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Changes in Endogenous Growth Hormone Secretion and Onset of Puberty in Transgenic Rats Expressing Human Growth Hormone Gene

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Abstract. A chimeric gene comprising murine whey acidic protein (mWAP) and human growth hormone (hGH) was used to produce transgenic rats that express hGH and secrete it into the blood. Two lines of transgenic rats carrying the mWAP/hGH construct were established: Line 1 was characterized by relatively high levels of serum hGH, and Line 2 had relatively low levels. The secretory profiles of rat GH (rGH) as well as hGH, the transgene product, were obtained in transgenic males and females of Line 2; both hGH and rGH serum levels were flattened with no episodic fluctuations, and the overall mean concentration of rGH was significantly lower than in normal littermates. Although the animals of Line 1 showed an accelerated increase in growth rate, those of Line 2 did not. Nevertheless, the onset of puberty in females, as assessed by vaginal opening and occurrence of first ovulation, advanced by 7–8 days in both Lines of animals. Accordingly, the body weight at puberty of Line 2 transgenic females was much lower than that of normal littermates, indicating that continuous hGH expression could induce precocious puberty without enhancing the growth rate.

Key words: Transgenic rat, GH, Puberty

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GROWTH hormone (GH), which is secreted from the anterior pituitary gland, stimulates postnatal somatic growth, and alters carbohydrate and lipid metabolism [1]. Some of these effects are brought about by enhancing liver cell production of insulin-like growth factor (IGF)-I [2]. Recently, a number of transgenic mice were produced that express GH genes derived from different mammalian species [3–7], and these studies contributed new understanding of the effects of GH on various tissues and cells.

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Whey acidic protein (WAP) is a mammary gland specific protein in rodents that is secreted into the milk [8]. A complex of lactogenic hormones regulate WAP gene expression [9]. Using the regulatory sequence from the murine WAP (mWAP) gene, several lines of transgenic animals were produced that secrete exogenous proteins into the milk [10–14]. It is known, however, that transgenic mice carrying the mWAP/human GH (hGH) construct secrete hGH not only into the milk but also into the blood, although hGH secretion into the blood is not as intense as in the milk [15]. Furthermore, blood hGH level in these mice are lower than in transgenic mice carrying hGH chimeric genes comprised of other regulatory sequences from other protein genes, such as the metallothionein gene [15]. In the latter, blood hGH levels are usually so high that metabolic and/or

reproductive disorders occur soon after birth [3, 6, 16, 17]. Thus, the WAP gene regulatory sequence could be evaluated as a promoter to produce transgenic animals expressing moderate blood levels of GH.

Because more background data concerning GH actions are accumulated for rats than mice, we produced transgenic rats in this study that express hGH under the control of the regulatory sequence from the murine gene encoding WAP and secrete it moderately into the blood. The effects of chronically secreted GH on somatic growth, endogenous GH secretion, and onset of puberty in female animals were investigated.

Materials and Methods

Preparation of the mWAP/hGH construct

The construct used for microinjection was prepared according to standard recombinant DNA procedure [18]. A 4.6 kb structural gene sequence was removed by digestion with KpnI and EcoRI from pWAPE7.2neo [19] which contains the 7.2 kb genomic mWAP gene. Subsequently, the KpnI site was converted into a BamHI site by addition of BamHI linkers, leading to a EcoRI/BamHI vector fragment that contains a vector sequence and 5′ flanking region of the mWAP gene. The coding region of the hGH gene was isolated as 2.1 kb BamHI/BamHI fragment from pMThGH [20] and was inserted into the BamHI site of the pBR327/5′ WAP plasmid from which the protein coding region of mWAP gene was removed.

