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Functional Alteration of the Somatotrophic Axis in Transgenic Mice with Liver-Specific Expression of Human Insulin-like Growth Factor Binding Protein-1

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ABSTRACT

In earlier work, postnatal growth restriction (more marked in males) was observed in a model of transgenic mice with liver-specific expression of human IGF binding protein-1. This was associated with diminished plasma IGF-I levels, the cause of which remained unexplained. Subsequently, abnormalities of CNS development were ascertained, justifying investigation of the somatotrophic axis. Pituitary gland weight in transgenic animals was reduced proportionally to body weight. Immunohistochemical examination of the pituitaries in 3- to 4-mo-old mice revealed somatotrophs of normal size in homozygotes, but density was decreased to approximately two thirds of that in wild-type siblings ($p = 0.001$). The same was true of lactotrophs. The GH content of the pituitary was significantly reduced in heterozygotes ($p < 0.02$) and more so in homozygotes ($p < 0.0003$), although the GH/total protein ratio was similar to that in wild types. Pituitary perifusion experiments showed that *in vitro* the amounts of GH secreted under basal conditions and under GH-releasing hormone stimulation were similar in transgenic and wild-type mice. Ten days of treatment with human GH (100 μg/d) in 45-d-old transgenic and wild-type mice provoked significant weight gain ($p = 0.02$) in all animals, the means being 12.4% for homozygotes and 10.4% in heterozygotes, as opposed to 5.8% in wild-type mice. The increase in weight tended to correlate with an increase in plasma IGF-I. From these results, we conclude that the reduced plasma IGF-I in IGF binding protein-1 transgenic mice may result from insufficient GH production by the depressed number of somatotrophs, possibly associated with functional alteration of hypothalamic control. **(*Pediatr Res* 52: 168–174, 2002)**

Abbreviations

IGFBP, IGF binding protein
hIGFBP, human IGF binding protein
GHRH, GH releasing hormone
ALS, acid-labile subunit
DAB, 3,3'-diaminobenzidine tetrahydrochloride dihydrate

A model of transgenic mouse was established in our laboratory, with liver-specific expression of hIGFBP-1 under the control of the α_1-antitrypsin promoter. The repercussions of this expression, which begins during fetal life, were particularly marked among homozygotes in one of the lines. The signs included reproductive abnormalities, a high incidence of ante- and perinatal mortality, and postnatal growth restriction that was more pronounced in males (1).

Plasma concentrations of hIGFBP-1 resulting from expression in the liver, the normal site of synthesis, remained within the physiologic range (3.0 ± 0.4 ng/mL in heterozygotes, 13.7 ± 1.4 ng/mL in homozygotes), but at constant levels, without the insulin-dependent fluctuations seen in the normal state (2). Although IGFBP-1 levels in the transgenic mice were low, they were higher than those in wild-type animals in which the strong signal in Western ligand blotting during the perinatal period was no longer detectable beyond the age of 6 d, unlike that in the transgenic mice (1). It therefore seemed possible that the growth restriction in the transgenic animals resulted from decreased bioavailability of free IGF-I sequestered by IGFBP-1, particularly in view of the unexpected, concomitant, and significant reduction of total plasma IGF-I in adult homozygotes (370 ± 75 ng/mL) by almost half compared with

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that in controls (630 ± 56 ng/mL), whereas in heterozygotes IGF-I levels were only slightly lower (544 ± 95 ng/mL) (1).

It did not seem likely that the reduced IGF-I was attributable to enhanced clearance. IGFBP-3 concentrations estimated from Western ligand blotting were in normal proportion to IGF-I, which, in largest part, is associated with IGFBP-3 and the ALS in 150-kD complexes that do not cross the capillary endothelium (3). The ALS, which is GH-dependent, could not be measured in these mice, but it has been demonstrated that even in GH-deficient rats, the ALS circulates in excess (4). IGFBP-2 and IGFBP-4 are also capable of forming short-lived binary complexes with IGF-I. However, their concentrations in the transgenic mice were similar to those in wild types as determined by ligand blotting, and in all cases were very low compared with those of IGFBP-3 (1).

