Influence of Estrogen Administration on the Growth Response to Growth Hormone (GH) in GH-Deficient Mice

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In women who are growth hormone (GH) deficient, exogenous estrogens increase the dosage of GH that is needed to normalize circulating levels of insulin-like growth factor (IGF-1). Serum IGF-1 derives mostly from the liver, and it is unknown whether the peripheral effects of GH are also impaired by estrogens. Because the ultimate effect of GH is longitudinal growth, we have investigated the influence of estrogen administration on the growth response to recombinant mouse GH therapy in prepubertal GH-deficient (GHD) GHRH knockout (GHRHKO) female mice. Twenty-four GHRHKO female mice (4 animals/group) were treated for 4 weeks (from the second to sixth week of age) with the following schedules: Group I, GH only (25 μg/day); Group II, subcutaneous (sc) ethynil estradiol (EE) (0.035 μg/day); Group III, GH + scEE; Group IV, oral (po) EE (0.035 μg/day); Group V, GH + poEE; Group VI, placebo. At the end of the treatment period, we measured uterine weight, total body weight (TBW), body length (nose-anus, N-A), and femur length. In addition, serum IGF-1 levels were measured. Uteri of mice treated with oral or scEE showed similar increases in weight. There was no difference in the increase in longitudinal growth parameters between mice treated with GH alone or with GH in association with oral or scEE. Serum IGF-1 decreased in animals treated with GH + scEE, compared with GH group, but no group was significantly different from placebo. These results show that subcutaneous or oral EE does not reduce the growth response to GH in female GHD mice. Exp Biol Med 230:715–720, 2005

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Growth hormone (GH) stimulates longitudinal somatic growth, acting both directly and through the mediation of insulin-like growth factor-1 (IGF-1) (1). Circulating IGF-1 is mostly produced by the liver (2), and it is routinely used as a marker of endogenous GH secretion or to titrate the dose of GH therapy in patients with GH deficiency (GHD). There is now clear evidence that locally produced IGF-1 (rather than circulating IGF-1) is the main mediator of the peripheral effects of GH (3–5), questioning the reliability of serum IGF-1 as a marker of GH effect. Indeed, in GHD children the increment in serum IGF-1 during GH therapy does not necessarily correlate with height increase (6).

A sexual dimorphism exists in GH secretion and action. Women have higher mean 24-hr GH level than men (7–9), but serum IGF-1 are not different between genders, implying relative GH resistance. Estrogen may be the cause of such resistance. Indeed, women with GHD who also receive estrogen therapy require significant higher doses of GH to maintain equivalent serum IGF-1 levels as men (7, 10). This effect is more marked with oral than transdermal estrogen. Although it is clear that oral estrogen lowers serum IGF-1 levels (11–13), the effect of transdermal estrogen is less clear, with IGF-1 reported as unaffected (11), increased (14), or reduced (15).

Although there is some evidence that oral estrogen antagonizes the metabolic effect of GH in women affected by GHD (16, 17), the use of serum IGF-1 to titrate the dose of GH therapy contrasts with the experimental evidence in animals that serum IGF-1 does not reflect the peripheral effects of GH (18). The ultimate effect of GH is growth, so we decided to investigate how estrogen administration (oral or parenteral) would influence the effect of GH in GHD mice. We reasoned that this approach would address the question of whether estrogens can alter the efficacy of GH on peripheral tissues. To this end, we treated prepubertal female mice with targeted ablation (knockout, KO) of the GHRH gene (GHRHKO) (19) with recombinant mouse GH, alone or in combination with estrogen. Our study shows that

estrogen therapy, either oral or parenteral, does not influence the GH effect on linear growth in GHD mice.

Materials and Methods

Animals. We used 24 female GHRHKO mice on a hybrid C57BL/6-SV129 background, born from KO breeding pairs to avoid the need for early genotyping. Animals were all weaned at 4 weeks of age and housed based on sex and treatment. All mice experienced a controlled environment with 14 hrs light/10 hrs dark cycles at 21°C and 23% humidity, with standard mouse/rat food (Prolab RMH2500, PMI Nutrition International, Brentwood, MO) and water *ad libitum*. All procedures were approved by Johns Hopkins Institutional Animal Care Committee.

