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REVIEW

High-Level Secretion of Growth Hormone by Retrovirally Transduced Primary Human Keratinocytes

Prospects for an Animal Model of Cutaneous Gene Therapy

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Abstract

A gene therapy clinical trial for treatment of growth hormone (GH) deficiency has not been reached yet, but several strategies using different gene transfer methodologies and animal models have been developed and showed successful results. We have set up an ex vivo gene therapy protocol using primary human keratinocytes transduced with an efficient retroviral vector (LXSN) encoding the human (hGH) or mouse GH (mGH) genes. These stably modified cells presented high in vitro expression levels of hGH (7 μg/10^6 cells/d) and mGH (11 μg/10^6 cells/d) after selection with geneticin. When the hGH-secreting keratinocytes were grafted onto immunodeficient dwarf mice (*lit/scid*), hGH levels in the circulation were about 0.2–0.3 ng/mL during a 12-d assay and these animals presented a significant body weight increase ($p < 0.01$) compared to the control. Substitution of conventional grafting methodologies with organotypic raft cultures revealed a peak value of up to 20 ng mGH/mL in the circulation of grafted *lit/scid* mice at 1 h postimplantation, followed by a rapid decline to baseline (~2 ng/mL) within 24 h. One week after grafting, however, the cultured excised implants still presented approx 45% of their original in vitro secretion efficiency. Further studies are being carried out to identify the main factor(s) that still constitute one of the major impediments to the success of this promising model of cutaneous gene therapy.

Index Entries: Primary human keratinocytes; growth hormone; retroviral vector; immunodeficient dwarf mice; cutaneous gene therapy; transduced stem cells; circulatory half-life; high-level secretion.

1. Introduction

A gene therapy clinical trial for the treatment of growth hormone (GH) deficiency has not yet been set up, but several strategies using different gene transfer methodologies and animal models have been developed and showed successful results, as recently reviewed by our research group *(1)*. In this review about 40 studies were analyzed considering ex vivo and in vivo methodologies of GH gene transfer, type of vectors, cells and animals utilized, and regulation systems, to highlight the most promising approaches to establish GH gene therapy as a realistic option for patients in the near future.

Concerning gene therapy, clinical trials already approved worldwide, more than 900 trials have been carried out in 24 countries between the first performed by Rosenberg et al. in 1989 *(2)* and the beginning of 2004 *(3)*. Some of the most successful results include the treatment of severe com-

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bined immune deficiencies (SCID-X1 and ADA-SCID), from the first therapeutic trial in 1990 *(4)* to the more recent trials developed in Italy and France *(5–8)*. The initial excitement became a serious concern when a leukemia-like condition was developed in 2/11 children treated in France because of integration of the retrovirus vector near the LMO2 proto-oncogene promoter *(9,10)*. This incident, together with the death of an 18-yr-old patient in 1999 caused by an overwhelming inflammatory reaction to an adenoviral vector *(11)*, had a strong impact and a drastic reduction on the number of trials developed. In 2003, only 53 new trials were approved worldwide, the lowest number since 1996 *(3)*.

Despite these setbacks, gene therapy continues to have potential advantages, such as the possibility of circumventing repetitive injections and laborious isolation and purification steps required for a recombinant protein, providing, in principle, a mechanism of protein delivery and regulation, which may closely resemble the physiological process *(1,12–15)*. These points can thus create conditions for applying this relatively new strategy to many human disorders for which the conventional treatment is still nonexistent, too expensive, or unsatisfactory.

As in any other experimental procedure, with inherent successes and failures, gene therapy is gradually restoring its confidence based on recent studies *(16)*. One of the most interesting results was the first nonviral somatic cell gene therapy trial in patients with hemophilia A, in which partial correction of this genetic disorder was obtained without serious side effects *(17–18)*. Another case of success was related to the use of cutaneous gene therapy to treat devastating inherited skin disorders, especially junctional epidermolysis bullosa and xeroderma pigmentosum, which have entered clinical trials based on reconstitution of a new dermis consisting of autologous keratinocytes transduced by retroviral transfer of the corrective gene (laminin-5) and autologous fibroblasts *(19–21)*. These trials also suffered a halt after the described adverse events and were resumed in 2004 *(22)*.

In the present, work we intend to review the results already obtained with epidermal keratinocytes transduced with the GH gene, including our studies which led to a high-level secretion in vitro, using an efficient retroviral vector (LXSN) encoding the hGH or mGH genes. These studies, together with a determination of the percentage of transduced stem cells and of the circulatory half-life of mGH and hGH in mice, are being carried out to identify the main factor(s) that still constitute one of the major impediments (i.e., poor sustainability of transgene secretion in vivo) to the success of this promising model of cutaneous gene therapy.

