Normalization of growth in hypophysectomized mice using hydrodynamic transfer of the human growth hormone gene

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DAILY EXOGENOUS, SUBCUTANEOUS administration of human growth hormone (hGH) is still the only treatment for patients suffering from GH deficiency (GHD). These patients lack, for a variety of reasons, the ability to produce hGH and will, untreated, suffer from retarded growth, abnormalities in body fat distribution and abundance, muscle growth, and bone mineralization. Because the first trial was performed using hGH extracted from the pituitary gland in 1958 (17), it has been known that injection of hGH can reverse these symptoms. The development of recombinant hGH (rHGH) in 1986 (10) has greatly improved the availability and treatment possibilities and is today the standard treatment of GHD. However, because of substantial costs associated with life-long hGH substitution and the need for daily subcutaneous injections, alternative strategies, such as somatic gene therapy, should be considered, in particular with regard to obtain controlled in vivo synthesis.

Considerable progress has been made in the development of stable and safe modes of transferring genes to somatic tissues. Although efficient in transferring foreign genes in vivo, the current virus-based systems have limitations. Vectors based on murine retrovirus cannot transduce nonproliferating cells, which makes it difficult to target cells with a low cell turnover. In addition, viral vectors have been associated with severe immune responses, making these unsafe and repeated administration complicated. Lentiviral vectors with the ability of transferring genes into quiescent cells deserve further investigations for liver gene therapy, although no clinical experience has been made yet.

Ex vivo techniques can be used to obtain circulating levels of exogenous proteins. In 1994, Heartlein et al. (7) were able to achieve stable and long-term (>500 days) levels of hGH in mice using modified autologous fibroblasts (transkaryotic implantation), but the levels obtained were relatively low (i.e., 1–4 μg/l), and no measurable effect on the body weight was observed. In 1995, al Hendy et al. (1) introduced microencapsulated myoblasts engineered to produce mouse growth hormone (mGH) in the Snell dwarf mice. The procedure resulted in an increased body size and growth rate for the initial 3–5 wk, but a significant difference between the mGH in treated and untreated mice could not be detected, and there was only a partial correction of the growth defect.

Systemic hydrodynamic (large volume injection) gene transfer is a promising new strategy to deliver and express foreign genes in vivo (2, 14, 20). Although the first reports showed a rapid decline in expression within days, new reports show that stable levels of gene expression after a single administration (2, 16) or after repeated injections (14, 19) can be achieved. The slow turnover of transfected hepatocytes in vivo in mature animals explains the long-term transgene expression, although the vectors are mainly present as...
nonintegrated episomes (2, 16). Modification of the hydrodynamic procedure using catheter-mediated delivery to the isolated liver may lead to a clinically practicable gene transfer method (4).

We have previously shown that hydrodynamic gene transfer of an hGH expression plasmid results in a high and sustainable expression of exogenous hGH in normal mice, despite the presence of anti-hGH antibodies (2). In this paper, we show that a single administration of a plasmid vector containing an hGH gene results in normalization of longitudinal growth and serum insulin-like growth factor I (IGF-I) in hypophysectomized mice.

MATERIALS AND METHODS

Plasmid construct. The pUC-UBI-hGH plasmid has been described previously (2). It derives from a standard pUC-19 cloning plasmid in which an Ubiquitin C promoter has been included upstream of a 2,152-bp BamHI-EcoRI fragment of the hGH gene containing four introns and a polyadenylation sequence. The plasmid pEGFP-N1 is commercially available (Clontech Laboratories, Palo Alto, CA). Large-scale purifications of the plasmids were prepared using a Plasmid Maxi Prep kit (Qiagen, Valencia, CA).

Animals. The animal experiments were approved by the Danish Animal Experiments Inspectorate. Male outbred mice (Bom:NMRI; M&B, Ry, Denmark), ~8 wk old, were used for DNA injections. The animals were hypophysectomized 3 wk before DNA injection by the transauricular method (5). In brief, a modified hypodermic needle was inserted through the auditory canal in the osseous bulla on both sides of the head of mice (Bom:NMRI; M&B, Ry, Denmark) for blood sampling and killed by cervical dislocation before recovery.

