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Partial correction of the dwarf phenotype by non-viral transfer of the growth hormone gene in mice: Treatment age is critical

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ABSTRACT

Non-viral transfer of the growth hormone gene to different muscles of immunodeficient dwarf (lit/scid) mice is under study with the objective of improving phenotypic correction via this particular gene therapy approach. Plasmid DNA was administered into the exposed quadriceps or non-exposed tibialis cranialis muscle of lit/scid mice followed by electroporation, monitoring several growth parameters. In a 6-month bioassay, 50 μg DNA were injected three times into the quadriceps muscle of 80-day old mice. A 50% weight increase, with a catch-up growth of 21%, together with a 16% increase for nose-to-tail and tail lengths (catch-up = 19–21%) and a 24–28% increase for femur length (catch-up = 53–60%), were obtained. mIGF1 serum levels were ~7-fold higher than the basal levels for untreated mice, but still ~2-fold lower than in non-dwarf scid mice. Since treatment age was found to be particularly important in a second bioassay utilizing 40-day old mice, these pubertal mice were compared in a third bioassay with adult (80-day old) mice, all treated twice with 50 μg DNA injected into each tibialis cranialis muscle, via a less invasive approach. mIGF1 concentrations at the same level as co-aged scid mice were obtained 15 days after administration in pubertal mice. Catch-up growth, based on femur length (77%), nose-to-tail (36%) and tail length (39%) increases was 40 to 95% higher than those obtained upon treating adult mice. These data pave the way for the development of more effective pre-clinical assays in pubertal dwarf mice for the treatment of GH deficiency via plasmid-DNA muscular administration.

1. Introduction

Plasmid-based gene therapy is among the most popular non-viral systems used in clinical trials, being particularly effective when DNA injection is followed by electroporation. Its utilization has continued to increase, representing 18.3% of all gene therapy clinical trials in 2012, close behind adenovirus (23.3%) and retrovirus (19.7%) applications [1,2]. Concerning systemic protein delivery, quite successful clinical improvements have been reported for the treatment of peripheral artery diseases and critical limb ischemia [3–5], but the utilization of naked DNA is, as far as we know, still at the preclinical level for the treatment of systemic diseases [6]. The first clinical tests for electroporation in humans, mostly related to DNA vaccination, were carried out by injection into the deltoid muscle [7–9] or into the anterior thigh [10], a muscular compartment that includes the quadriceps. This type of treatment is in general safe, well tolerated and clinically acceptable, even if a small percentage of the subjects may experience some adverse reaction, pain or discomfort [7].

In order to develop appropriate preclinical testing protocols for non-viral gene therapy in skeletal muscle, in our recent studies we have utilized the immunodeficient (lit/scid) and the immunocompetent (lit/lit) dwarf mice as suitable models of systemic diseases like growth hormone deficiency (GHD), since these mutants have an inherited growth defect strictly resembling human isolated type 1B GHD [11–13]. Indeed, our research group has found significant body weight increases of approximately 23% and 34% in 1 month- and 3 month-bioassays in which hGH- or mGH-plasmid DNA were injected into the exposed quadriceps muscle of lit/scid and lit/lit mice, respectively [14–16]. Because the saline-treated lit/scid mice presented a more stable body weight and a higher catch-up growth was obtained upon GH-DNA treatment (27% in the 1 month-treatment) in comparison with lit/lit mice (16% in the 3 month-treatment), the former model was chosen for the present work, the goal of which was to maximize the phenotypic correction of dwarfism via a non-viral GH gene transfer. This was tried first by increasing treatment time (up to 6 months), with repeated injections, always monitoring hGH and mIGF1 levels, and then

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by testing different age groups. A comparative study of different growth parameters was also carried out, especially considering those directly related to linear growth.

2. Materials and methods

2.1. Animals

The mutant strains of CB17-Ghrhr lit/+ Prkdc scid/Bm (lit/scid) and scid mice were obtained from Dr. W. Beamer (The Jackson Laboratory, Bar Harbor, ME, USA) [17]. The animals were maintained on a vented shelf and used to breed colonies of lit/scid and scid mice, as previously described [14]. Animals between 40 and 80 days of age were used for the experiments, which were approved by the local animal ethics committee.

2.2. Plasmid

The plasmid pUC-UBI-hGH was derived from a standard pUC-19 cloning plasmid by including the ubiquitin C promotor (position -1464 to - 15) upstream to a 2152 bp BamHI - EcoRI fragment of the hGH gene containing 4 introns and polyadenylation sequences [18]. The ubiquitous expression of various genes driven by this promoter has been reported by different authors [19,20] and its efficiency in hGH skeletal muscle expression already demonstrated by us in previous work [14]. The plasmid was multiplied in DH5alfa bacteria and purified using the DNA Xtra Midi/Maxi-Nucleobond Machnerey-Nagel purification system (Duren, Germany).

