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Different *ex Vivo* and Direct *in Vivo* DNA Administration Strategies for Growth Hormone Gene Therapy in Dwarf Animals

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1. Introduction

Recombinant growth hormone (GH) was one of the first proteins to be synthesized via DNA recombinant techniques in the late seventies. It was also one of the first proteins to be used in studies of animal models for gene therapy, already in the eighties. This was due to the real therapeutic need for GH, combined with the fact that its detection by well-known immunoassay methods is facile and sensitive. Moreover, evident phenotypic effects can be observed and measured in several animal models (e.g., dwarf mice), some of which have GH deficiencies that closely resemble their human counterparts.

GH gene therapy has the potential advantage of circumventing laborious and expensive purification processes, quality control procedures and the repetitive injections that are required in the conventional treatment. The ideal situation would, of course, be to introduce the deficient protein into the circulation via a mechanism that resembles the natural process. These treatments have not yet reached the clinical stage for humans, the major challenge being to achieve a sustainable and regulated *in vivo* GH secretion. However, several interesting and promising *ex vivo* and direct *in vivo* DNA administration strategies for GH gene therapy have been developed and studied using animal models. These studies obviously open the way for the systemic delivery of other therapeutic proteins in addition to GH.

The various *ex vivo* models for GH gene therapy are based on the use of target cells, such as keratinocytes, fibroblasts, endothelial cells, peritoneal mesothelial cells, or skeletal myoblasts. These cells can also be encapsulated to prevent rejection when implanted in allogeneic hosts. The majority of the methodologies are carried out via cell isolation and *in vitro* cultivation, genetic modification by viral or non-viral vectors containing the GH gene and re-implantation of the secreting cells onto the animal.

Primary keratinocytes are one of the most attractive vehicles for gene transfer and gene therapy. They are among the most accessible cells in the body and can be serially propagated in culture; the procedure for their transplantation is already well established, e.g., for burn patients, and the therapy can be reversed by excision of the genetically modified tissue. Cutaneous gene therapy has already been demonstrated to be a powerful

tool for the successful treatment of severe skin disorders such as epidermolysis bullosa ¹. Keratinocytes can also act as cellular bioreactors, secreting GH or other proteins systemically. When primary human keratinocytes were retrovirally transduced with the human (hGH) or mouse (mGH) growth hormone genes in our laboratory, they exhibited high and stable *in vitro* secretion. When these hGH-secreting keratinocytes were grafted onto immunodeficient dwarf mice (lit/scid), a significant body weight gain was obtained, despite low hGH serum levels. When the conventional epithelial sheet grafting methodology described by Ian Barrandon and coworkers in 1988 ² was replaced by the implantation of an organotypic raft culture, peak values of up to 20 ng mGH/ml were observed in the circulation of lit/scid mice. However, this was followed by a rapid decline of the levels to a baseline value of ~2 ng/ml ³. For comparison, the mGH serum concentration in heterozygous lit/+ mice is of the order of 6 ng/ml.

Among the various *ex vivo* methodologies for GH gene therapy, the implantation, by a Canadian research group ⁴, of encapsulated mGH-secreting myoblasts into the peritoneal cavity of Snell dwarf mice should be mentioned. Using this procedure, the recombinant allogeneic cells were protected from rejection and remained functional for at least 6 months. The animals showed a remarkable increase in body weight and the increase in body length doubled compared to the control group.

The GH gene therapy approach based on *in vivo* gene transfer via adenoviral injection developed by a Houston (TX) group led to a complete phenotypic correction of dwarfism in little mice (lit/lit) ⁵. Nonetheless, this strategy suffers from several potentially serious problems that require more profound investigation prior to its adaptation to human therapy. One of these problems is the high expression of the transgene, mostly by hepatocytes, leading to continuously elevated GH and IGF-I circulating levels that may cause dysfunctions or cancer.