The decrease in IGF-I was surprising, in that oversecretion of GH would have seemed more probable, resulting from depression or suppression by excess IGFBP-1 of the IGF-I–mediated negative feedback on GH secretion (5). There were two possible explanations: either IGFBP-1 exerted inhibitory effects on hepatic IGF-I synthesis, both peptides being produced by hepatocytes (6), or IGFBP-1 damaged the hypothalamo-pituitary system, considering the anomalies of CNS development affecting the cerebral cortex, corpus callosum, and hippocampus subsequently discovered in the transgenic mice (7). The present study was undertaken to identify the point(s) at which the somatotrophic axis is affected, to account for the depressed plasma IGF-I and growth restriction.

METHODS

Animals and biologic samples. Transgenic B6/CBA mice carrying the human α_1-antitrypsin promoter fused to hIGFBP-1 cDNA have been described elsewhere (1). Northern blotting revealed transgene expression exclusively in the liver during fetal life and unchanged through to adulthood. Between the two lines obtained initially, line 149 exhibited more developmental abnormalities, the severity of which was directly related to the level of transgene expression. This was the line subjected to further investigation. Heterozygous and homozygous animals and nontransgenic litter mates were identified on the basis of Southern blot analyses of DNA samples from tail biopsies (1).

Pituitaries were excised after decapitation of 3- to 4-mo-old mice for purposes of GH measurement, immunohistochemical analysis, and perifusion experiments.

Six-week-old mice were treated with GH (hGH Norditropine, 24 U/mg, NovoNordisk, Bagswaerd, Denmark). Blood samples for the IGF-I assays were taken by intraorbital puncture and collected in tubes rinsed with 0.1M EDTA. Plasma samples were stored at −20°C.

All protocols were approved by the institutional review committee.

Pituitary immunohistochemistry. Rabbit antibodies against rat prolactin and rat GH (which cross-react well in the mouse) were kindly provided by A.F. Parlow (NIDDK, Bethesda, MD, U.S.A.) and J. Trouillas (Lyon, France), respectively. Goat peroxidase-labeled antibody against rabbit IgG was purchased from Interchim (Montlucon, France) and DAB from Aldrich Chimie (L'Isle d'Albeau Chesnes, France).

Pituitaries embedded in paraffin were serially sectioned at a thickness of 10 μm. Sections were incubated for 30 min in PBS containing 0.3% H_2O_2 to remove endogenous peroxidase activity and then incubated for 15 min in PBS containing 7% sheep serum to saturate nonspecific binding sites. Thereafter, they were incubated overnight at 4°C with PBS containing 0.1% BSA and either anti-rat prolactin antibody (1:40,000) or anti-rat GH antibody (1:2,000). After washing, the sections were incubated for 6 h at room temperature with the peroxidase-labeled anti-rabbit IgG antibody (1:20,000). Staining was achieved by incubating the sections in Tris-HCl (20 mM, pH 7.8) containing 0.2 mg/mL DAB and 0.0036% H_2O_2 (vol/vol). Negative controls were obtained by replacing primary antibodies with nonimmune serum. The number and sizes of cells immunoreactive for prolactin and GH were assessed using a ×100 objective lens within an ocular micrometer.

Pituitary perifusion experiments. In each experiment, the pituitaries from three wild-type males and three homozygous males were perifused simultaneously. Immediately after excision, pituitaries were washed in perifusion chambers (0.5 mL volume) for 30 min at 37°C in oxygenated Dulbecco's modified Eagle's medium (with L-glutamine, 4.5 g/L glucose, 25 mM HEPES) containing 0.1% BSA, and superfused at a rate of 0.1 mL/min with the same medium. After a 120-min equilibration period, effluents were collected every 5 min. Mouse GHRH (Phoenix Pharmaceuticals, Belmont, CA, U.S.A.; 10^{-7} M) and 0.03 M KCl was added to the medium for 15- and 20-min periods, respectively, separated by perifusion with medium alone. Samples were frozen until GH determination.

Pituitary extracts. After excision, pituitary glands were frozen in liquid nitrogen and stored at −80°C. Extracts were obtained by sonication in 0.1 M $NaHCO_3$, followed by centrifugation to remove tissue debris. The Bradford assay (Bio-Rad protein assay, Munich, Germany) was used to determine protein concentration, using BSA as the standard.