Tail vein injections. Before the injection procedure, the animals were kept at a high ambient temperature to dilate the tail veins. Anesthesia was carried out in a chamber with 3.75% (vol/vol) isoflurane (Baxter Medical, Kiåa, Sweden) in air until digital reflation was absent. Naked DNA was administered to the mice by injection of 45 μl of the supernatant were subsequently diluted 1:200 before analysis. Serum IGF-I levels were measured by RIA using a polyclonal rabbit antibody (Nichols Institute Diagnostics, San Capistrano, CA) and recombinant human IGF-I as standard (Amersham International). Monoiodinated IGF-I (125I-labeled [Tyr31]IGF-I) was obtained from Novo-Nordisk (Bagsvaerd, Denmark). Absorption was performed with 1% BSA containing 1% Tween 20 (both from Merck, Darmstadt, Germany) followed by centrifugation at 3,600 rpm for 18 min. The presence of significant hGH antibody formation was defined as a precipitation percentage larger than 0.1 SD of that obtained in control sera. This method relies on precipitation of large proteins (molecular mass greater than ~70 kDa) and is therefore not specific for γ-globulins. However, PEG has been used for decades in RIA for precipitation of labeled antigens bound to specific antibodies (3, 12).

Serum IGF-I. Serum IGF-I was measured after extraction with acid-ethanol. The extraction mixture was incubated for 2 h at room temperature followed by centrifugation, and 25 μl of the supernatant were subsequently diluted 1:200 before analysis. Serum IGF-I levels were measured by RIA using a polyclonal rabbit antibody (Nichols Institute Diagnostics, San Capistrano, CA) and recombinant human IGF-I as standard (Amersham International). Monoiodinated IGF-I (125I-labeled [Tyr31]IGF-I) was obtained from Novo-Nordisk (Bagsvaerd, Denmark). The intra- and interassay coefficients of variance were <5 and <10%, respectively.

Serum IGF-binding protein-3. SDS-PAGE and Western ligand blotting were performed according to the method of Hossenlopp et al. (8) as previously described (6). Serum (2 μl) was subjected to SDS-PAGE (10% polyacrylamide) under nonreducing conditions. The electrophoresed proteins were transferred by electroelution on nitrocellulose paper (Schleicher & Schuell, Munich, Germany), and membranes were incubated overnight at 4°C with ~500,000 cpm 125I-IGF-I (sp act 2,000 Ci/mmol) in 10 ml 10 mmol/l Tris-HCl buffer containing 1% BSA and 0.1% Tween (pH 7.4). Membranes were washed with Trish-buffered saline and dried overnight, and the nitrocellulose sheets were subsequently autoradiographed with Kodak X-AR film and exposed to DuPont-NEN (Boston, MA) enhancing screens at ~80°C for 3–7 days. Specificity of the IGF-binding protein (IGFBP) bands was ensured by competitive coinubation with unlabeled IGF-I purchased from Bachem (Bubendorf, Switzerland). On Western ligand blotting (125I-IGF-I as ligand), IGFBP-3 appears as a 38- to 42-kDa doublet band corresponding to the intact acid-stable IGF-binding subunit of IGFBP-3. Western ligand blots were quantified by densitometry using a Shimadzu CS-9001 PC dual-wavelength flying spot scanner.
RNA extraction and RT-PCR. RNA samples were isolated from liver, heart, lung, spleen, kidney, intestine, brain, and skin tissue from killed mice using a High Pure RNA Tissue Kit (Roche, Mannheim, Germany). Samples were collected 30 days after the hydrodynamic gene transfer. cDNA was prepared from total RNA by (oligo)dT primed RT synthesis [First-Strand Synthesis Kit for RT-PCR (AMV); Roche]. hGH cDNA was amplified by PCR using primers spanning a 187-bp region between the sense primer 5'-agaagttcag-acaaactca-3' and the antisense primer 5'-gcaagttcag-ccaaactca-3'. A control for template abundance was performed in parallel reactions using β-actin primers spanning a 196-bp region between sense primer 5'-CTGTGCTGTCCCTGTATGCC-3' and antisense primer 5'-GTGGTGGTGAAGCTGTAGCC-3'. PCR amplification with either set of primers was carried out with a thermal program consisting of 30 cycles of 94°C for 30 s, 59°C for 30 s, and 74°C for 1 min.

Monitoring of growth. The tail length was measured with a calliper every week, and the body weight was measured two times a week. Measurement started 10 days before injection and continued throughout the study (68 days). At the time of death, the tibia was isolated, and the length was measured using a calliper. Selected organs (spleen, heart, lungs, and liver) were isolated and weighed.