Ethynil Estradiol (EE) and Recombinant Mouse GH Schedules. In preliminary experiments we established that the minimal dose of mouse GH necessary to increase growth of GHRHKO mice was 25 μg/day subcutaneously (sc). We have used mouse GH (National Hormone and Peptide Program, Harbor UCLA Medical Center, Torrance, CA) because treatment with non-species-specific GH may induce the production of antibodies in rodents and thereby reduce the effectiveness of long-term treatment (20).

In preliminary experiments we verified that a dose of ethynil-estradiol (EE) of 0.035 μg/day (based on previously published rodent literature; Refs. 21, 22) was sufficient to obtain significant uterine growth in prepubertal mice.

Twenty-four 2-week-old GHRHKO female mice were divided into six groups and treated for 4 weeks as follows: Group I received GH (25 μg sc, once/day), Group II received scEE (0.035 μg once/day), Group III received a combination of GH and scEE (at the same dosages), group IV received oral EE (poEE) (0.035 μg once a day), Group V received GH and poEE (at the same dosages), and group VI received placebo (sc normal saline).

For oral treatment, EE (Sigma Aldrich, St. Louis, MO) was dissolved in 95% ethanol (1 mg/ml), diluted 1:1000 in olive oil, and administered by gavage (35 μl) as previously published (23). For sc treatment, EE was diluted in 95% ethanol (0.01 mg/ml) and then diluted daily 1:10 in normal saline before injection (35 μl).

Auxological Data. Total body weights (TBW) and body length (nose-to-anus distance, N-A) were measured at the beginning of the treatment and thereafter once a week 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, animals were sacrificed by halothane overdose (Sigma Aldrich, St. Louis, MO). Blood was obtained by cardiac puncture, and serum stored at –20°C for IGF-1 measurement. Femurs were harvested, carefully cleaned, and measured with an electronic caliper. Uteri were cut at the level of the cervix, isolated from parametrial fat and from ovaries and their wet weight measured using a precision electronic balance (AG104, Mettler-Toledo, Columbus, OH). Uterine weights were expressed as a proportion of body weight using the formula (weight [g]/TBW [g]) ×100.

Serum IGF-1 Measurement. Serum IGF-1 was measured using mouse/rat IGF-1 Radioimmunassay kit (DSL-2900, DSL Webster, TX), after acid ethanol extraction, following manufacturer recommendations. All the samples were assayed together. Each sample was assayed in duplicate. The IGF-1 assay included quality controls provided by the manufacturer. The standard curve of the assay was performed in accordance to the manufacturer's provided samples. Intra-assay coefficient of variation was 11%.

Statistical Analysis. Results are expressed as means ± SE. Because our data met the assumptions of a normal distribution, parametric statistics were used. There was sufficient power to detect biological differences (the power of our tests ranged between 0.957 and 1). Changes in body length and TBW over the course of the experiment were analyzed with mixed ANOVA with one between-subjects variable (i.e., treatment) and one within-subjects variable (i.e., weeks of treatment). Because we hypothesized that GH would affect body length and weight *a priori*, multiple pairwise comparisons were used for *post-hoc* analyses. Femur length, uterine weight, and IGF-1 concentration were analyzed using one-way ANOVA with one between-subjects variable (i.e., treatment). Significant effects were further analyzed using Tukey's *post-hoc* comparisons. *P* values less than 0.05 were considered significant.

Results

Body Length (N-A). Body length curves are shown in Figure 1. At the end of the study all the GH-treated mice (Groups I, III, and V) achieved a significantly higher length compared with the animals not receiving GH (Groups II, IV, and VI). There was no statistically significant difference in

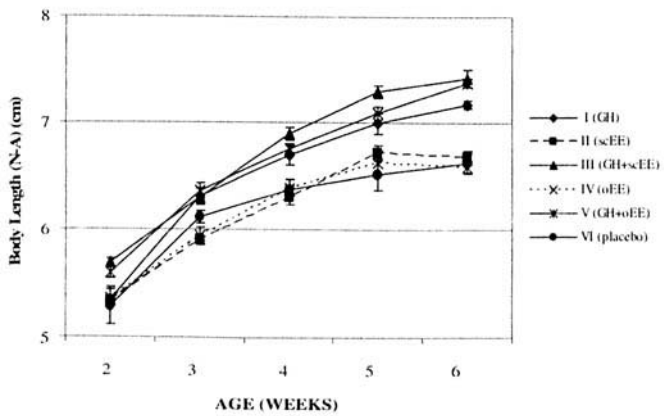

Figure 1. Effect of 4 weeks of treatment with recombinant mouse GH, alone or associated with subcutaneous (sc) or oral (o) ethynil estradiol (EE), on body length (expressed as nose-to-anus distance, in cm), compared to scEE- or poEE- or placebo-treated mice. Shown are the means of each group of animals ($n = 4$) ± SE.

