Once daily administration of CJC-1295, a long acting growth hormone (GH) releasing hormone (GHRH) analogue, normalizes growth in the GHRH-knock out mouse

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Running head: CJC-1295 treatment in GHRHKO mice

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Abstract

Although the majority of children with isolated GH deficiency have a good growth response to GHRH, the use of this therapeutic agent is limited by its very short half-life. Indeed, we have shown that in mice with GHRH gene ablation (GHRH knock-out- GHRHKO) even twice/day injections of a GHRH analogue are unable to normalize growth. CJC-1295 is a synthetic GHRH analogue that selectively and covalently binds to endogenous albumin after injection thereby extending its half-life and duration of action. We report the effects of CJC-1295 administration in GHRHKO animals.

Three groups of 1-week old GHRHKO mice were treated for 5 weeks with 2 µg of CJC-1295 at intervals of 24h, 48h and 72h. Placebo-treated GHRHKO mice and mice heterozygous for the GHRHKO allele served as controls.

GHRHKO animals receiving daily doses of CJC-1295 exhibited normal body weight and length. Mice treated every 48h and 72h reached higher body weight and length than placebo-treated animals, without full growth normalization. Femur and tibia length remained normal in animals treated every 24h and 48h. Relative lean mass and subcutaneous fat mass were normal in all treated groups. CJC-1295 caused an increase in total pituitary RNA and GH mRNA suggesting that proliferation of somatotroph cells had occurred, as confirmed by immunohistochemistry images.

These findings demonstrate that treatment with once daily administration of CJC-1295 is able to maintain normal body composition and growth in GHRHKO mice. The same dose is less effective when administered every 48h or 72h.

Keywords: GH deficiency, GHRH therapy, growth
Introduction

Human growth hormone (GH) releasing hormone (GHRH) is a 44-amino acid peptide that stimulates GH secretion and somatotroph cell proliferation in the pituitary gland through its active 1-29 N-terminus peptide (10). Although daily injection of recombinant human GH is presently the only therapeutic option available for patients with GH deficiency (GHD), GHRH may represent a more valid and physiologic approach, especially for children with isolated GHD, in whom this condition seems to be related to altered hypothalamic function rather than to impaired GH secretory capacity (16). However, GHRH has limited therapeutical use in children, due to its very short half-life (9), requiring multiple daily injections or infusion by a pump. Similarly, we have previously demonstrated that long-term treatment with twice-daily injection of a GHRH super-analogue is only partially able to restore somatotroph hypoplasia and growth in the GHRH knock out (GHRHKO) mouse (2).

CJC-1295 is a synthetic human GHRH analogue that selectively and covalently binds to endogenous albumin after subcutaneous (s.c.) administration, thereby extending its half-life and duration of action (11, 12). Studies in animals (rats, pigs and dogs) have shown that several days after a single administration of CJC-1295, serum IGF-1 levels are still increased (5). In a recent report, CJC-1295 administered to healthy subjects has been proven to be effective in causing a sustained dose-dependent stimulation of serum GH and IGF-1 levels, when injected at intervals of one to two weeks (14).

The primary aim of this study was to determine the minimum frequency of injections that could correct the GH deficiency phenotype in the GH deficient GHRHKO mice, as measured by improvement of auxological parameters and body composition. Secondly, we wanted to assess the stimulatory effect of CJC-1295 on somatotroph cells proliferation. We show that once-daily injections of 2 µg of CJC-1295 are able to completely normalize growth. Furthermore, administration of the same dose of CJC-1295 every 48 or 72 hours produced intermediate results, indicating an interval-dependent effect.
Materials and methods

Animals

Male offspring derived from KO breeding pairs on a hybrid C57BL/6-SV129 background were used for all GHRHKO groups, while male heterozygous animals (HTZ) (also on a C57BL/6-SV129 background) served as a control group for normal growth and GH status. HTZ rather than wild-type C57Bl/6 or SV129 mice were used to ensure a closer match between the genetic background of GHRHKO mice and controls. Mice were weaned during the treatment onto a standard mouse/rat chow (Prolab RMH2500, PMI Nutrition International Brentwood, MO) at 4 weeks of age, and were housed in groups of 4 animals based on genotype and treatment. All animals experienced a controlled environment with 14h light/10h dark cycles at 21°C and 23% humidity. Food and tap water were supplied *ad libitum*. All procedures were approved by the Johns Hopkins Institutional Animal Care Committee.