A much better option would appear to be the direct administration of naked plasmid DNA, a methodology that has been successfully adopted by several authors in the last decade. Analysis of growth parameters such as body weight, organ weight (quadriceps muscles, liver, kidneys, heart and spleen) and total (nose-to-tail) length of the animals have been used to study the endocrine and local (autocrine/paracrine) effects of hGH in greater depth after intramuscular DNA administration in immunodeficient dwarf mice (lit/scid) ⁶. Although the majority of the strategies described for GH gene therapy are still limited by the absence of an appropriate mechanism for regulating *in vivo* hormone expression and secretion, a number of factors favor *in vivo* drug generation as the best alternative. In particular, this would avoid costly industrial productions, very expensive treatments and would improve the quality of life of patients (children and adults) suffering from GH deficiency (GHD).

In this chapter, we review the results reported in five studies from our laboratory, primarily involving *ex vivo* methodologies based on primary human keratinocyte retroviral transduction and direct plasmid DNA administration in immunodeficient (lit/scid) and immunocompetent (lit/lit) dwarf mice. We also analyze the data reported in nine studies by seven other research groups using *ex vivo* techniques based on transduced fibroblasts and myoblasts and *in vivo* methodologies based on direct adeno and adenoassociated viral vectors or on naked DNA administration. The animal models used by these authors include lit/lit, Snell dwarf mice, hypophysectomized rats and mice, growth hormone-releasing hormone knockout (GHRHKO) mice and growth-retarded swine.

2. *Ex vivo* strategies

In 1995, the Canadian group mentioned above reported that the growth defect of dwarf mice (Snell dwarf) could be partially corrected by implanting microencapsulated allogeneic myoblasts engineered to secrete mouse growth hormone. The encapsulation of these mouse myoblasts into GH-deficient Snell dwarf mice provided a completely homologous system⁴. The plasmid pKL-mGH, encoding mGH cDNA under the regulation of human β -actin promoter and also containing the neomycin resistance gene, was used to transfect the mouse myoblast cell line C2C12. G418 resistant clones were selected and screened for the level of mGH in the culture medium. Clone Myo-45, which secreted 147 ng of mGH/ 10^6 cells/day, was selected for encapsulation. Microcapsules were implanted via a 22G catheter into the peritoneal cavity and, by the end of the 3rd week, the body weight of the dwarf mice had increased about 1.6-fold and the increase in body length had doubled compared to the control group. There were also significant increases in the levels of non-esterified free fatty acids (a measure of the lipolytic effect of the capsule-delivered mGH), while peripheral organ weights and tibial growth plate thickness were also significantly greater. The authors hypothesized that most of the capsule-derived mGH was sequestered in the liver through the hepatic GH receptors, inducing the secretion of hepatic insulin-like growth factor I (IGF-I), which mediates most of the GH-dependent systemic metabolic effects. After 5 weeks, however, a lack of further growth in weight or length was evident in all of the mice. According to the authors, this was not due to the absence of mGH transgene expression by the encapsulated cells, but rather to the non-responsiveness of the mice to the hormone at this age (13-15 weeks). In fact, a second implantation of freshly-prepared capsules on day 42 did not result in any further growth enhancement. Moreover, encapsulated cells retrieved at the end of the experiment (178 days) continued to secrete *in vitro* >200 ng/ 10^6 cells/day of mGH. Interestingly, all the different groups of dwarf mice had low or undetectable GH levels in their plasma, i.e. 1.32-1.42 ng/ml at best, comparable to the detection limit of ≤ 0.62 ng/ml of the mGH RIA. The authors' assumption was that the capsules probably delivered circulating levels of mGH below the detection limit of the assay. Nonetheless, this study demonstrated the clinical efficacy of this non-autologous system and its potential for widespread application in therapies requiring a continuous systemic supply of recombinant gene products.