Generation of transgenic rats

All the rats used were Wistar strain purchased from Imamichi Institute for Animal Reproduction (Tsuchiura, Japan). Animals were housed at 23 ± 1°C on a lighting schedule of 14 h light/10 h darkness (lights on 0500 h). The 4.7 kb mWAP/hGH construct was isolated from vector sequences by EcoRI digestion. After purification by agarose gel electrophoresis, DNA at a concentration of 1 μg/ml was microinjected into the pronuclei of fertilized eggs harvested from naturally mated female rats. Eggs that survived after microinjection were implanted into the oviducts of pseudopregnant foster females. After offspring were evaluated to confirm hGH gene expression, the mature male transgenic rats were mated with normal females. The resulting transgenic and nontransgenic littermates were used in the present study.

DNA analysis

To identify pups carrying the mWAP/hGH construct, DNA was isolated from tail biopsies as previously described [6] and analyzed by Southern blotting [21] using radio-labeled 1.2 kb PvuII fragments of the hGH structural gene as hybridization probes (Fig. 1).

Blood collection

To sample blood from a freely moving animal, a silastic cannula was implanted through the right jugular vein to the right atrium. After surgically implanting the cannula under ether anesthesia on

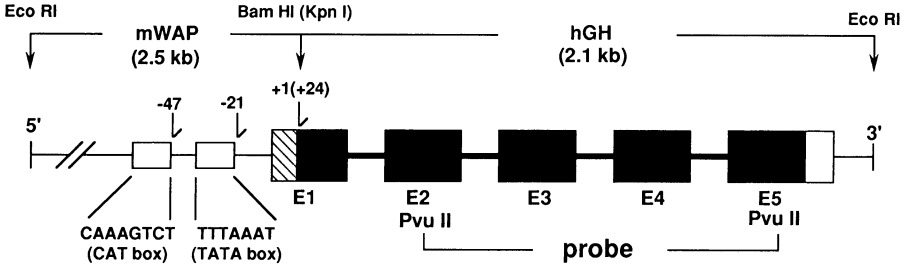

Fig. 1. Construct of mWAP/hGH gene introduced into rats. Solid boxes represent exons; horizontal bars, introns; horizontal lines, 5′ and 3′ flanking sequences; hatched and open boxes at 5′ and 3′ ends, untranslated sequences.

the day before the sampling experiment, the rats were housed individually. On the day of the experiment, animals were moved to the experimental room and allowed a 2 h adaptation period before the start of blood sampling. To determine rGH and hGH secretory profiles, blood samples (0.25 ml) were drawn every 20 min over a 4 h sampling period (1300–1700 h).

Hormone measurements

Serum rGH concentrations were determined by double antibody RIA using materials supplied by the NIDDK Hormone Distribution Program. The reference standard for the rGH assay was NIDDK-rGH-RP-2. The standard curve was linear between 3.75 and 240 ng/ml. Serum hGH concentrations were measured by double antibody RIA with a commercial kit (Dinabot, Tokyo), and the standard curve was linear between 0.5 and 80 ng/ml. The antibody against rGH did not cross-react with hGH, and vice versa, within the dose range of the standard curves used in this study.

Determination of the onset of female puberty

The rats were weighed and examined daily for vaginal opening, an initial indicator for the time of puberty [22]. Vaginal smears were taken daily after vaginal opening (which usually occurred on the day of proestrus or estrus), and ovaries were dissected on the first day of diestrus. The presence of corpora lutea in the ovaries was considered indicative that ovulation, and hence puberty, had occurred.

Statistical analysis

Statistical comparisons were made with Student's t test or Wilcoxon's rank sum test. A difference was considered significant if $P<0.05$.

Results

Two male rats (No. 7–6–3 and No. 7–6–5), out of 53 born following transfer of the microinjected eggs, carried the mWAP/hGH construct in the genomic DNA as confirmed by Southern blot analysis and secreted hGH into the blood as confirmed by RIA. F_1 and F_2 rats for this study were generated from each of the two founder transgenic rats by mating with normal female rats. The transgenic descendants of the rat No. 7–6–5 and No. 7–6–3 were defined as Lines 1 and 2, respectively. As shown in Table 1, transgenic rats of Line 1 were characterized by relatively high levels of serum hGH, while those of Line 2 had relatively low levels. Only Line 1 transgenic rats showed a marked increase in body weight at 9 weeks of age, although females of Line 2 showed a slight but significant increase.