GH RIA. GH concentrations in pituitary extracts and perifusion media were determined by RIA using reactants developed and generously provided by A.F. Parlow (Harbor-UCLA Medical Center, Torrance, CA, U.S.A.). Purified mouse GH preparations were used for iodination by the chloramine T method and for the standard curves. Anti-rat GH antibody was used at 1:3,200,000 final dilution. Samples were studied at three dilutions, each in duplicate. Incubation was conducted for 3 d at 4°C in a total volume of 800 μL in 0.01 M PBS, 0.25% BSA, and 0.01 M EDTA. Separation of bound and free hormone was achieved by immunoprecipitation using polyethylene glycol and bovine immunoglobulin. Intra- and interassay variations were approximately 3% and 10%, respectively.

Plasma IGF-I assay. This was performed as previously reported (1). Briefly, 25-μL samples were incubated in 0.01 M HCl and ultrafiltered on Centricon 30 (Amicon, Epernon, France) to dissociate and separate IGFs from IGFBPs. IGF recovery is close to 100% in the ultrafiltrate in which neither IGFBPs nor their proteolytic fragments are detectable (8). The ultrafiltrates were lyophilized, then taken up in 0.1 M phosphate buffer, 0.1% BSA, and incubated for 3 d at 4°C in a final

volume of 400 μL with ^{125}I-IGF-I and a polyclonal anti-hIGF-I antibody (1:100,000) that cross-reacts with murine IGF-I. The standard curve was established with the same preparation of recombinant hIGF-I as that used for iodination. Unknown samples were tested at two concentrations plus one blank (without antibody), each in duplicate. After incubation, free and bound IGFs were separated using albumin-coated charcoal. Intraassay variation was less than 5% and interassay variation, 10%.

Statistics. Conventional methods were used for comparisons of the means (Student's *t* and Mann-Whitney *U* tests) and linear regression analysis. Probability values ≤ 0.05 were regarded as significant.

RESULTS

GH content of pituitary glands. The pituitary glands of homozygous transgenic mice were decreased in weight approximately proportionally with their body weights, as compared with wild-type siblings (Table 1). Figure 1 shows the GH content of the pituitaries of heterozygous, homozygous, and wild-type mice. Although the values were widely dispersed in both transgenic and wild-type animals, on average they were higher in males than in females, the means for heterozygotes were significantly lower than those for wild types, and the means for homozygotes were significantly lower than those for heterozygotes. There was nevertheless a positive correlation between GH values and total protein content (Fig. 2), and the ratios of GH/total protein were of the same order of magnitude in transgenic and wild-type mice (Table 2).

Immunocytochemistry. Compared with those in wild-type animals, the somatotrophs and lactotrophs of homozygotes appeared of normal size, but density was reduced (Fig. 3). There was no significant difference between males ($n = 3$) and females ($n = 4$). For all the hypophyses examined, the number of somatotrophs in the same area was decreased by 28% (41 ± 2 versus 57 ± 3 per 19,000 μm^2 surface area in wild types, $p = 0.001$) and that of lactotrophs, by 35% (42 ± 2 versus 66 ± 1 per 19,000 μm^2 surface area in wild types, $p = 0.0001$).

The observation of reduced cell population but normal GH/total protein ratio would indicate *in vivo* stimulation of GH secretion. It was therefore necessary to assess the secretory capacity of the pituitary glands.

Perifusion of the pituitary glands. The results shown in Figure 4 represent the GH secretion profiles *in vitro* by pituitaries of male homozygotes and wild-type mice (means for three pituitaries in the same experiment). The quantities of GH secreted were similar, both under basal conditions and under stimulation. The means of the cumulative GH concentrations in the eluates collected between 35 and 80 min of perifusion, corresponding to the peaks induced by GHRH, were 1384 ± 128 ng (±SEM) for 500 μL in wild types and 1385 ± 296 ng in homozygotes. The means for the cumulative concentrations corresponding to the peaks induced by potassium depolarization (eluates collected between 85 and 130 min of perifusion)