Fibroblasts are also a potentially interesting cell type for *ex vivo* gene delivery because of their easy accessibility, facile culture *in vitro*, convenient re-implantation and their potential for delivering proteins to the circulation. In 1995 a research group at the Taiwan National University employed hypophysectomized rats as an animal model to explore the feasibility of using genetically engineered fibroblasts for growth hormone gene therapy⁷. A bicistronic retroviral vector controlled by the LTR promoter, which contained a porcine growth hormone (pGH) cDNA at the first cistron and a neomycin resistance gene at the second cistron, was used to infect primary rat embryo fibroblasts. The transduced cells exhibited an *in vitro* expression level of up to 1.18 μ g pGH/ 10^6 cells/day and 5×10^6 cells/animal were injected directly into the peritoneum of hypophysectomized rats, leading to a significant growth of the tibia when tested at days 15 and 57 post-implantation. Alternatively, 1×10^6 cells were cast in collagen matrices (0.5 cm in diameter) and implanted underneath the skin on the back of the rats: a semi-quantitative RT-PCR confirmed that the pGH expression by these cells lasted up to 70 days. Unfortunately, a sensitive ELISA system was not available and pGH could not be detected in the sera of the animals. However, the tibia growth

bioassay in hypophysectomized rats did provide clear evidence for the *in vivo* activity of pGH in a direct functional assay and confirmed that fibroblasts are indeed capable of persistently expressing foreign genes *in vivo*.

The same Chinese research group subsequently used primary porcine fetal fibroblasts, transduced with the same pGH-carrying vector described above, to enhance the weight gain of growth-retarded Tao-Yuan Swine, a local breed in Taiwan that is slow growing and fat, but palatable⁸. Immortalized fibroblasts were avoided because of their tumorigenic potential, even though they have been used quite successfully in several different studies in mice. The transduced primary cells were encapsulated with the same type of alginate-poly-L-lysine-alginate membranes used previously for mice myoblasts⁴ and then implanted into the peritoneal cavity of the swine, resulting in a significant increase in weight gain already on day 16 post-implantation, even though no increase in serum pGH could be detected. The use of immunoprotective microcapsules thus constitutes a simple method of delivering recombinant genes *in vivo* and proved to be a valid approach for the improvement of the growth of these animals. Since the microcapsules obviate the need for patient-specific *ex vivo* preparations and are amenable to industrial-scale production and quality control, this approach is also potentially economically viable.

Our research group has focused its *ex vivo* activities mainly on the use of human primary keratinocytes that were retrovirally transduced with the human (hGH) or the mouse (mGH) GH genes, implanting them into immunodeficient dwarf (lit/scid) mice^{3,9-11}. Epidermal keratinocytes were chosen as the target of our gene therapy methodology because they are among the most accessible cells in the body and can be serially propagated in culture, following the pioneering work of Rheinwald and Green¹². When these cells were stably transduced with the hGH gene (under control of the retroviral LTR promoter), a high *in vitro* secretion of up to 7 μg hGH/ 10^6 cells/day was obtained⁹. Their grafting onto lit/scid mice led to circulating hGH serum levels of 0.2-0.3 ng/ml during a 12-day assay (peak value, 1.5 ng/ml at 4h). Grafted mice also showed an increase in body weight (0.060 g/animal/day), which was significantly higher ($P < 0.01$) than that of the controls (0.023 g/animal/day). This was the first time that continuous *in vivo* secretion of the hormone and subsequent phenotypic alterations due to the grafting of transduced, hGH-secreting primary human keratinocytes had been demonstrated in lit/scid mice, an animal model known to be very sensitive to low concentrations of hGH¹³.

Conventional epidermal sheets of these mGH-secreting keratinocytes, prepared by us using the classical technique of Barrandon et al.², showed a drop in secretion rates of >80% simply due to detachment of the epithelium from its substratum. Replacement of this conventional grafting methodology by organotypic raft cultures¹⁴ completely overcame this problem. Employing a similar *ex vivo* strategy and this modification of the grafting resulted in a very high, stable *in vitro* secretion of up to 11 μg mGH/ 10^6 cells/day. Moreover, the amazing value of ~ 26 μg mGH/ 10^6 cells/day could be reached by clonal selection¹¹, performed by seeding the mGH-secreting keratinocytes at low densities (~ 500 cells/10 cm culture dish) and isolating colonies with a diameter of ~ 5 mm by the use of cloning cylinders. Cells were expanded four times by serial passage (approximately one passage/week), counted and the medium collected for mGH determination by radioimmunoassay. Clonal selection revealed that, after four serial passages (equivalent to > 30 cumulative cell doublings or > 10^7 cells), approximately 30% (7 out of 24) of the isolated clones maintained or presented increased mGH expression. This percentage represents the fraction of transduced keratinocyte stem

