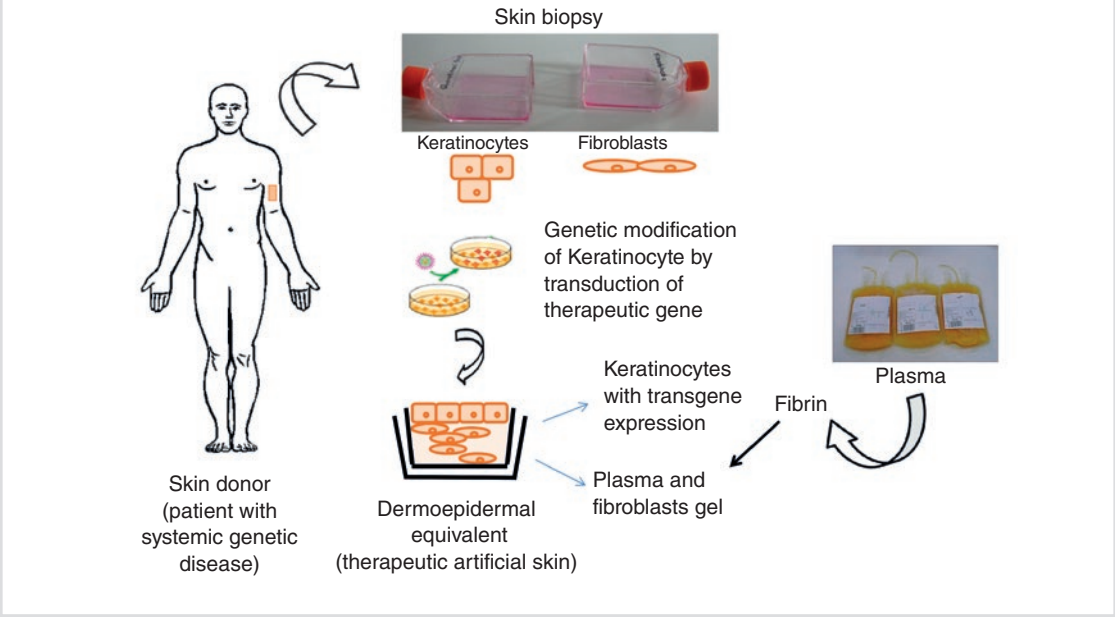

Figure 1. *Generation of therapeutic gene-producing dermoepidermal equivalents. After the establishment of fibroblasts and keratinocytes primary cultures, obtained from donor's skin biopsies, fibroblasts embedded in a fibrin's blood plasma gel form a tridimensional matrix on which genetically modified keratinocytes by insertion of the transgene are seeded, to generate the therapeutic gene-producing dermoepidermal equivalent.*

Keratinocyte culture to obtain artificial skin

The skin as a therapeutic vehicle can be produced by obtaining and handling keratinocytes from a biopsy. Both for allotransplantations (transplantations between different organisms, but from the same species) and xenotransplantations (between different species), as well as for autologous transplantations (the donor is the same as the receptor), keratinocyte culture has been developed and improved through different techniques, thus offering increasing benefits in terms of its extensive viability and attainment of typical characteristics of skin *in vivo*[1].

Dead cells are naturally detached from the skin's surface and are continuously replaced by epithelial stem cells-derived keratinocytes, which comprise a subpopulation of basal keratinocytes that have the capability of renewal and expansion. This specific type of cells has numerous advantages for its use in the production of artificial skin due to its proliferative characteristics, which contribute to prolong the life of the graft[1].

A variant of keranocyte cultures for use in gene therapy are the dermoepidermal equivalents or "artificial skins", which are made up of keratinocytes cultured in a fibrin and fibroblast matrix that results in a structure similar to the skin, with typical characteristics of each cell stratum. Dermoepidermal equivalents (Fig. 1) have particular features and benefits provided by this matrix, which favors both epithelial and mesenchimal cell migration, as in the natural healing and cell proliferation process. This way, fibrin constitutes a reservoir of different factors (cytokines and growth factors) and provides a tridimensional structure that promotes differentiation, proliferation and migration. With these properties, the use of the fibroblast and fibrin matrix allows for the use of a biopsy of small dimensions to obtain a bioengineered tissue with increased resistance, simpler handling and reduced costs[3].

This artificial skin or dermoepidermal equivalent has been assessed after being transplanted using immunohistochemical techniques, and phenotypical restoration, very similar to normal tissue, as well as synthesis of structural proteins and basal components, such as laminin, have been found. After four weeks of grafting, a structure very similar to human skin with adequate vascularization is observed *in vivo* and tissue

architecture is preserved healthy and mature for up to 16 weeks after transplantation[2].