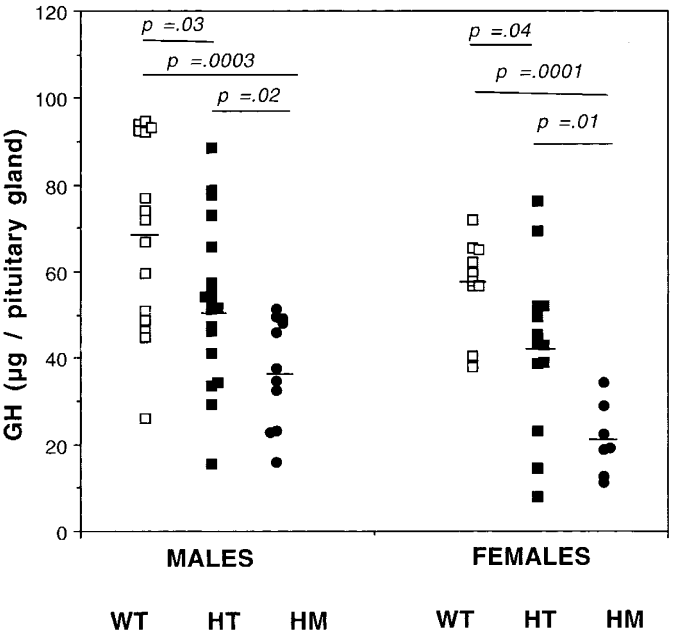

Figure 1. GH content of the pituitary glands of heterozygous (*HT*) and homozygous (*HM*) IGFBP-1 transgenic and wild-type (*WT*) mice aged 3–4 mo (*p* values obtained using the *t* test).

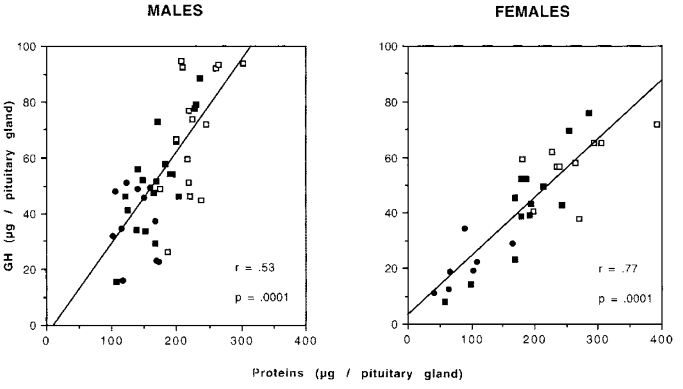

Figure 2. GH as related to total protein content in the pituitary glands of IGFBP-1 transgenic mice (● homozygotes, ■ heterozygotes) and wild types (□).

Table 1. *Body and pituitary weights of 6-mo-old wild-type and IGFBP-1 transgenic mice*

	Wild types ($n = 7$)	Heterozygotes ($n = 6$)	Homozygotes ($n = 11$)
Body weight (g)	35.4 ± 1.8	32.8 ± 3.4	26.8 ± 0.6*
Pituitary weight (mg)	1.90 ± 0.18	1.62 ± 0.11	1.30 ± 0.08**
Pituitary weight (mg)/body weight (g) × 100	5.34 ± 0.45	4.75 ± 0.62	4.94 ± 0.37

Results are mean ± SEM.

* $p = 0.002$ versus heterozygotes and 0.0001 versus wild types (Student's *t* test).

** $p = 0.03$ versus heterozygotes and 0.002 versus wild types (Student's *t* test).

Table 2. *GH to total protein ratio in the pituitary glands of homozygous and heterozygous IGFBP-1 transgenic and wild-type mice*

	Males	Females
HM	0.28 ± 0.03	0.24 ± 0.03
HT	0.30 ± 0.02	0.21 ± 0.02
WT	0.30 ± 0.02	0.23 ± 0.02

Results are mean ± SEM.
Abbreviations used: HM, homozygous; HT, heterozygous; WT, wild type.