cells present, a very important parameter for attaining the desired persistent gene therapeutic effect. The stable long-term grafting of such cultures onto *lit/scid* mice could be followed for more than 4 months, with a significant weight increase over the control group being observed in the first 40 days. Circulating mGH levels reached a peak of 21 ng/ml just 1h after grafting but, as already mentioned, these levels fell rapidly (in ~24 h) to baseline values³. Thus, in this study, an animal model for cutaneous gene therapy based on *lit/scid* mice and on the grafting of organotypic raft cultures was developed and the mGH-secreting primary human keratinocytes employed exhibited the highest *in vitro* secretion ever reported for any form of GH in these cells. However, the desired sustainability of the *in vivo* secretion of the transgene was not achieved.

Several hypotheses can be raised as the possible cause(s) of this immediate suppression or blocking of exogenous GH in the circulation of *lit/scid* mice after the grafting. These include: (i) a limited mGH circulatory half-life in *lit/scid*; (ii) a rapid clearance from the bloodstream due to a specific binding or selective transfer; (iii) impediments due to poor vascularization, to a fast inflammatory process or to an unidentified specific barrier; (iv) the occurrence of extremely efficient apoptotic events, transgene inactivation or promoter failure; (v) partial immune reactivity spontaneously developed by the immunodeficient mice, leading to some production of B and T cells (i.e., "leakiness"). Although several of these hypothetical mechanisms could be rationally or even experimentally excluded, so far we have been unable to positively prove the existence of any one of them. This led us think that comparative tests carried out by injecting mGH-expressing naked DNA, enhanced by *in vivo* electroporation, at approximately the same epidermal site as keratinocyte grafting, might prove to be informative. This resulted in studies from our laboratory of the *in vivo* administration of naked DNA to *lit/lit* and *lit/scid* mice, with the goal of comparing the circulating hormone levels obtained by this type of GH delivery with those obtained via implantation of GH-secreting cells.

3. *In vivo* approach

Adenovirus administration is an effective way of delivering therapeutic genes since they can be produced and purified in a concentrated form, which facilitates *in vivo* delivery. Another advantage is that potential oncogenic effects resulting from viral integration into critical chromosomal regions are unlikely because adenoviruses do not integrate into host chromosome DNA⁵. Since it is well-known that adenoviruses can elicit multiple innate immune responses after systemic administration, there are several approaches under investigation that attempt to overcome this limitation. These include modification of the adenoviral vector design, the use of tissue specific promoters and local administration routes and the utilization of immunosuppressive drugs or specific compounds to block important immune pathways known to be induced by adenovirus¹⁵.

Concerning GH deficiency and dwarf animals, an adenoviral vector containing rat GH (rGH) cDNA was used in 1996 by the Houston group mentioned above to induce constitutive GH expression in hepatocytes of GH-deficient *lit/lit* mice⁵. When the recombinant adenoviral vector, controlled by the human elongation factor 1- α (EF1 α) promoter, was administered via the tail vein of this animal at a dose of 10^8 pfu (plaque-forming units), high levels of GH were detected in the serum for at least 7 weeks. This viral dose led to an unbelievably high peak of the serum GH level of 1.9 $\mu\text{g/ml}$, which then decreased to ~125 ng/ml during the next 2 weeks, remaining at that level for the duration of