The profiles of serum rGH in normal rats and of serum hGH and rGH in Line 2 transgenic rats are shown in Figs. 2a and b, respectively. In normal rats, episodic peaks in serum rGH exceeded 100–200 ng/ml and occurred at approximately 3 h intervals in males; more frequent but smaller peaks were observed in females (Fig. 2a). These profiles are consistent with previous reports [23]. On the

Table 1. Features of transgenic rats produced in this study

	Sex	Serum hGH (ng/ml)[a]	Serum rGH (ng/ml)[a]	Relative growth ratio[b]
Line 1	male	157.0 ± 9.69[c] (n=3)[d]	NA[e]	1.350††
	female	468.3 ± 153.5* (n=4)	NA	1.788††
Line 2	male	11.03 ± 0.91 (n=4)	24.76 ± 2.20† (n=4)	0.968
	female	21.37 ± 1.15** (n=4)	17.29 ± 1.67† (n=4)	1.068†
Normal	male	ND[f]	75.16 ± 15.20 (n=3)	1
	female	ND	92.54 ± 47.20 (n=3)	1

a, Value in Line 2 and Normal animals represent the overall means of the data shown in Figs. 2 and 3; b, Relative weight of transgenic rats compared with sex-matched normal littermates at 9 weeks of age; c, Mean ± SEM; d, Number of rats; e, Not available; f, Not detected; *$P<0.05$, **$P<0.01$ *vs.* male rats of the same line; †$P<0.05$, ††$P<0.01$ *vs.* sex matched normal littermates.

other hand, in transgenic males, no episodic peaks in serum rGH were discernible over the 4 h period (Fig. 2b), and the overall mean rGH level was significantly lower than for normal males (Table 1). In transgenic females, the secretion of rGH also was suppressed, but small peaks were observed occasionally (Fig. 2b). In both transgenic males and females, there was no obvious fluctuation in serum hGH levels (Fig. 2b), but the overall mean level in males was significantly lower than in females (Table 1).

Age and body weight at vaginal opening of normal and transgenic females are shown in Fig. 3a and b, respectively. Normal females showed vaginal opening at 36.8 ± 0.3 days of age. In contrast, the day of vaginal opening advanced by 7–8 days in both Line 1 and 2 transgenic females. The presence of corpora lutea in the ovary on the first day of diestrus, 1–2 days after vaginal opening, was confirmed in all the animals examined. Because Line 2 transgenic animals did not show an increase in growth rate, body weight at the day of vaginal opening for Line 2 transgenic females was significantly lower than that of either normal or Line 1 transgenic females.

Discussion

In this study, two lines of transgenic rats with measurable serum hGH were obtained. Line 1 was characterized by relatively high levels of serum