Treatment with CJC-1295

Three groups of 1-week old GHRHKO mice were treated for 5 weeks with 2 µg of CJC-1295 at intervals of 24h (CJC/24h; n = 8), 48h (CJC/48h; n = 8) and 72h (CJC/72h; n = 7). Two placebo (pbo) treated groups (treated daily) were included to allow comparison with the active treated animals: GHRHKO mice (GHRHKO/pbo; n = 8), and mice heterozygous for the GHRHKO allele that have normal growth parameters (HTZ/pbo). Drug or placebo was injected in the morning between 0800 and 0900 h. At the end of the treatment period CJC/24h, CJC/48h, and CJC/72h were sacrificed 24h, 48h and 72h after their last dose respectively, in order to determine the time-dependent effect of CJC-1295 on GH and IGF-1 production. Due to the delicate abdominal wall structure in 1-week old mice, the drug was injected subcutaneously (s.c.) in the interscapular area during the first week of treatment and in the abdominal area starting from the second week of
treatment. Mice within the same litter were assigned to different groups to avoid any influence of litter size and maternal breast feeding behaviour.

**Auxological parameters**

Body length, naso-anal length (N-A), and total body weight (TBW) were measured at baseline and weekly thereafter, using a daily calibrated electronic balance (Scout Pro Balance, Ohaus Corp, Pine Brook, NJ) and an electronic digital caliper (Control Company Friendswood, TX). At the end of the treatment period, measurements of femur and tibia length were obtained by dissection of the surrounding muscle tissues and disarticulation without disturbing the articular cartilages, using an electronic digital calliper. Femur length was measured as the maximal distance between the head of the great trochanter and the distal condyles, while the tibia length was considered as the maximal distance between the proximal condyles and malleolus.

To determine the effects of treatments on body composition, weights of subcutaneous fat pad (SF), visceral fat (VF), and lean mass (LM) were assessed separately using a precision electronic balance (AR1140, OHAUS Corp, Pinebrook, NJ). After removal of skin, the peri-renal and epidydimal fat pads were pooled for VF measurement and the sum of the fat pads from the interscapular and axillary region, thighs, and inguinal region were used for SF measurement.

Lean mass was determined by weighing animals deprived of tail, skin, adipose tissue and organs. LM, VF and SF weights of each animal were normalized to total body weight (TBW) by calculating the percentage as follows: \[
\frac{\text{weight (g)}}{\text{TBW (g)}} \times 100.
\]

**Pituitary total RNA and GH and Prolactin mRNA**

Due to the extremely small pituitary size (~1mm), the weight of the gland could not be measured directly. Similarly, three glands from each group were pooled for RNA extraction. Quantification of pituitary mass was extrapolated by measuring the mean total RNA obtained from a pool of three pituitary glands of each group. Total RNA was isolated using TRIzol reagent (Invitrogen, Life
Technologies, Carlsbad, CA) according to manufacturer’s recommendations. The average total RNA yield for each group was quantified spectrophotometrically at 260/280 nm (DU 640 Spectrophotometer, Beckman-Coulter, Fullerton CA).

Pituitary GH and prolactin mRNA content were measured by Northern analysis as previously described using 3 µg of total RNA (1) for each group. Results were normalized to mouse GAPDH mRNA expression after stripping of the membrane and quantified by phosphor imager (Molecular Imager FX, Bio-Rad, Life Science Group, Hercules CA) as previously described (1).

**Pituitary GH protein:**

Pooled pituitary extracts used for RNA isolation were also used for protein extraction according to TRIzol manufacturer’s recommendations. Equal amounts of protein from each group were resolved on 15% SDS-PAGE, and electro-transferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA). After overnight blocking in Tris-buffered saline, 0.02% Tween 20, and 5% milk, at 4°C, the membrane was incubated for 2 h at room temperature with rabbit anti-mouse GH antibody (National Hormone and Peptide Program, Harbor UCLA Medical Center, Torrance CA) at a 1:80,000 dilution. After washing, the membrane was incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:3,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Band size was determined by comparison with a full range protein weight marker (Precision plus protein, Biorad, Hercules, CA).

**Pituitary immunohistochemistry:**

After removal of skin from the heads, parietal and temporal bones were removed and tissues were fixed in 10% buffered formalin for 24 h and decalcified (Cal-Ex, Fisher Scientific, Fair Lawn, NJ) for 1 week. After bisecting the brain through the dorsal sagittal sinus, the hemispheres were embedded in paraffin and cut into 5 µm sections. The sections were then immuno-stained for GH and prolactin with rabbit anti-mouse GH and prolactin antibodies (National Hormone and Peptide Program, Harbor UCLA Medical Center, Torrance, CA) (1:3,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Band size was determined by comparison with a full range protein weight marker (Precision plus protein, Biorad, Hercules, CA).
dilution) using the avidin-biotin-peroxidase complex technique with DAB substrate (DAKO Co, Carpinteria, CA) (brown). Cell nuclei were counterstained using hematoxylin (blue).