the experiment (7 weeks). A viral dose of 10^9 pfu induced an even higher serum GH level of ~ 35 $\mu\text{g}/\text{ml}$, which then decreased to ~ 2.5 $\mu\text{g}/\text{ml}$ over the next two weeks and stabilized at 1 $\mu\text{g}/\text{ml}$ for the duration of the study. The authors attributed the decrease in GH expression in part to the ability of the host's immune system to recognize and subsequently eliminate the virally transduced cells. Little dwarf mice treated with 10^8 pfu of rGH adenovirus showed an increase in circulating IGF-I levels (from 61 to 238 ng/ml in 3 weeks). Although the serum GH levels increased dramatically, the serum IGF-I levels did not follow the same pattern. This may be due to the presence of reduced IGFBP-3 in GH-deficient humans and animals. A rapid weight gain, resulting in a body weight comparable to that of normal age-matched lit/+ animals, was achieved by 5 weeks of treatment. Total body length was also indistinguishable from that of lit/+ mice within 7 weeks of treatment and body composition was normal, with a reduction in the percentage of fat and increase in the water and protein contents. The animals treated with rGH adenoviruses exhibited slight, but significant, increases in liver and kidney sizes and a tendency to increase fasting blood glucose and insulin levels, which are known effects in response to prolonged exposure to high levels of GH. It was suggested, however, that the induced hepatic and renal hyperplasia warranted further investigation. According to the authors, the little mice dwarf phenotype could be corrected, with minimal side-effects, by constitutive GH expression achieved through *in vivo* recombinant adenoviral delivery. Because the correction of GH insufficiency by gene transfer may represent in the future an alternative to the conventional treatment, the importance of this work is undeniable since it was the first study to demonstrate a complete phenotypic correction of dwarfism. Nonetheless, a constitutive adenoviral-induced GH expression in hepatocytes producing extremely high GH serum concentrations, would meet serious obstacles to its application in humans.

In a subsequent study, carried out in 1999 by groups at the National Institute of Health (NIH, Bethesda, MD), an adenovirus encoding mGH cDNA was injected into the quadriceps muscle or submandibular ducts of mGH-deficient Snell dwarf mice to obtain a homologous system and thus avoid possible side effects resulting from species differences¹⁶. When the adenoviral vector was used *in vitro* to infect SMIE (submandibular immortalized epithelial) cells, an epithelial cell line derived from the adult rat submandibular gland, the highest *in vitro* expression level obtained was ~ 184 ng mGH/ml at 72h post infection for cells infected at a MOI (multiplicity of infection) of 300. To test its *in vivo* efficacy, the virus was injected into the quadriceps muscle of Snell dwarf mice (5×10^9 pfu/animal), resulting in an average serum level of 42 ± 29.7 ng mGH/ml at 4 days post injection. Submandibular administration provided widely varying serum levels of 64.1, 3.4 and 11 ng mGH/ml in three individual animals. The intramuscular protocol was more efficient, providing an average 8% increase in body weight of the dwarf mice just 4 days post treatment and close to 100% by 30 days. This indicates that skeletal muscle gave a fairly stable transgene expression and suggests that it can be a highly useful tissue for the delivery of circulating proteins. To obtain enough serum to determine the various metabolic parameters, the adenoviral vector was also administered to young rats via the intravenous route. The main effects were an increase of $\sim 35\%$ in serum IGF-I levels, $\sim 60\%$ in cholesterol and $\sim 40\%$ in triglycerides, all changes that are consistent with systemic mGH action and anabolic effects. The mice and rats utilized in these studies received dexamethasone to limit the host immune response of these immunocompetent animals. The authors concluded that the adenoviral vector could be a valuable tool in preclinical mouse model studies. Even though administration via muscle is

somewhat milder than via other tissues (e.g., the liver), we believe that most of the limitations related to adenoviral gene therapy are still present in this approach.

More recently, in 2008, a new generation of double-stranded adeno-associated viral vectors (dsAAV) encoding mGH cDNA driven by a universal promoter (CMV) were used by a group coordinated by Johns Hopkins University School of Medicine to prepare viral particles that were injected into GHRHKO mice, a model of isolated GH deficiency due to generalized ablation (knock-out, KO) of the GHRH gene^{17,18}. These genetically modified dsAAV can infect dividing and non-dividing cells *in vitro* and *in vivo* with a long-term transgenic expression (up to 1 year) and elicit a lower toxicity and cellular immune response, therefore being generally considered to be safer than adenoviral vectors.