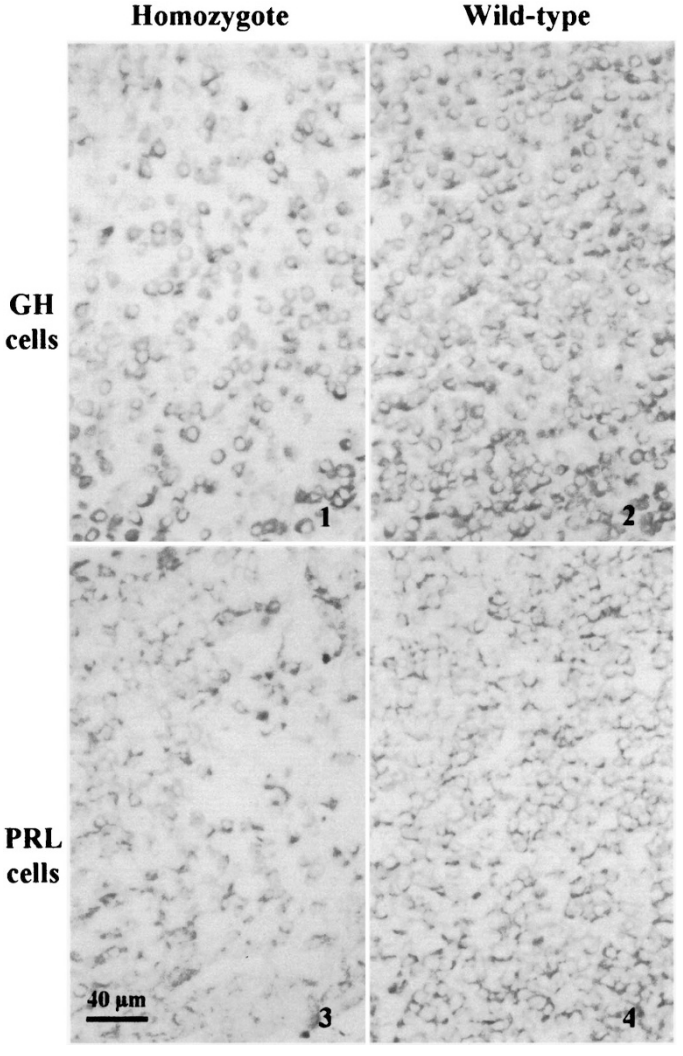

Figure 3. GH- and prolactin (PRL) immunoreactive cells in the pituitaries of adult homozygous IGFBP-1 transgenic and wild-type mice. Original magnification ×400. Cell size was similar in the two groups, but the number of somatotrophs per 19,000 μm^2 surface area was 41 ± 2 in homozygotes (n = 7 pituitaries), as opposed to 57 ± 3 in wild types (n = 6, p = 0.001), and the number of lactotrophs was 42 ± 2 in homozygotes (n = 6), as opposed to 66 ± 1 in wild types (n = 6, p = 0.0001) (t test).

were 3924 ± 293 ng for 500 µL in wild types and 4497 ± 773 ng in homozygotes.

hGH treatment. Forty-five-day-old transgenic and wild-type mice were treated simultaneously for a period of 10 d (with a break at d 6). hGH was administered by i.p. injection (50 µg in saline solution) twice daily, yielding a total dosage of 900 µg. Controls consisted of wild-type mice treated in parallel with saline solution.

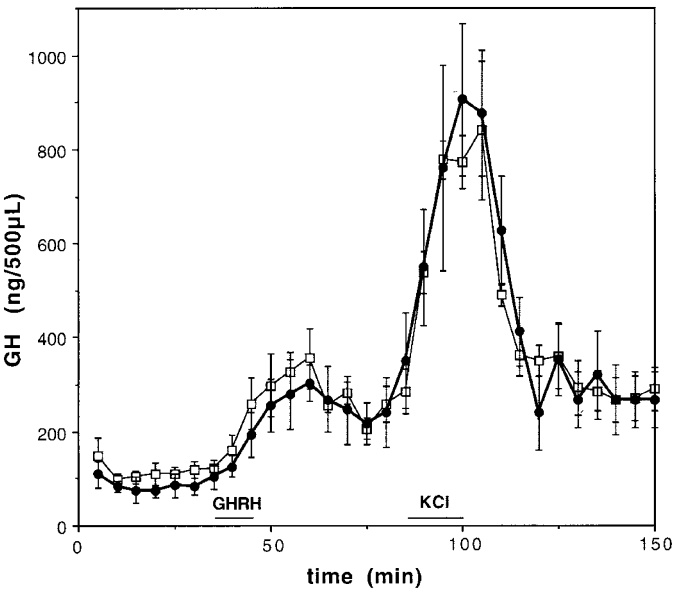

Figure 4. GH secretion by pituitary glands of male homozygous IGFBP-1 transgenic (●) and wild-type (□) mice superfused at 0.1 mL/min in perifusion chambers. Effluents were collected every 5 min for GH measurement. Mouse GHRH (10^{-7} M) was added to the medium for 15 min and KCl (0.03 M) for 20 min. Each curve represents the means of secretion by three pituitaries in the same experiment. Similar results were obtained in another experiment.