**Serum IGF-1**

On the day of sacrifice, blood was obtained by eye-bleeding. After centrifugation, sera from all groups were stored at -20°C and assayed together. Serum IGF-1 was measured using a rat-mouse IGF-1 RIA (DSL-2900, DSL Webster TX), after acid ethanol extraction, following the manufacturer’s recommendations. All samples were analyzed in duplicate.

**Statistical analysis**

Results are expressed as means ± SEM. Parameters were statistically analyzed by ANOVA using an SPSS statistical package, with posthoc analysis by Bonferroni’s method. Results were considered statistically significant at p <0.05.
Results

Auxological data and body composition

CJC-1295 was well tolerated in all animals with no evidence of either local or systemic toxicity. Animals receiving a daily dose of CJC-1295 maintained normal TBW (fig.1) and N-A measurements (fig.2) through the end of the 5-week treatment period. Animals treated every 48h and 72h had significantly greater TBW and N-A as compared to GHRHKO/pbo but remained significantly smaller compared to both heterozygous mice and CJC/24h. Final femur and tibia lengths in both CJC/24h and CJC/48h groups were comparable to HTZ/pbo but were reduced in CJC/72h despite being greater than in GHRHKO/pbo (tab.1).

Results of the final body composition showed that LM% and SF% remained normal in all active treated groups while no difference was observed in VF among all 5 groups (tab. 1).

Total pituitary RNA, GH mRNA and GH protein content

CJC-1295 caused a 13, 9 and 7 fold increase in total pituitary RNA in the CJC/24h, CJC/48h and CJC/72h groups, respectively, when compared to GHRHKO/pbo animals. This was reflected in an 11, 8 and 6 fold increase in GH mRNA levels measured by Northern analysis (fig. 3a). If one corrects for the daily dose of drug, CJC/24H, CJC/48H, and CJC72H increased GH mRNA levels at comparable levels (13, 15 and 16 fold/day of treatment), These findings were paralleled by an elevation in GH protein content (fig. 3b) in treated animals compared to GHRHKO/pbo as measured by Western Immunoblot.

A much less dramatic effect was observed on prolactin mRNA, with increases of 2.4, 2.5 and 2.3 fold in the three treated groups when compared to placebo (tab. 2).

Pituitary immunohistochemistry
Prolonged treatment with CJC-1295 caused an increase in pituitary size and GH positive staining cells in all three treated groups (fig 4). No clearly evident difference was observed in the lactotroph cell mass (data not shown).

**Serum IGF-1**

Serum IGF-1 levels measured 48h and 72h after injection of CJC-1295 were not significantly different from those of GHRHKO/pbo, despite the significant changes in the auxological parameters described above. In contrast, CJC/24h animals had significantly higher levels of circulating IGF-1 compared to HTZ/pbo, not reflected by a significantly greater final N-A or bone measurements compared to HTZ animals (fig. 5).
Discussion

GHRHKO mice have targeted disruption of the GHRH gene that causes pituitary hypoplasia due to a reduced somatotroph cell population, proportionate dwarfism, and alterations in body composition (1, 3). We have previously demonstrated that in these animals somatic growth can be increased by post-natal treatment with JI-38, a super active GHRH analogue, although a completely normal phenotype could not be restored even with multiple daily injections (2).