In an initial study, GHRHKO mice were injected intraperitoneally with either a single dose (low dose) or two doses (high dose) of 1×10^{11} viral particles at the 10th and 11th days of age and were followed up to the 6th or 24th week of life¹⁷. Body weight and length of both viral-treated groups became normal at 6 months of age and normal femoral and tibial lengths, body composition and weight of organs (liver, spleen, heart and kidney) were also obtained. At week 6, serum GH levels were higher in mice receiving both virus doses compared with controls, while they were normal at week 24. This is consistent with the results of previous studies showing that long-term expression by this type of viral vector is limited to the liver and skeletal and cardiac muscle. This was confirmed by the detection of GH mRNA in these same tissues of the GHRHKO mice. Nevertheless, serum IGF levels were significantly higher in both virus-treated groups compared to the control group at week 24, showing that the expressed GH is still functional in GH-deficient and immunocompetent mice and that no resistance to its effect was developed over time, since a species-specific GH cDNA was used. The use of a universal promoter is obviously a limitation for any clinical application of this approach because of the well-known long-term risks associated with excessive unregulated GH secretion. The authors concluded that, while the applicability of these findings is still very far from any possible clinical trial in humans, these new AAV vectors offer a good starting point for the development of novel regulated viral gene delivery systems for GH administration. Such systems could be based on the use of inducible promoters that can be regulated at will or of tissue-specific promoters, providing good systemic delivery together with limited local expression.

In a subsequent study, the same group utilized a dsAAV expressing mGH cDNA under the control of a muscle creatine kinase regulatory cassette in order to ensure adequate systemic delivery in conjunction with muscle-specific expression¹⁸. A low-dose (0.5×10^{11} pfu) and a high-dose (1×10^{11} pfu) of virus were injected into the right quadriceps muscle of GHRHKO mice at the age of day 10. Virus-injected GHD mice showed a significant ($P < 0.05$) increase in body length and weight, however without becoming fully normal, and a significant ($P < 0.05$) reduction in visceral fat at week 6 of age. Quantitative RT-PCR showed that GH mRNA expression in the quadriceps muscle of animals treated with the high-dose of virus was significantly higher than in the gastrocnemius and cardiac muscles or the kidney and liver of the same mice. At 6 weeks of age, serum GH and IGF-I levels in both treated groups were not significantly higher than those in the control mice. This study showed that, although the strategy of vector-mediated GH therapy is still not applicable at the clinical stage, systemic GH delivery to GHD animals is possible via a single injection of viral particles derived from dsAAV using an approach that limits GH expression to skeletal muscle. In fact, it is known that widespread gene expression can result in toxicity¹⁹.

An alternative system for delivering genes *in vivo* is the administration of naked plasmid DNA. When carried out in certain tissues, particularly muscle, this is considered to be a practical method that can produce significant levels of gene expression, although lower than those achieved with viral vectors. The simplicity of this methodology has made it the most utilized non-viral system in clinical trials of gene therapy, representing as much as 18% of current trials ²⁰.