Statistical analyses. A two-tailed Student's t-test was used for statistical analyses.

RESULTS

To investigate the therapeutic possibilities of nonviral hGH gene transfer, we used hypophysectomized mice as a model for GHD. The mice were hypophysectomized at 5 wk of age and treated with plasmid injection at 8 wk of age. The hypophysectomized mice were injected with the hGH plasmid (pUC-UBI-hGH; n = 9), the control GFP plasmid (pEGFP-N1; n = 7), or left untreated (n = 7). Furthermore, we included normal (nonhypophysectomized) mice (n = 7) in the study.

Serum hGH and hGH antibodies. After administration of the pUC-UBI-hGH plasmid, high levels of hGH protein could be detected in serum. After an initial increase to 67.1 μg/l 13 days after injection, the concentration remained stable at a level of ~50 μg/l throughout the study (Fig. 1A). Interference of endogenous mGH was avoided by the use of an hGH specific assay. As expected, hGH could not be detected in mice receiving pEGFP-N1, untreated mice, or in the normal mice. Mice injected with the hGH plasmid developed antibodies against hGH. In a blood sample taken 68 days after injection, the hGH antibodies were detected using a method based on PEG precipitation of radiolabeled hGH (data not shown). However, this had no apparent impact on the effect on serum IGF-I, longitudinal growth, or increase in body weight (see below).

Serum IGF-I and IGFBP-3 levels. To investigate the physiological effect of the elevated hGH levels, we measured serum IGF-I, the major physiological mediator of GH actions. After an initial rise of IGF-I to
above the levels found in normal mice (599 ± 33 compared with 373 ± 29 μg/l 13 days after DNA injection), serum IGF-I stabilized at a level comparable with that found in normal mice (Fig. 1B). From 40 days after treatment and onward, no significant difference in serum IGF-I between intact mice and mice treated with pUC-UBI-hGH plasmid was detected. As expected, the IGF-I levels were very low in mice receiving pEGFP-N1 and in uninjected mice. It is known that GH, in addition to stimulating IGF-I synthesis, stimulates the formation of ternary IGF-binding complexes, including IGFBP-3 (13). IGFBP-3 was measured in samples taken 7 days before treatment, and at death. A normalization in IGFBP-3 was observed in mice treated with pUC-UBI-hGH, whereas mice treated with pEGFP-N1 and untreated mice remained stable at the initial low level (Fig. 1C). Interestingly, IGFBP-3 also increased in the normal mice over time, probably because of a pubertal/postpubertal surge.

RNA analysis. To investigate plasmid DNA expression in various tissues, a subset of animals was killed and analyzed 27 days after injection. RNA was purified from tissues and subsequently used as template in RT-PCR reactions to examine the presence of hGH mRNA. After 27 days, hGH mRNA was detected exclusively in the liver (Fig. 1D), in agreement with previous findings (2, 16).

Body weight gain, longitudinal growth, and organ weight. Within few days after plasmid injection, an increase in body weight and tail length was observed in pUC-UBI-hGH plasmid-injected mice, whereas mice injected with pEGFP-N1 plasmid and uninjected mice maintained a stable body weight and tail length throughout the study period (Fig. 2, A, B, and D). After

Fig. 2. Comparison of different growth parameters. ●, pUC-UBI-hGH (n = 9); ■, pEGFP-N1 control plasmid (n = 7); ▲, not injected (n = 7); X, normal mice (n = 7). Body weight (A), tail length (B), and tibia length (C) at day 68 pUC-UBI-hGH (open bar), pEGFP-N1 (light gray bar), mice not injected (dark gray bar), and normal mice (filled bar). Data are presented as means ± SE. D: photo taken 60 days after injection. From left to right: hypophysectomized mouse treated with pUC-UBI-hGH, hypophysectomized mouse treated with pEGFP-N1, and normal mouse.
a catch-up period of ~3 wk for the body weight, and 5 wk for the tail length, the treated mice stabilized at a growth rate similar to normal nonhypophysectomized mice. Tail length was normalized and showed no significant difference in comparison with normal mice, whereas body weight stabilized at a level of 4–5 g below that of normal mice. A different measure for longitudinal growth, i.e., tibia length, was determined at the end of the study. Tibia length was also normalized, and no significant difference was found between mice treated with pUC-UBI-hGH and normal mice. Mice injected with the pEGFP-N1 plasmid and uninjected mice had significantly shorter tibia length (Fig. 2C). The effect of the hydrodynamic gene transfer on the weight of different organs was assessed after 68 days, when all remaining mice were killed (Fig. 3). In hypophysectomized mice treated with pUC-UBI-hGH, the average weight of all investigated organs was significantly higher than in the corresponding mice treated with pEGFP-N1 and untreated mice. Compared with normal mice, no significant difference in organ weight was found for the liver, lungs, and spleen. However, the weight of the heart was significantly higher in normal mice than in mice treated with pUC-UBI-hGH.