The curves for weight gain presented in Figure 5 show an acceleration of growth during GH treatment, which was modest in wild types and more pronounced in homozygotes and heterozygotes, followed by slower growth after the end of treatment. Figure 6 shows the means (±SEM) of the percentage weight increases between the beginning and end of treatment (24 h after the last injection), compared with those for

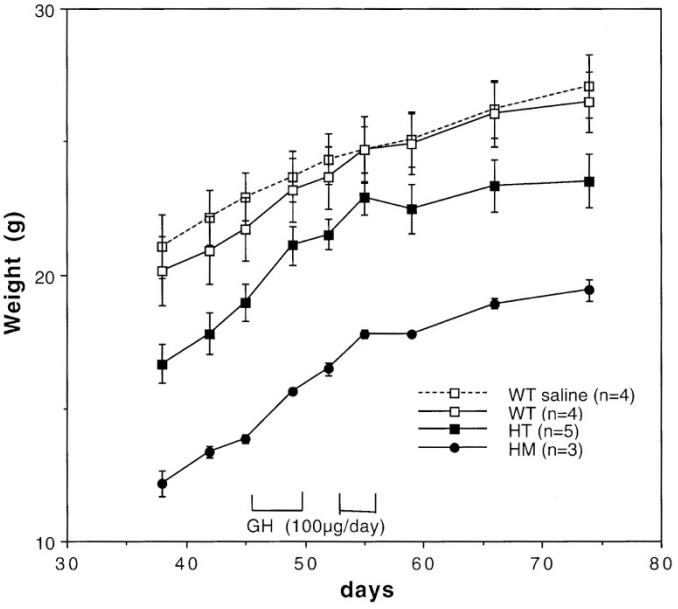

Figure 5. Weight curves for IGFBP-1 transgenic mice (homozygotes, *HM*; heterozygotes, *HT*) and wild types (*WT*) treated from the age of 45 d with human GH for a period of 10 d. Numbers of animals per group are indicated in parentheses. Wild-type controls were treated with saline solution (vehicle).

untreated animals of the same age. Among untreated animals, mean weight gain was lesser in wild types than in heterozygotes and homozygotes, the weights of which at the age of 45 d were considerably lower than those of wild types (Fig. 5). Injection of saline (vehicle) in control wild types had no effect on growth. Weight gain with GH treatment was 28.5 ± 0.5% in homozygotes, as compared with 20.7 ± 2.9% in heterozygotes ($p = 0.025$) and 13.7 ± 0.8% in wild types ($p = 0.001$). In all three treated groups, growth stimulation by GH was significant ($p \leq 0.02$) when compared with the spontaneous growth of untreated animals. After subtracting the weight gain of untreated mice, growth that could be attributed specifically to GH treatment was on average 12.4% in homozygotes and 10.4% in heterozygotes, as opposed to 5.8% in wild types.

Plasma IGF-I was assayed before and 24 h after GH treatment. These levels (in nanograms per milliliter) are shown in Figure 7. Calculations of percent variation showed that in wild types injected with saline solution, there was minimal change (−7 to +14%). In GH-treated wild types, IGF-I increased in two of four cases (+33 and +47%). Among heterozygotes, four of five of which had basal IGF-I levels at the lower limit or below those of corresponding wild types, plasma IGF-I after GH treatment remained stable in the mouse with the highest basal level and in the others, it increased (28%, 53%, 154%, and 457%). In the three homozygotes investigated, plasma IGF-I before treatment was below that of wild types and rose after treatment by 19%, 43%, and 92%. Statistical analysis revealed a significant difference in IGF-I levels before and after treatment in heterozygotes ($p = 0.025$) and in the transgenic mice (heterozygous and homozygous) taken as a group ($p = 0.015$, *t* test). For all animals, it appeared that there was a relationship between percentage weight gain and IGF-I levels, as suggested by the correlation between the two variables when the two extreme increases of 154% and 457% were excluded ($r = 0.56$, $p = 0.002$, not shown).