A research group from a company in Texas has been a pioneer in GH naked DNA administration. They designed a muscle-specific gene medicine, composed of a hGH expression plasmid containing the chicken skeletal α -actin promoter complexed with a protective, interactive, non-condensing (PINCTM) delivery system, to be administered intramuscularly in hypophysectomized rats ²¹. This polymeric PINC gene delivery system consists of polyvinylpyrrolidone (PVP), which protects plasmids from extracellular nuclease degradation and facilitates the uptake of the plasmid by muscle cells. To test the *in vitro* hGH expression, C2C12 myoblasts were transfected with the muscle-specific hGH plasmid, showing a secretion rate of ~ 43 ng hGH/ml. The plasmid, formulated in saline or complexed to PVP, was then injected into the tibialis cranialis muscle of normal and hypophysectomized rats. The levels of hGH in normal rat muscle injected with 150 μ g of hGH plasmid complexed with PVP were ~ 3 ng/gr muscle, i.e., approximately 10- to 15-fold higher than the control (plasmid in saline). Comparable hGH levels were detected in muscle extracts for up to 14 days, showing a decline 21 days after the injection. In hypophysectomized rats, a single but relatively high intramuscular dose (1.8 mg DNA/rat) of the hGH plasmid/PVP complex resulted in ~ 1.5 ng hGH/gr muscle (21 days post injection). The animals also showed a significant increase in growth, while serum IGF-I levels reached a value of 145.4 ± 77 ng/ml (vs. 34.7 ± 2.0 ng/ml before injection) at 21 days, declining thereafter. It is noteworthy that, after a single intra-muscular dose of this gene medicine, the increase in growth and the serum IGF-1 levels were comparable to those obtained with daily injections of recombinant hGH. Anti-hGH antibodies appeared in the serum at 14 days post injection, this antibody response being completely blocked by the administration of cyclosporine. hGH was not detectable in the sera of the injected animals, in spite of the increases in serum rat IGF-I levels and growth. This was attributed by the authors to the possibility that hGH levels were below the detection limit of the radioimmunoassay and/or that the human hormone was rapidly cleared from the circulation due to its short half-life in rodents. This gene medicine was shown to be effective over several weeks at a single intramuscular dose. Future work by the authors will be devoted to optimizing both the delivery system and the gene expression plasmid in order to produce the sustained levels of therapeutic protein necessary to permit the use of this specific gene-therapy approach in humans.

Another strategy used for *in vivo* GH delivery by a Danish group with which we are collaborating is hydrodynamic gene transfer. This transfer strategy is based on a rapid tail vein injection of naked plasmid DNA contained in a volume of buffer that corresponds to approximately 10% of the body weight of the animal. Although the exact mechanism is not clear, the rapid injection of a large amount of aqueous solution is likely to cause a transient right-side congestive heart failure with back-flow to the liver vessels. The liver is thus the target organ in this therapy, although the transfected vectors are mainly present as non-integrated episomes. This methodology was used to deliver hGH into hypophysectomized mice ²². The entire plasmid pUC-UBI-hGH solution, containing the ubiquitin C promoter

and the genomic hGH sequence, was injected (40-50 μ g DNA/1.5-1.9 ml) within 3-9 seconds. High levels of hGH were obtained in the circulation of the mice, reaching a peak value of 67.1 ng/ml 13 days post injection and remaining stable at ~50 ng/ml throughout the study (68 days), with a concomitant normalization of circulating IGF-I (from a maximum of 599 ng/ml on day 13) and IGF-binding protein (IGFBP)-3 levels. Furthermore, longitudinal growth became normal in terms of tibia length and tail length, while body weight stabilized at a level 4-5 g below that of normal mice. Weights of the liver, spleen and lungs were normal, whereas that of the heart was only partially normal, with hGH mRNA being expressed exclusively in liver tissue. Although the hGH levels were ~20-fold higher than physiological baseline levels, the IGF-I and IGFBP-3 levels were comparable to those found in normal mice. The authors suggested that a modification of the hydrodynamic procedure, for example by using a catheter-mediated delivery to the isolated liver, could make the protocol feasible for future human applications. These data strongly suggest that non-viral hGH gene transfer could be a feasible alternative to daily hGH injections in GHD patients, but again there are serious obstacles that must be overcome before the procedure can be used clinically. Hydrodynamic transfer has been successfully applied in dogs²³, but, as pointed out by the authors, other routes for gene transfer can be more convenient for humans.