2. Grafting of GH-Secreting Keratinocytes Onto Different Animal Models

Epidermal keratinocytes, the main cell of skin, are considered an attractive target population for gene transfer because they can be serially propagated in culture *(23)* and their transplantation is already optimized *(24–26)*. These cells may be engineered as bioreactors to secrete gene products that have local or systemic effects *(27,28)*. In the latter case, proteins secreted by genetically modified keratinocytes could reach the circulation via a mechanism resembling the natural process *(12–14,29,30)*.

Keratinocytes are generally used in ex vivo gene therapy strategies and the main studies carried out on GH gene transfer using these vehicles are presented in **Table 1**. As we can observe, the achievement of a sustainable transgene secretion in vivo is one of the principal obstacles with these cells and it is a factor that still seriously hampers their utilization in the therapy for GH deficiency. TABLE 1

Morgan et al. *(31)* carried out one of the first studies using human primary keratinocytes transduced by a retroviral vector encoding the hGH gene and the in vitro secretion rate was 0.072 μg hGH/10^6 cells/d *(32)*. Despite the fact that no hGH was detected in the circulation of the grafted athymic mice, the authors emphasized that the cultured keratinocytes reconstituted a normal epidermis which was then able to undergo terminal differentiation. An established keratinocyte cell

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Table 1
Overview of the Main Growth Hormone Gene Therapy Studies Based on Epidermal Keratinocytes

Reference	Vector (promoter)	Target cell	Animal	In vitro expression (mg/10^6 cells/d)	GH circulatory levels (ng/mL)	In vivo duration (d)
31	DOL-hGH[a] (LTR)		nude mice	0.072		
32	pTKGH (TK)[c]					
			nude mice	5.3	0.6–1.0	28
34	EBV-based (SV-40)		nude mice	0.7	2.6 (n = 1) 0.6 (n = 5)	4
35	a-SGC-hGH[a]	pig kerat.	pig	~3 ng/mL	0.35[d]	10
15	pLhGHSN[a] (LTR)	HPK[b]	lit/scid mice	7	0.2–0.3	
Peroni, in preparation	pLmGHSN[a] (LTR)	HPK[b]	lit/scid mice	11	20	

AU please update Peroni and add to list

[a]Retroviral vector. [b]Human primary keratinocytes. [c]Thymidine kinase promoter. [d]In wound fluid.

line (SCC-13), derived from a squamous carcinoma of epidermis *(33)*, was then transfected by the same research group with the hGH gene inserted in a nonviral vector *(32)*. Although a relatively high expression of 5.3 μg hGH/10^6 cells/d was obtained in vitro, the levels of the secreted hormone in circulation in the mice were not constant, declining from initial values of approx 0.6–1.0 ng/mL to undetectable values 4 wk postgrafting.

Using primary human keratinocytes transfected via lipofection with an expression vector carrying the hGH gene regulated by the SV40 promoter, Jensen et al. *(34)* did not get secretion of the hormone for more than 4 d, even though they obtained the highest hGH circulatory level reported for grafted keratinocytes at that time (up to 2.6 ng/mL for n = 1 mice with an average of 0.6 ng/mL for n = 5 mice). At day 10, though, the circulatory levels were undetectable in all five mice, matching the loss of the β-galactosidase reporter gene that occurred in a parallel experiment. In another study *(35)*, keratinocytes derived from pigs were retrovirally transduced with the hGH gene and transplanted into pig wounds creating an in vivo cell culture system. Human GH was detected for 10 d in the wound fluid with a peak value of 0.35 ng/mL on day 6. The disappearance of the hormone from the fluid on day 10 coincided with the reestablishment of an epithelial barrier that could prevent its diffusion. The authors suggested that this model could be useful for healing wounds in which only a temporary production of growth factors or therapeutic proteins is required.

In our laboratory we have also set up an ex vivo gene therapy protocol using primary human keratinocytes transduced with an efficient retroviral vector (LXSN) based on the LTR promoter *(15,36)* and encoding the hGH or mGH genes. These stably modified cells presented high in vitro expression levels of hGH (7 μg/10^6 cells/d) and mGH (11 μg/10^6 cells/d) after selection with geneticin. We observed, however, that these epidermal sheets prepared following a classical method *(37)* displayed a secretion loss >80% already in vitro, simply a result of the enzymatic detachment of the epithelium from its substratum by using dispase as reported by other authors *(32)*. The same problem was also observed when a similar strategy with the mGH gene was used, in an attempt to study a more homologous system. Nonetheless, when the hGH-secreting keratinocytes were grafted onto immunodeficient dwarf mice (*lit/scid*), hGH levels in the circulation did not go below 0.2–0.3 ng/mL during a 12 d assay (peak value, 1.5 ng/mL at 4 h) and these animals presented a significant body weight increase ($p < 0.01$) compared to the control *lit/scid* mice implanted with nontransduced keratinocytes *(15)*.