Cutaneous gene therapy applied to skin conditions

Gene therapy in combination with tissue engineering represents a promising treatment approach for different monogenic diseases, including those directly affecting the skin integrity, such as epidermolysis bullosa, and other systemic diseases such as hemophilia.

Searching for knowledge to optimize cutaneous engineering and tissue engineering-derived products, the Del Río group[3] has conducted multiple investigations directed to cell repair and regeneration, with promising results in the development of treatment strategies with clinical application. This bioengineered skin has enabled the consolidation of a preclinical platform in gene and cell therapy that is an important alternative in the field of cutaneous regeneration, applicable to different diseases such as psoriasis, xeroderma pigmentosum, ichthyosis, and the group of ampullar conditions, including epidermolysis bullosa[1].

One of the most widely studied applications within therapy with tissue engineering is that developed in epidermolysis bullosa, a genetic-origin disease where the integrity of the skin is seriously compromised, with blisters, skin lesions and ulcers formed with the slightest trauma or friction, and even spontaneously, due to the lack of type VII collagen. The patients experience constant pain and frequent infections, among other complications; they require multidisciplinary mangement and constant surgical procedures. For this condition, gene therapy, in combination with tissue engineering, has found a very favorable therapeutic option by performing biopsies in patients with this entity and producing genetically modified dermoepidermal equivalents with insertion and expression of the deficient collagen gene. These have been grafted into mice and have shown epidermal characteristics similar to those of grafts performed with healthy tissue, without affecting the dermoepidermal junction presented by skin lacking type VII collagen.

The dermoepidermal equivalent employed as autologous transplantation has been succesfully used in the mangement of extensive burns, necrotizing fascitis, giant nevus removal, among other entities[3]. Preclinical studies of this kind have had an impact on the advance of gene and cell therapy models in monogenic diseases, both cutaneous and systemic.

Cutaneous gene therapy for systemic monogenic entities: hemophilia B as a model

Hemophilia B is one of the most common inherited bleeding disorders, caused by a functional deficiency of coagulation factor IX. Individuals with < 1% factor IX clotting activity show serious clinical manifestations with spontaneous hemorrhages since early childhood. Hemophilia B is caused by alteration of a single gene, which affects the amount or functional activity of the protein, thus making it susceptible to significant improvement with replacement therapy and, therefore, gene therapy is an alternative for its management, since a slight increase in plasma levels of the protein (1-5%) is able to decrease disease severity and reduce or eliminate spontaneous bleeding. In the development of gene therapy models, important advances have been observed in the use of skin as a bioreactor to produce therapeutic proteins that are systemically secreted, as in the case of coagulation factor IX.

With no doubt, the most significant advances in the development of the skin model for gene therapy in hemophilia B are owing to the Gerrard and Brownlee group in Oxford, England, who, since 1993, obtained the first results with the grafting of retroviral vector-transduced keratinocytes into mice until they achieved optimization with long-term expression of the transgene in 1998. In 1996, Ann Gerrard demonstrated that human keratinocytes, genetically modified with viral vectors and transplanted as grafts into mice, were able to produce human factor IX (hFIX) at the systemic level and biologically active; however, although considerable amounts were achieved *in vitro,* they turned out to be quite reduced in the systemic production *in vivo*[4].

In 1998, White et al. optimized the procedure by achieving long-term expression of hFIX owing to two fundamental factors: 1) the use of a “bi-layer” dermoepidermal equivalent produced from a primary culture of keratinocytes transduced on a fibroblast and collagen matrix, and 2) optimization of the used retroviral vector (MGF), which, by eliminating the antibiotic resistance selection gene and by modifying the clonation site of the hFIX gene, achieved a 5-fold increase in the already processed messenger RNA level, which was more efficient in plasma protein expression[5]. After producing the dermoepidermal equivalent and transplanting it in nude mice, they observed that the grafts became vascularized and incorporated to the skin of the mouse, reaching an expression between 0.1 and 2.75 ng/ml of human factor IX in plasma (potentially

feasible to reach 3% with a graft of 1.2% of the rodent's body surface area), which would allow for severity of hemophilia to be reduced to a moderate range. hFIX levels in mouse plasma stabilized at between 40 and 50 days on average after transplantation and detectable factor IX levels were present for over a year[5].

Challenges and perspectives for cutaneous gene therapy in hemophilia and other systemic entities

Even with the achievements obtained with the development of dermoepidermal equivalents demonstrating characteristics of skin *in vivo,* achieving for artificial skin formed by a "critical" amount of epithelial stem cells remains an important challenge, which could be warranted with enrichment of this cell population by means of assessment of specific surface markers. Generation of skin using a keratinocyte culture in a plasma and fibroblast matrix allows for epithelial stem cells proliferation to be favored. This model, together with a minimally invasive procedure that contemplates an autologous graft, will reduce the risks for rejection, whereas optimization of therapeutic viral vectors will be able to warrant therapeutic functionality in the long term.

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