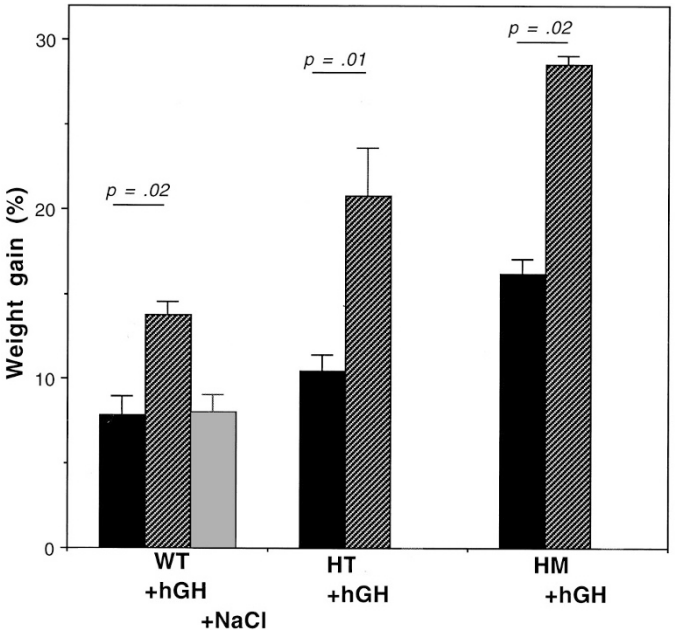

Figure 6. Mean weight gain in GH-treated transgenic (homozygotes, *HM*; heterozygotes, *HT*) and wild-type (*WT*) mice (*hatched bars*). Wild-type controls were treated with saline (*shaded bar*). The means are compared with those for untreated animals of the same age (*filled bars*). Weight gain was calculated on the basis of the difference between weight at the beginning and 24 h after the end of treatment as a percentage of weight at the beginning of treatment (*p* values obtained using the Mann-Whitney *U* test).

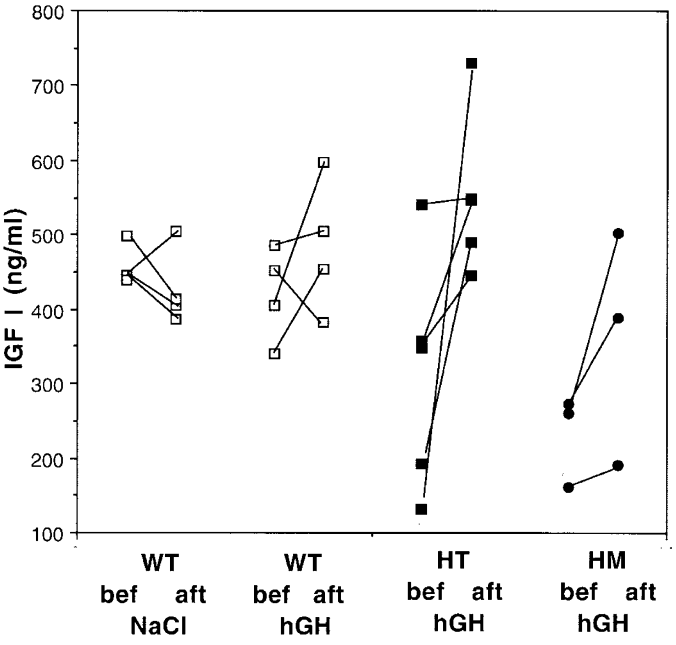

Figure 7. Plasma IGF-I levels in mice before (*bef*) and 24 h after the end of (*aft*) GH treatment (wild types, *WT*; heterozygotes, *HT*; homozygotes, *HM*). *NaCl*, wild-type mice injected with saline solution.

DISCUSSION

In this line of transgenic mice with liver-specific hIGFBP-1 expression, investigation of the cause of the drop in plasma IGF-I levels and its effects on growth restriction revealed a 28% reduction in somatotroph density in the pituitary glands of homozygotes, as compared with wild-type animals. However, assays of pituitary GH content showed that the GH/total protein ratios in transgenics and wild types were similar. This would suggest increased GH synthesis by the smaller somatotroph population, which reflects an appropriate response of the hypothalamo-pituitary system to the sequestration of free IGF-I by excess IGFBP-1. The perifusion experiments indicated that there was little difference between transgenics and controls in the amounts of GH secreted *in vitro* under basal or GHRH-stimulated conditions. In the absence of specific antibody, we were unable to measure the GHRH content of hypothalami. Nevertheless, the data for pituitary GH content and GH/total protein ratios indicate that the somatotrophs must be stimulated by GHRH *in vivo*.