2.3. Plasmid administration and electroporation

The animals were anesthetized with xylazine and ketamine, followed by a hyaluronidase (20 U/20 μL) injection into the exposed quadriceps muscle region, as described [14]. After 30 min, 50 μg/20 μL of purified plasmid was administered in the same region, followed by electrotransfer with eight 90 V/cm pulses of 20 ms, separated by 0.5 s intervals, using an ECM-830 electroporator and a caliper electrode with a 3 mm distance between the plates (length/size 1.0 × 1.0 cm), both from BTX (Holliston, MA, USA). Saline was used as the control in all assays. For non-exposed tibialis cranialis electroporation, mice were treated with hyaluronidase as the above followed by one 800 V/cm pulse of 100 μs and by one 100 V/cm pulse of 400 ms.

2.4. Bioassay procedures

Three different Bioassays were carried out. In Bioassay I (6-month assay) all animals were ~80 days old at the onset of the experiment. One group of lit/scid (n = 26) received doses of 50 μg (1st and 2nd administration) or 100 μg/mouse (3rd administration) of pUC-UBI-hGH, while a second group (n = 24) received saline. Both groups were submitted to electroporation into the right (day 1) or into the left (day 104 and 161) exposed quadriceps. A third group (n = 21), composed of co-aged scid mice, was used as the positive control and for catch-up growth calculation.

In Bioassay II (1-month assay) one group of lit/scid, 40-day old at the onset of the experiment, was treated only once with 50 μg of pUC-UBI-hGH via quadriceps administration. A second group, also 40-day old, received only saline and was also submitted to electroporation. All groups consisted of 7 mice.

In Bioassay III (2-month assay) one group of lit/scid was 40-day old and the other 80-day old at the onset of the experiment. Both groups received twice (on day 1 and on day 41) 50 μg/20 μL of pUC-UBI-hGH, injected into each tibialis cranialis muscle. A third and a fourth group, with either 40- or 80-day old mice, received only saline and were also electroporated. A fifth and a sixth group consisting of co-aged scid mice was used as the positive control and for catch-up growth calculation. The number of mice per group is reported in Table 3.

The body weight of the animals was determined throughout the entire assay period and used to calculate the average daily weight variation. The animals whose blood was withdrawn from the retro-orbital cavity during the experiment were sacrificed in order to continue the experiment with only completely healthy mice. The tail and nose-to-tail lengths were measured with an electronic caliper before and at the end of the experiment. Blood was collected, femurs were dissected and measured with the same caliper. Serum mIGF1 levels were measured using the Quantikine mouse-rat IGF1 kit (R&D Systems, MN, USA), while serum hGH concentrations were determined utilizing an in-house radioimmunoassay, based on NIDDK reagents (Dr A.F. Parlow, National Hormone and Pituitary Program, Torrance, CA, USA) [21].

2.5. Catch-up growth calculation

Catch-up growth (C-uG) was calculated, as previously reported [16], using the body weight (g), nose-to-tail (cm), tail (cm), femur (mm) lengths or mIGF1 concentration, according to the following formula:

$$C\text{-}uG = (Wt - Wc)/(Wn - Wc) \times 100.$$

where:

Wt = final weight or length or mIGF1 concentration of the treated group;

Wc = final weight or length or mIGF1 concentration of the control (saline − treated) group;

Wn = final weight or length or mIGF1 concentration of a normal co − aged animal group (scid mice in this case).

2.6. Statistical analyses

Quantitative variables, reported as the mean ± SD, were analyzed by the unpaired Student's t test. Growth equations were generated by fitting the data to a quadratic relationship. The quadratic, linear and independent coefficients, calculated from the different experimental groups, were compared via the F-test method included in the Prism 5.0 package (GraphPad Software Inc., La Jolla, CA, USA). Means and curves were considered to be statistically different, representing distinct treatment effects, when the P value was <0.05.

3. Results

The results of the 6-month assay (Bioassay I) in which three progressive administrations of hGH-DNA were applied to the right and left quadriceps of lit/scid mice DNA are shown in Fig. 1. The weight increase reached about 50% on the initial value, body and tail length presented the same increment of 16%, and the right and left femurs increased 28% and 24%, respectively (Table 1). Fig. 2 illustrates the different dissected femurs where the length increase due to hGH-DNA treatment in lit/scid mice, as well as the natural growth occurring in non-dwarf scid mice, can be visually appreciated. Based on all the parameters presented in Table 1, the catch-up growth over the 6-month bioassay was between 19 and 60%, particularly high especially considering femur measurements. An integrated statistical analysis based on the catch-up growth parameter is also presented: it shows a highly significant difference in the increase between weight, nose-to-tail and tail versus right and left femur measurements. As shown in Fig. 1, treated lit/scid and co-aged scid mice underwent similar growth in terms of absolute body mass variation. Considering that the non-dwarf mice were already ca. 3-fold heavier than dwarf mice at the beginning of the experiment and that both groups consisted of adult animals, it was then decided to experiment the DNA treatment on younger animals. In Table 2 the corresponding mIGF1 determinations point to an increase in the