CJC-1295 is a synthetic analogue of the human GHRH with substitution of four amino acids that render the compound more resistant to proteolytic inactivation by dipeptidyl-peptidase IV (DPP-IV). This core therapeutic moiety is linked to a Drug Affinity Complex (DAC) that allows covalent binding (conjugation) of the CJC-1295 to endogenous albumin once injected, thereby extending its half-life to duration similar that of albumin. The aim of this study was to determine the effect of long-term treatment with CJC-1295 on somatotroph cell proliferation, GH production and IGF-1 secretion and, ultimately, growth and body composition in the GHRHKO mouse. In order to determine the minimum frequency needed to achieve significant results, we administered CJC-1295 at three different time intervals. Since mouse albumin has a half-life of approximately 1 day (13), we injected CJC-1295 at time intervals of 24h, 48h and 72h. The rational for selecting these time points was based upon studies which had shown that a single injection of CJC-1295 in rats produced the highest plasma AUC of GH within the first two hours, with a mean residence time in plasma of more than 30h, and levels still detectable at 72h (12).
We have previously shown that GHRHKO animals become significantly smaller than controls by the second week of life (4). To avoid a further delay in growth which could have precluded meaningful interpretation of the data across treatment groups, treatment with CJC-1295 was started at 1 week of age. After 5 weeks of treatment, mice receiving the compound at 24h intervals maintained completely normal growth parameters (N-A, TBW, femur and tibia lengths) and body composition when compared to HTZ/pbo mice. Interestingly, mice in the CJC/48h group displayed a mixed phenotype, reaching significantly higher N-A and TBW compared to GHRHKO/pbo but significantly lower than HTZ/pbo, although femur, tibia and body composition were normal. At the end of the treatment period, the CJC/72h group reached an intermediate phenotype, showing growth parameters significantly higher when compared to GHRHKO/pbo, although none were normal. The reduced efficacy of CJC-1295 in the groups receiving injections at 2 and 3 day intervals was likely due to the fact that the quantity of drug remaining in circulation after 24 hours was insufficient for full bioactivity, because of the short t1/2 of mouse albumin [20 to 24 hours, as compared to about 48 hours in rats and up to 19 days in humans (13)]. In addition, the quantity of drug administered to these groups was only one-half to one-third of that administered to the group receiving daily injections. Despite the failure of complete growth normalization in the CJC/72h group, CJC-1295 was still able to prevent the alterations in body composition seen in the GHRHKO/pbo group. This may indicate higher thresholds for GH effects on longitudinal growth than on body composition.
Since the somatotrophs of the GHRHKO mice are hypoplastic due to chronic absence of GHRH stimulation, we analyzed the efficacy of long-term therapy CJC-1295 on somatotroph cell proliferation and GH secretion. Measurement of total RNA showed an interval-dependent increase in the overall yield, indicating an increase in cell population. Northern Blot analysis and Immunoblot confirmed that increases in GH transcription and translation had occurred. Final proof for the proliferative effects of CJC-1295 on somatotroph cells was derived from immunohistochemistry studies of the pituitaries, which demonstrated that in active treatment groups the glands were increased in size and that the increment was due mainly to somatotroph proliferation. Immunohistochemistry did not show a clear evidence of significant lactotroph cell proliferation. The observed increase in prolactin RNA was much less marked than the increase in GH mRNA, and was not dose-dependent. Nevertheless, CJC-1295 normalized prolactin mRNA levels. This increase likely reflects some degree of GHRH-induced proliferation of the mammotroph cells, previously reported in mice over expressing GHRH (7).

Although assessment of the serum IGF-1 (measured at the maximum time distance from the last injected dose at day of sacrifice) showed an interval-dependent increase of the hormone that mirrored the growth patterns of each group, the statistical analysis failed to show any significant difference between GHRHKO/pbo, CJC/48h and CJC/72h groups. This apparent inconsistency between improvements in auxological parameters and body composition seen in CJC/48h and CJC/72 in face of the absence of significant elevation in serum IGF-1 has been extensively reported by others and our group (2, 3, 8, 17). In this
particular case, the interval-dependent decline in serum IGF-1 levels in animals receiving CJC-1295 may be explained by the half-life of the compound. Pharmacokinetics analysis in healthy men treated with CJC-1295 showed a dose-related increase in GH AUC regardless of whether the dose had been given only once or as multi-doses. In contrast, serum IGF levels, although dose-dependent, showed progressive elevation over time when CJC-1295 was given repeatedly, indicating cumulative pharmacokinetic effects (14). Although we have no data to support this hypothesis, it is reasonable that CJC-1295 may have determined similar effects in our animals, causing serum IGF-1 to reach lower plateaus as the time interval between consecutive injections increased. In addition, we measured IGF-1 at the time of sacrifice, therefore at longer interval from the last CJC-1295 dose in mice treated with less frequent dosing.

This study has demonstrated, through the use of the GHRHKO model, that CJC-1295 has important trophic effects on GH secreting cells of the pituitary. This finding is of particular clinical interest since children with isolated GH deficiency often do not have any alteration in the somatotrophs but rather have impaired hypothalamic function causing de-regulation in GHRH secretion (16). Indeed GHRH treatment of these children accelerates growth (6, 15), suggesting that GHRH is an ideal and more physiologic treatment when GH deficiency is due to hypothalamic dysfunction rather than to primary pituitary disease.

In conclusion, our study shows that a once-daily injection of CJ-1295 is sufficient to maintain normal growth parameters and body composition. Although the efficacy of the same dose is reduced when administered every 48h or 72h, it is possible that higher doses may elicit effects similar to those of daily treatment.
These findings also suggest that the determination of dosing intervals of this compound in other species must take into consideration the pharmaco-dynamics of the drug, which are dependent on the species-specific half lives of albumin.
References


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bone mineral content in dogs. 87th Annual Meeting of The Endocrine Society, Abstract P1-78, 2005.