Table 1. Effect of GH and EE on Final N-A Length and Final TBW[a]

Group	Final N-A length (cm ± SE)	Final TBW (g ± SE)
I (GH)	7.18 ± 0.04[b]	12.52 ± 0.23[d]
II (scEE)	6.67 ± 0.06	10.52 ± 0.31
III (GH + scEE)	7.43 ± 0.08[b]	15.57 ± 0.32[c,d]
IV (poEE)	6.59 ± 0.06	10.1 ± 0.43
V (GH + poEE)	7.39 ± 0.04[b]	14.07 ± 0.31[d]
VI (placebo)	6.62 ± 0.09	10.95 ± 0.39

[a]GHRHKO mice received recombinant mouse GH alone or associated with subcutaneous (sc) or oral (po) ethynil estradiol (EE) or only EE or placebo. Shown are means of each group of animals ($n = 4$) ± SE.
[b]$P < 0.001$ versus Groups II, IV, and VI.
[c]$P < 0.001$ versus Group I.
[d]$P < 0.05$ versus Groups II, IV, and VI.

final length among all animals receiving GH, regardless of whether GH was given alone or in association with EE, po or sc (Table 1).

Total Body Weight (TBW). Total body weight curves are shown in Figure 2. After 4 weeks of treatment, body weights were significantly higher in the groups of animals that received GH, with or without EE (Groups I, III, V), than in animals treated only with EE or placebo (Groups II, IV, VI). Mice treated with GH plus scEE (Group III) achieved a significantly higher TBW compared with mice treated with GH alone (Group I) (Table 1).

Femur Length. Similar to what was observed for body length, femur length was significantly higher in GH-treated animals (Groups I, III, and V) than in animals not receiving GH (Groups II, IV, and VI). There was no statistically significant difference in femur length among all animals receiving GH, regardless of whether GH was given alone or in association with EE, oral or sc (Fig. 3).

Uterine Weight. At the end of the treatment period, uteri of mice treated with EE, with or without GH, showed a similar weight increase, regardless of the route of administration, confirming comparable degree of estrogenization. The uterine weights among groups receiving EE were higher than those of placebo-treated animals. There was no significant difference between the placebo group and GH-only-treated animals (Fig. 4).

Serum IGF-1. Among GH-treated animals, serum IGF-1 was lower in the animals treated with GH and scEE (Group III) compared with mice receiving only GH (Group I). Animals treated only with oral EE (Group IV) had significantly lower IGF-1 levels compared with those given GH alone (Group I) and GH + poEE (Group V) (Table 2).

Discussion

Exogenous and endogenous estrogen levels influence GH and IGF-1 levels in normal women (7, 9) and can affect the response of serum IGF-1 to GH therapy in women with GHD (7, 12). The degree of the effects of exogenous estrogens depends on the dose and the route of administration (11, 13). Women on oral estrogens need a higher dose of GH replacement therapy than those on transdermal estrogens therapy to normalize serum IGF-1 (12). Hepatic inhibition of GH action by estrogens seems mostly mediated by the modulation of JAK/STAT pathway by up-regulation of the inhibiting proteins SOCS-2 and –3 (24).

Although the level of circulating IGF-1 (derived mostly from the liver) is routinely used to titrate GH dosage in GHD patients, this is only a marker of GH effect, and, at least in animals, does not necessarily reflect GH peripheral effects (3–5, 18, 25). Therefore, if GH resistance occurs mostly in the liver, it is conceivable that titrating GH therapy according only to serum IGF-1 may be done at the expenses of reaching supraphysiologic GH levels in the peripheral tissues.