As already mentioned, our group started working with the administration of naked DNA and has described a strategy for *in vivo* GH gene therapy based on electroporation of hGH-coding plasmid in the quadriceps muscle of lit/lit and lit/scid mice⁶. Muscle was chosen for the establishment of the methodology since it has been demonstrated that gene delivery vectors can be stably maintained in postmitotic fibers after their injection and that this tissue is able to provide a long-term release of the therapeutic protein. The utilization of gene electrotransfer must also be emphasized because it is considered to be one of the most efficient, safest, most practical and less expensive non-viral methods of DNA delivery and also generally decreases the inter-individual variability. Using the pUC-UBI-hGH plasmid²², we found the optimal electroporation conditions to be eight 50 V pulses of 20ms each at 0.5 s intervals. Employing these conditions, various amounts (12.5, 25, 50, 75 and 100 μ g) of the purified plasmid were administered into the exposed right quadriceps muscle. Serum hGH levels, determined 3 days after DNA injection, provided a dose-response curve with a highly significant ($P < 0.01$) linear correlation in the range of 0-50 μ g of injected plasmid. Because 50 μ g of DNA produced circulating hGH levels of 2-3 ng/ml for at least 12 days, a long-term body weight gain assay was carried out in lit/scid mice. After 60 days of continuous secretion of hGH, ranging from 1.5-3 ng/ml, the DNA-treated group increased from an average weight of 8.92 ± 1.29 g/mouse to 11.87 ± 0.53 g/mouse, corresponding to an average weight gain of 33.1% ($P < 0.001$). In contrast, the control group (mice injected with saline followed by electroporation) decreased from 9.08 ± 0.95 g/mouse to 8.70 ± 0.88 g/mouse, for a statistically non-significant weight loss of 4.2%.

The DNA-injected quadriceps muscles presented a 48.1 % weight increase versus the saline-injected control ($P < 0.001$), whereas the weight increase of non-injected quadriceps of treated animals was 31.0 % ($P < 0.005$). DNA-injected quadriceps showed a 45.5 % increase compared to the non-injected quadriceps of the same treated mice ($P < 0.001$). These data point to local (autocrine and/or paracrine) and systemic (endocrine) effects as a result of hGH DNA injection.

Another long-term experiment carried out with immunocompetent dwarf little mice (lit/lit), fail to produce the same degree of statistical significance. The body weight increase was somewhat lower (~21%) and the increase in body weight ceased after approximately one month. However, up to the 32nd day, the slope of the growth curve was 0.048 ± 0.038 g/mouse/day and the difference relative to the control curve (slope = 0.038 ± 0.016) was statistically significant ($P < 0.01$).

This treatment with a single injection of 50 μ g of pUC-UBI-hGH in lit/scid mice was also compared to regular injections of recombinant hGH (5 μ g/twice a day/animal) during 30 days. The two different strategies provided a similar response in terms of weight variation, when comparing the body weight gain of 0.094 g/mouse/day for the naked DNA system and of 0.095 g/mouse/day for recombinant protein injected daily, while the slope for the control (saline injection followed by electroporation) was 0.22 g/mouse/day (manuscript in preparation)⁸.

We have thus shown that intramuscular naked DNA hGH administration can be effective for promoting the growth of dwarf "little" mice, a model of human isolated growth hormone deficiency (IGHD). More must still be done, however, especially in terms of achieving long-lasting, sustained serum levels of the therapeutic protein.

4. Conclusions

As far as we know, the treatment of systemic protein deficiencies via gene therapy has not yet reached the stage of successful clinical applications, even for other diseases like hemophilia²⁴. As shown here, however, total or partial correction of growth defects has been achieved in several animal models for GH gene therapy by the use of a variety of different *ex vivo* or *in vivo* methodologies, all of which have the potential for future developments. We believe that, among these, the intramuscular administration of hGH-coding naked DNA, followed by a properly adapted electroporation technique, is probably the most promising therapy for GH deficiency due to its simplicity, practicality and safety. The fact that the plasmid is maintained in an episomal state results in a lower expression, but at the same time it offers the possibility of avoiding dangerously high hormone levels and undesirable chromosomal integration. Obviously, more must still be done in the direction of obtaining a long-lasting *in vivo* expression of the transgene.

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This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

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