Figure legends

1. Effect of CJC-1295 on total body weight (mean ± SEM). At the end of the 5 week treatment period, CJC/24h group exhibited completely normal TBW. CJC/48h and CJC/72h groups reached significantly higher TBW when compared to GHRHKO/pbo, although remained smaller than HTZ/pbo.

   * p< 0.01 vs. CJC/48h, CJC/72h and GHRHKO/pbo
   ** p< 0.001 vs. GHRHKO/pbo

2. Effect of CJC-1295 on naso-anal length (mean ± SEM). At the end of the 5 week treatment period, CJC/24h group exhibited completely normal body length. CJC/48h and CJC/72h groups grew significantly more GHRHKO/pbo, although remained shorter than HTZ/pbo.

   * p < 0.001 vs. CJC/48h, CJC/72h and GHRHKO/pbo
   ** p < 0.01 vs. GHRHKO/pbo

3. Panel a: GH mRNA measured by Northern blot analysis. Results are expressed as arbitrary units and normalized with GAPDH after stripping of the membrane (three glands were pooled from each group).

   Panel b: Pituitary GH protein content by Western blot analysis. The expected size of mouse GH is 22 kDa.
4. Effect of CJC-1295 on pituitary morphology. Immuno-histochemical identification of GH positive cells (brown) counterstained with hematoxylin (blue). The arrows point to the few somatotroph cells present in the GHRHKO/pbo animals.

5. Effect of CJC-1295 treatment on serum IGF-1 (mean ± SEM). Shown are of serum IGF-1 levels on the day of sacrifice. CJC/24h, CJC/48h and CJC/72h had received their last dose of CJC-1295 respectively 24h, 48h and 72h prior.

* p < 0.01 vs CJC/48h, CJC/72h and GHRHKO/pbo
Table 1 Tibia and femur length and body composition data after 5 week treatment

<table>
<thead>
<tr>
<th></th>
<th>HTZ/pbo</th>
<th>GHRHKO/pbo</th>
<th>CJC/24h</th>
<th>CJC/48h</th>
<th>CJC/72h</th>
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<tbody>
<tr>
<td>Tibia (mm) (mean ± SEM)</td>
<td>16.6 ± 0.1</td>
<td>13.8 ± 0.1†</td>
<td>16.9 ± 0.1</td>
<td>16.4 ± 0.1</td>
<td>16.1 ± 0.1‡</td>
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<tr>
<td>Femur (mm) (mean ± SEM)</td>
<td>13.7 ± 0.1</td>
<td>10.7 ± 0.1†</td>
<td>13.8 ± 0.2</td>
<td>13.4 ± 0.1</td>
<td>13.0 ± 0.1‡</td>
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<tr>
<td>% Lean mass (mean ± SEM)</td>
<td>44.9 ± 0.5</td>
<td>37.5 ± 0.6†</td>
<td>43.3 ± 0.7</td>
<td>43.8 ± 0.8</td>
<td>43.7 ± 0.7</td>
</tr>
<tr>
<td>% Visceral fat (mean ± SEM)</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.6 ± 0.2</td>
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<tr>
<td>% Subcutaneous fat (mean ± SEM)</td>
<td>2.5 ± 0.2</td>
<td>5.6 ± 0.4§</td>
<td>2.6 ± 0.3</td>
<td>2.9 ± 0.4</td>
<td>3.5 ± 0.6</td>
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</table>

† p< 0.0001 vs. all groups
‡ p< 0.05 vs HTZ/pbo and CJC/24h
§ p< 0.01 vs. all groups
**Table 2** Prolactin mRNA levels (in arbitrary units) measured by Northern blot and normalized by GAPDH RNA. In parenthesis the fold increase compared to placebo-treated GHRHKO animals.

<table>
<thead>
<tr>
<th></th>
<th>HTZ/pbo</th>
<th>GHRHKO/pbo</th>
<th>CJC/24h</th>
<th>CJC/48h</th>
<th>CJC/72h</th>
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<tr>
<td></td>
<td>3972</td>
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<td>3991(2.4)</td>
<td>4155 (2.5)</td>
<td>3846 (2.3)</td>
</tr>
</tbody>
</table>
Fig. 3

GH mRNA (arbitrary units)

GHRHKO/pbo  HTZ/pbo  CJC/24h  CJC/48h  CJC/72h

21 KDa

Fig. 3
Serum IGF-1 (ng/ml)

GHRHKO/pbo  HTZ/pbo  CJC/24h  CJC/48h  CJC/72h

Fig. 5