Plasma GH levels had been measured in mice of this line during the earlier study. No difference was found between transgenics and wild types (9). However, these measurements are not informative regarding production in the pituitary, GH clearance being rapid, unlike that of IGF-I (3). The fact that plasma IGF-I levels in homozygous males were only half those of wild types (1) means that hepatic production of IGF-I was defective. Moreover, the negative correlation seen between

IGF-I and IGFBP-1 levels in adult heterozygous and homozygous animals (1) clearly demonstrated the link between IGF-I levels and transgene expression.

It seemed possible that IGFBP-1 could directly inhibit IGF-I synthesis in the liver, inasmuch as both peptides are produced by hepatocytes (6). However, from the tests using GH treatment, which provoked a rise in plasma IGF-I and a marked acceleration of growth in transgenics, it is evident that their hepatocytes responded normally to their physiologic stimulus and that inhibition by IGFBP-1 is unlikely. It could therefore be concluded that insufficient GH production may account for the diminished IGF-I levels. The reduced population of somatotrophs could explain this. However, because the pituitary glands of homozygotes were capable of secreting as much GH *in vitro* as the wild-type counterparts, the question arises of a functional alteration of hypothalamic control that would result in insufficient production of GH *in vivo*. Circumstantial evidence would plead in favor of hypothalamo-pituitary dysfunction: *1*) the more pronounced growth retardation in homozygous males than in females (1) suggests a decrease in the frequency or amplitude of GH pulses, which are characteristic of the sexual dimorphism of the somatotrophic axis (10); and *2*) homozygotes exhibit reproductive abnormalities associated with severe alteration of ovulation in transgenic females. LH levels in the pituitaries of homozygotes have recently been shown to be more than eight times higher than those in wild types. This is accompanied by a slight increase in the number of LH-gonadotrophs and a reduction in their size, which would suggest some alteration of the pulsatile secretion of LH (P. Froment *et al.*, manuscript in preparation).

The reduced numbers of GH- and prolactin-producing cells would point toward a disturbance in adenohypophyseal development, associated with the other anomalies of the CNS observed in these transgenic mice (7). Somatotrophic and lactotrophic changes have been reported in a line of mice with a disrupted IGF-I gene, in which GH cell size, but not number, is significantly reduced, with a GH RNA signal stronger than in controls. In contrast, the number of lactotrophs and the prolactin hybridization signal are significantly diminished (11). Despite the differences, the abnormalities described in these two models of transgenic mice suggest that IGF-I is involved in the development of the adenohypophysis. Also, hypertrophy and hyperplasia of somatotrophs have been reported in transgenic mice overexpressing GHRH (12). Interestingly, reduced plasma GH and prolactin levels, decreased size and increased number of LH immunoreactive cells, and increased LH concentration in the pituitary have also been observed in streptozotocin-induced diabetic rats (13, 14), suggesting a similar alteration of the hypothalamo-pituitary axis.

The results of this study therefore indicate that insufficient GH production is implicated in the growth restriction of our transgenic mice. In two other models of IGFBP-1 transgenic mice obtained using the ubiquitous metallothionein (15) or phosphoglycerate kinase (16) promoters, growth retardation was observed only in the latter and the somatotrophic axis was not investigated. In our model, it is noteworthy that the initial disturbance responsible for the anomalies is overexpression of IGFBP-1 at its normal site of synthesis, the liver. The excess IGFBP-1 provided via the bloodstream to the adenohypophysis and to the CNS before the blood–brain barrier becomes functional may alter their development either by some intrinsic action at the cellular level or, more probably, by reducing IGF bioavailability.

It is tempting to draw a comparison between the changes in the somatotrophic axis in this model with those reported in intrauterine growth restriction in the rat and in man, in which the complex pathophysiology is governed by both maternal and fetal factors. Here, low IGF-I and high IGFBP-1 serum levels have been observed in the fetus and at birth (17–21). In children with persistent postnatal growth retardation, diminished spontaneous secretion of GH (22, 23) constitutes the rationale for GH treatment (24, 25).

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