Considering that most of GH secretory power of our epidermal sheets was already lost before in vivo grafting, we found that this loss of secretion could be completely circumvented by substituting the classical grafting technique by the construction of an organotypic raft culture *(38,39)*. Grafts of such cultured cells on *lit/scid* mice could be followed for more than 4 mo, but a significant weight increase relative to the control groups (animals implanted with nontransduced keratinocytes) could only be obtained during the first 40 d ($p < 0.05$). A study of the mGH levels in the circulation of grafted animals revealed a relatively high peak value of up to 20 ng mGH/mL at 1 h postimplantation, followed by a rapid decline to baseline (~2 ng/mL) within 24 h. One week after grafting, however, whole excised implants from sacrificed mice showed an average secretion of 191 ± 84 ng mGH/implant/d, which represented 45% of the average value of 424 ± 271 ng/d obtained for three analogous cultures kept in vitro for the same period of time **(Table 2)**, showing that the in vivo process does not shut off hGH expression, nor inactivates the promoter. We could also observe that trypsinization, as was done by Kolodka et al. *(39)* with the same type of cells, reduced even more mGH secretion but did not suppress it. This in vivo persistence of human cells is in the process of being confirmed by an immunostaining methodology for a human specific protein, e.g., involucrin.

TABLE 2

Table 2
mGH Levels Determined in Conditioned Medium (24 h) From Organotypic Sheets Maintained In Vitro or Recovered From Grafted *lit/scid* Mice

Organotypic raft culture condition	ng mGH/mL[a]	ng mGH/implant[a]
in vitro	212 ± 135	424 ± 271
grafted and excised	95.6 ± 41.7	191 ± 84
in vitro + trypsin	170 ± 31.2	340 ± 62
grafted and excised + trypsin	43.5 ± 38.1	89 ± 15.6

[a]Average mGH secretion ± SD; each value is derived from n = 3 culture dishes.

3. Determination of the Percentage of mGH-Transduced Stem Cells

Considering that transduction of stem cells seems to be a key requirement for sustained transgene expression in vivo *(29,40,41)*, we decided to determine the percentage of mGH-transduced stem cells in our keratinocyte population. This will provide a better control on the quality and reproducibility of our grafts and possibly will open the way to keratinocyte stem cells enrichment. The followed methodology *(39)* is based on the general agreement that an epidermal keratinocyte stem cell would exhibit clonogenicity and long-term growth potential in vitro *(42)*. Briefly, the mGH-secreting keratinocytes were seeded at low densities (~500 cells/10-cm culture dish) and colonies with the diameter of ~5 mm were isolated with cloning rings, trypsinized and expanded by serial passage. Cells were passaged four times (approximately one passage/week), counted, and the medium was collected for mGH determination by radioimmunoassay **(Fig. 1)**. Clonal analysis revealed that, after four serial passages (equivalent to >30 cumulative cell doublings or $>10^7$ cells), approx 30% (7 out of 24) of the isolated clones maintained or even presented an increased mGH expression. This percentage would represent thus the fraction of transduced keratinocyte stem cells, confirming our previous determination and possibly showing a small improvement compared to the value of 21.1–27.8% β-galactosidase transduced stem cells reported by Kolodka et al. *(39)*.

FIG 1

4. Determination of mGH and hGH Circulatory Half-Life ($t_{1/2}$) in lit/lit Mice

Because the circulatory half-life ($t_{1/2}$) is another parameter that can influence sustained in vivo secretion and the circulatory level of systemic proteins *(43,44)*, we carried out this determination for mGH and hGH in the little mouse. This was undertaken to find out if there was a significant difference between mGH $t_{1/2}$ and hGH $t_{1/2}$ in the little mouse. The mice were intravenously injected by tail vein with 1 µg of the respective hormone and the variation of circulatory levels with