DISCUSSION

Systemic injections of naked plasmid constitute a simple and efficient approach for the expression of high levels of exogenous hGH in vivo. After a single injection in hypophysectomized mice, we have observed a sustained elevation of serum hGH, normalization of IGF-I and IGFBP-3, and normalization of tibia length, tail length, and body weight gain.

Although hGH substitution in children and adult patients with GHD has been achieved successfully by daily subcutaneous injections of recombinant hGH (9), the search for alternatives to this treatment is still required. The GH levels in normal individuals are controlled through a complex interdependent secretion of hormonal factors, which is difficult to mimic by daily protein injections. Lifelong subcutaneous injections may be considered inconvenient by some patients with the risk of decreasing compliance (18). Furthermore, the cost of treatment by administration of rhGH is considerable, currently amounting to more than $10,000/patient annually. These concerns point toward in vivo synthesis by simple gene transfer as an appealing, potential alternative.

Using microencapsulated myoblasts, Al Hendy et al. (1) obtained partial correction of the growth abnormalities seen in the Snell dwarf mice. The protein resulted in an increase in length and body weight and increased size of the tibial epiphyseal cartilage in the treated group. In the first 3–5 wk, an increase in growth rate was observed, and the myoblast continued to be actively secreting mGH throughout the study (178 days). However, as described above, correction of the growth abnormality was only partially normalized; furthermore, there was no detectable rise in circulating GH, and finally IGF-I was not measured (1).

Normal mice have pulsatile GH levels (15). Baseline values are ~2 μg/l, whereas peak values may reach 100 μg/l. In the present study, hGH plasmid transcription and translation are independent of endogenous mediators and are therefore expected to be relatively stable. We found a concentration of 40–50 μg/l, which is below the peak levels in normal mice but ~20-fold higher than the baseline levels. The effects of constantly elevated hGH levels have to be investigated further, considering that high GH and IGF-I levels may be associated with the development of neoplasm (11). It is in-

Fig. 3. Wet weight of selected organs at day 68 pUC-UBI-hGH (open bars), pEGFP-N1 (light gray bars), mice not injected (dark gray bars), and normal mice (filled bars). A: liver; B: lungs; C: spleen; D: heart. *P < 0.01 for pUC-UBI-hGH-injected mice vs. normal mice. Data are presented as means ± SE.
triguing, however, that, although GH levels in the injected mice were 20-fold higher than physiological baseline levels, the IGF-I and IGFBP-3 levels were normalized and comparable to levels in normal mice. The regulatory mechanisms have to be investigated further, but these findings seem to indicate that, to achieve adequate treatment, strict regulation of expression is not necessary. Another possible explanation would be that hGH is less efficient than mGH. Unlike GH, IGF-I is not under pulsatile secretion in normal individuals, and serum IGF-I is normally relatively stable. Thus the presumably stable IGF-I levels found in hGH-injected mice mirror the physiological levels. We do not know why the hypophysectomized mice do not catch up in body weight, although length and weight of most internal organs are normalized. One explanation may be the known lipolytic effect of hGH, although the lack of the other pituitary hormones in treated animals cannot be ruled out.

Several groups have investigated the safety of the hydrodynamic gene transfer procedure, and no permanent damage on liver or other organs has been observed (14, 16, 19, 20). However, serious obstacles remain to be solved before the procedure can be used in a clinical setting. Hydrodynamic transfer has been performed successfully in larger animals [for example in dogs (21)] after open surgery, but ideally more convenient routes for gene transfer into humans are preferable. Access through the choledochus duct or the cava vein may be advantageous since these vessels are relatively easy to reach through either endoscopic retrograde cholangiopancreatography or the femoral vein.

Although the current protocol cannot directly be applied in humans, the data strongly suggest that nonviral hGH gene transfer may be a feasible alternative to lifelong, daily hGH injections in GHD patients. However, further studies are warranted to fully unravel the potential use in humans.

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DISCLOSURES

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