The aim of our study was to examine the influence that the administration of estrogens has on GH effect on growth in prepubertal GHD female mice. Although normal mice

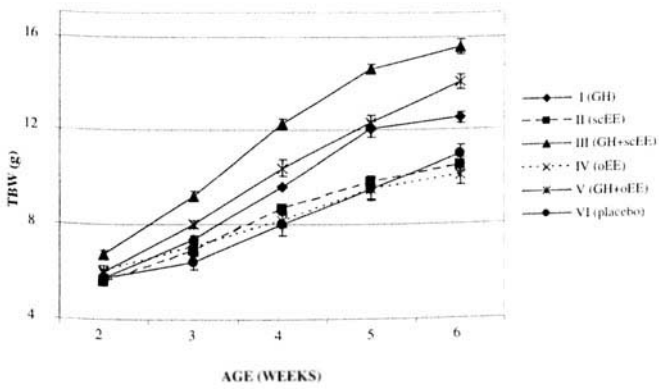

Figure 2. Effect of 4 weeks of treatment with recombinant mouse GH, alone or associated with subcutaneous (sc) or oral (o) ethynil estradiol (EE), on total body weight (TBW, grams), compared to scEE- or poEE- or placebo-treated mice. Shown are the means of each group of animals ($n = 4$) ± SE.

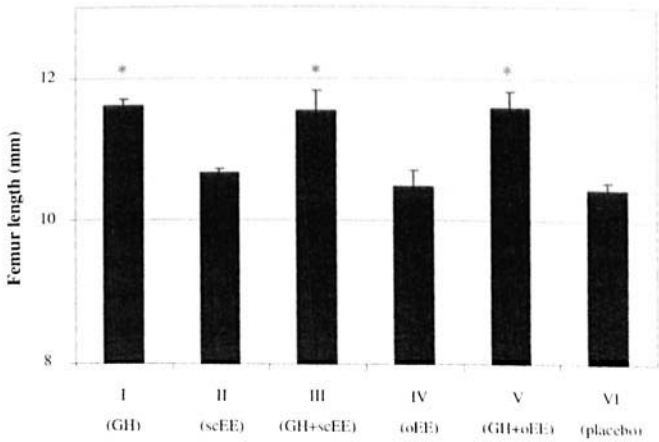

Figure 3. Effect of 4 weeks of treatment with recombinant mouse GH, alone or associated with subcutaneous (sc) or oral (o) ethynil estradiol (EE), on femur length (millimeters) compared to scEE- or poEE- or placebo-treated mice. Shown are means ± SE of each group of animals ($n = 4$). *Statistically higher ($P < 0.05$) compared to Groups II, IV, and VI.

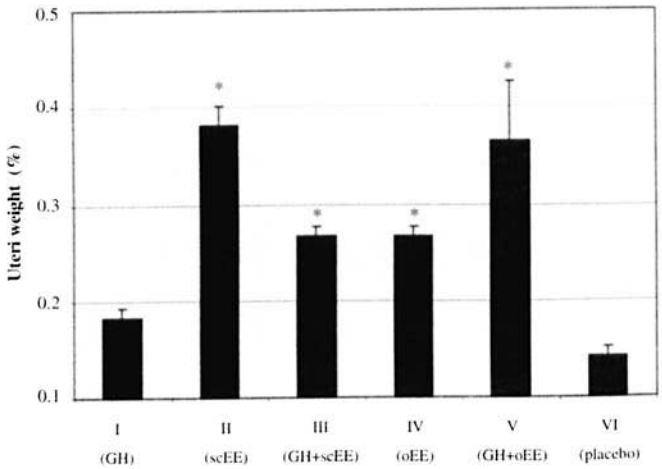

Figure 4. Effect of 4 weeks of treatment with subcutaneous (sc) or oral (o) ethynil estradiol (EE), alone or associated with recombinant mouse GH, on uterine wet weight, expressed as [uterus weight (g)/ total body weight (g)] × 100. Shown are means ± SE of each group of animals ($n = 4$). *Statistically higher ($P < 0.05$) compared to placebo group (Group VI).

complete vaginal opening during the fifth week of life, GHD or GH-resistant mice have delayed onset of completion of puberty (seventh week) (26–28). Unlike what happens in humans, mice do not fuse their epiphyseal cartilages when exposed to sex steroids (29) unless very high doses are given (e.g., 17β-estradiol 500 μg/kg/day) (30). We used a dose of EE of about 5 μg/kg/day, which we predicted would not arrest longitudinal growth but would be sufficient for adequate estrogenization. It is important to notice that the dose of EE is higher (per kilogram) than the dose of EE present in the estrogen preparations used routinely in women (approximately 0.3–0.5 μg/kg/day), making the possibility that we did not reach full estrogenic effect extremely unlikely. In addition, to avoid having any resistance masked by an excessive GH dose, we used the minimal dose of recombinant mouse GH that in preliminary studies had been able to increase growth of GHD mice. We administered GH either alone or associated with EE, po or sc. As controls, we used animals receiving placebo or EE alone, po or sc. Oophorectomy was not performed because this surgery is technically difficult in 2-week-old mice and requires an extended period of recovery. In addition, animals were treated during their prepubertal age, when they have low endogenous estrogen levels (26–28). Although this model does not mimic what happens in adult women, it allows us to study the effect of estrogen on animals that are at the age of maximal potential growth.