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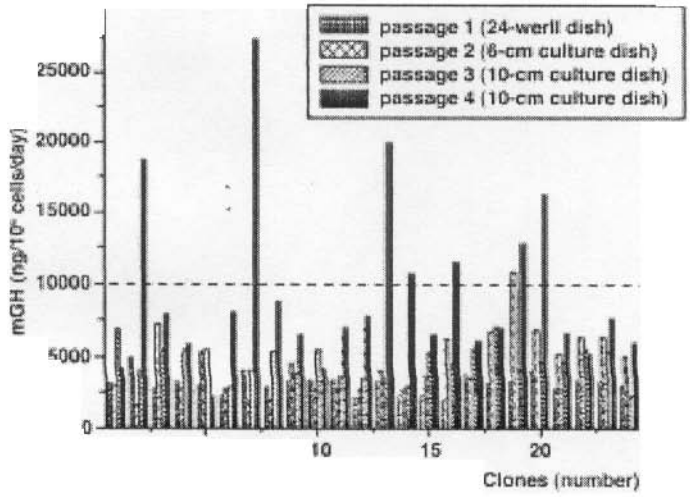

Fig. mGH levels obtained in vitro by clones isolated from a transduced keratinocyte population.

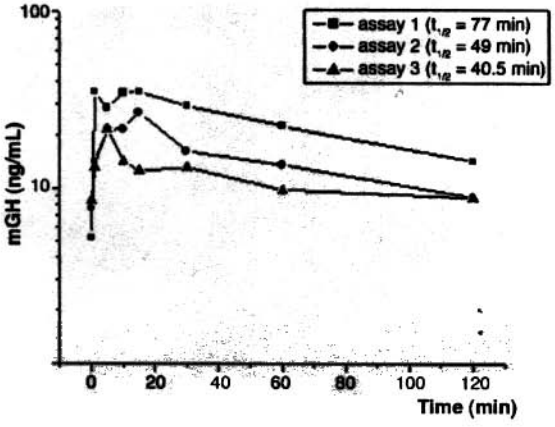

Fig. 2. mGH levels in sera from *lit/lit* mice (n = 4 animals/time) after intravenous administration of 1 µg mGH. Blood was removed from eye vein and mGH concentration was determined by radioimmunoassay. Values of circulatory half-life ($t_{1/2}$) obtained in three different assays are shown.

time was used to calculate the $t_{1/2}$. An example of the curves obtained in the assays using mGH is presented in **Fig. 2** and an average of $t_{1/2} = 55.5 \pm 19.1$ min ($n = 3$) was calculated for this hormone, against a $t_{1/2} = 39.5 \pm 2.1$ min ($n = 2$) for hGH determined previously in similar experiments.

FIG 2

The difference between the two hormones was, however, statistically insignificant ($p = 0.24$) and indicated that the utilization of mGH in mice will probably not significantly improve its $t_{1/2}$ in comparison with the utilization of hGH.

5. Conclusions and Future Trends

Based on the obtained results we can conclude that:

1. The rapid decline of circulatory levels of our transgene products expressed by genetically modified primary human keratinocytes can be attributed to several causes described in the literature. We are establishing an immunostaining methodology for a human specific protein (involucrin), to confirm the presence of human cells. The persistence of hormone secretion from the excised implants, however, already suggests this presence.
2. Clonal analysis of colonies isolated from transduced keratinocytes revealed that, after four serial passages (equivalent to >30 cumulative cells doublings or $>10^7$ cells), approx 30% of those clones maintained or even presented an increase in mGH expression. This percentage thus represents the fraction of keratinocyte stem cells that have been transduced, confirming previous results and literature data.

3. In the experiments carried out to determine mGH circulatory half-life ($t_{1/2}$) in the little mice, an average $t_{1/2} = 55.5 \pm 19.1$ min (n = 3) was obtained for mGH, against a $t_{1/2} = 39.5 \pm 2.1$ min (n = 2) for hGH obtained in previous assays. The difference between the two hormones was, however, statistically nonsignificant ($p = 0.24$) and indicated that this parameter is not responsible for the low circulatory levels of our transgene.

In conclusion, these studies are being conducted to identify the main factor(s) that still constitute one of the major impediments (i.e., limited sustainability of the transgene secretion in vivo) to the success of this promising model of cutaneous gene therapy. In our opinion, the loss of circulatory levels of the transgene produced by genetically modified primary human keratinocytes could still be owing inefficient transport of the protein from a subcutaneous site to the bloodstream, binding to endothelial cells or even to the effect of a barrier formed by the basement membrane that exists between epidermis and dermis. The immunogenicity of the vector elements and the type of cells producing the transgene cannot be totally excluded because scid mice might spontaneously develop a partial immune reactivity ("leakiness"). Naked DNA experiments could help to investigate these possibilities.

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