Table 2. Effect of GH and EE on Serum IGF-1[a]

Group	Serum IGF-1 (ng/ml ± SE)
I (GH)	230.21 ± 35.98
II (scEE)	143.39 ± 17.18
III (GH + scEE)	120.28 ± 7.22[b]
IV (poEE)	80.65 ± 9.26[c]
V (GH + poEE)	197.03 ± 29.56
VI (placebo)	136.99 ± 10.40

[a]GHRHKO mice received recombinant mouse GH alone or associated with subcutaneous (sc) or oral (po) ethynil estradiol (EE) or only EE or placebo. Shown are means of each group of animals ($n = 4$) ± SE.
[b]$P < 0.05$ versus Group I.
[c]$P < 0.02$ versus Groups I and V.

All the animals receiving EE, either orally or parenterally, achieved comparable degrees of estrogenization, as demonstrated by similar increases in uterine weights, proving EE absorption by both routes. Measurement of final N-A length and femur length after 4 weeks of treatment showed that the growth of animals receiving only GH was not different from that in animals that received GH in association with EE, either orally or subcutaneously. At the end of the treatment, all GH-treated groups achieved significantly greater N-A and femur lengths compared to placebo group and to mice receiving only EE. All animals treated with GH, whether associated with po or scEE, achieved a statistically higher TBW compared with mice receiving only EE or placebo. Taken together, these results demonstrate that, in female GHD mice, estrogen administration does not reduce the effect of GH therapy on linear growth, regardless of the route of administration. The fact that animals treated with GH + scEE increased their TBW more than mice receiving only GH, despite the absence of difference in length, may result from changes in body composition that are independent of longitudinal growth. Regrettably, we did not measure individual body compartments (fat mass and body mass) and therefore cannot draw any conclusions in this sense. Nevertheless, body length and femoral length are universally used as indices of GH growth-promoting activities (3, 5, 18, 28, 31).

Although the endpoint of our study was the effect of EE and GH on growth, we observed that mice receiving GH + scEE showed a statistically significant decrease (47.7%) in circulating IGF-1 compared to animals treated with GH alone, whereas in mice treated with GH + poEE, the decrease (14.4%) was not significant. We had expected an opposite result, reasoning that the first-pass effect of poEE would cause higher GH resistance in the liver. Nevertheless, the literature on the effect of different routes of administration on serum IGF-1 is quite controversial and limited to human studies. In women, different results have been described, with serum IGF-1 being unaffected (13), increased (14), or decreased (15) by transdermal estrogens. It has been suggested that the lower effect of transdermal estrogens on the GH milieu may depend on the lower dose of hormone delivered to the circulation. Therefore, administration of higher dose of estrogens even if they bypass the oral route may cause more evident inhibitory effects on hepatic GH responsiveness. In the absence of reliable "conversion" methods, we used identical doses of parenteral and oral EE. It is possible that parenteral administration of estrogens results in a higher adsorption rate than the

transdermal route. Furthermore, the effect of EE on liver has been shown to be greater than those of other estrogen types, and is not entirely eliminated when EE is administered parenterally (32, 33). Therefore, the effect of scEE on the liver (the main source of circulating IGF-1) may mimic those of poEE (despite the lack of first-pass effect). Nevertheless, despite different serum IGF-1 levels, neither EE regimen affected the growth response to GH.

We did not find a significant increase in serum IGF-1 in animals treated with GH when compared with placebo. Although this may seem surprising, the observation that serum IGF-1 levels do not directly correlate with the somatic growth response to GH administration in mice and children has been previously reported by others and us (5, 6, 18, 25, 34). We reported that GH replacement can fully normalize growth in GHRHKO mice without increasing serum IGF-1 (18). This observation suggests that GH may act on growth independently of circulating IGF-1.

In conclusion, our study demonstrates that EE administration, either oral or parenteral, does not affect the linear growth response to GH administration in GHD female mice. This finding challenges the concept that the action of GH therapy on peripheral tissues is influenced significantly by estrogen administration and raises doubts on the appropriateness of using serum IGF-1 levels to titrate GH dosing in women on estrogen replacement.

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