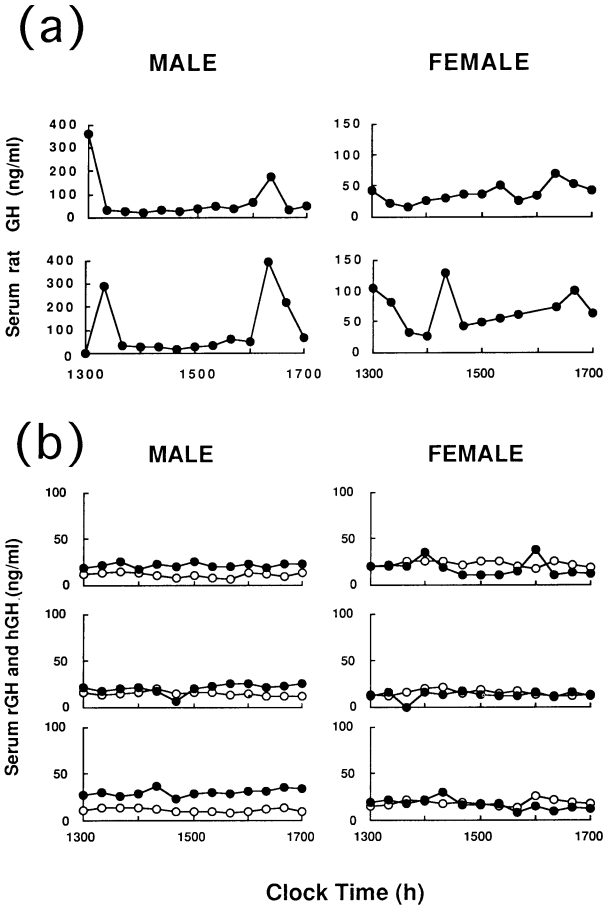

Fig. 2. Representative individual profiles of changes in serum rGH (closed circle) in normal rats (a) and those of serum rGH (closed circle) and hGH (open circle) in Line 2 transgenic rats (b).

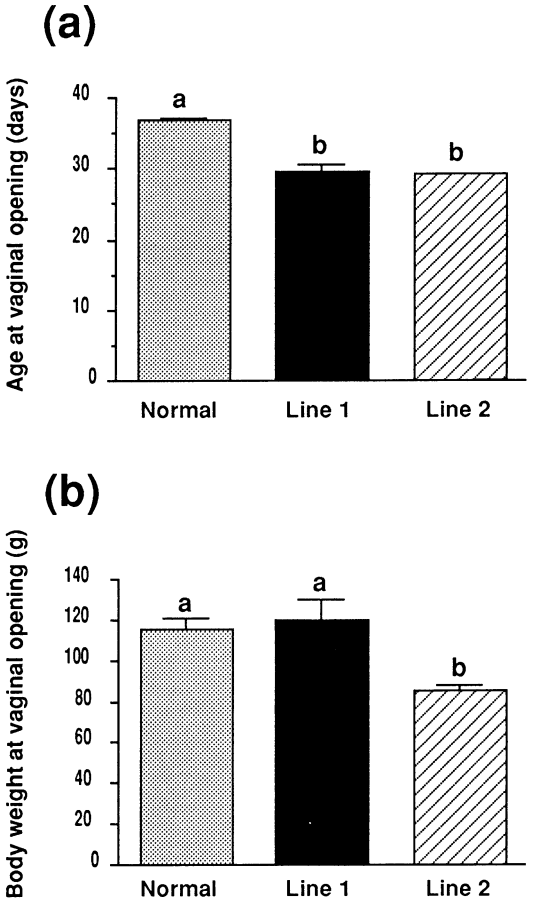

Fig. 3. Age in days (a) and body weight (b) at vaginal opening in normal and transgenic female rats. The data represent mean ± SE. Values having the same superscript are not significantly different ($P>0.05$), Stippled bar, normal rats (n=6); shaded bar, Line 1 transgenic rats (n=3); hatched bar, Line 2 transgenic rats (n=8).

hGH, and Line 2 had relatively low levels. Most mice expressing a transgene of GH from various species show an increase in growth rate [20]. In this study, transgenic rats secreting hGH at higher levels (Lane 1) had body weight increases that indicate a growth promoting effect of hGH on the rat. However, transgenic rats secreting hGH at lower levels (Line 2) had body weights similar to normal littermates. The total growth promoting activity shared by endogenous rGH and transgene-derived hGH may be equivalent to that of rGH in intact rats.

It is known that GH can regulate its own secretion via a negative feedback mechanism. Given peripherally, GH causes suppression of the spontaneous discharge of GH from the pituitary in male rats [24–26]. When 200 μg of hGH was exogenously injected, serum hGH levels rose to over 250 ng/m*l*, and endogenous rGH pulses were completely suppressed [24]. However, injecting 100 μg of exogenous hGH raised serum hGH levels to 150–200 ng/m*l* and was not sufficient to completely suppress episodic rGH secretions in male rats [26]. In Line 2 transgenic male rats, hGH at a concentration of approximately 10 ng/m*l* considerably suppressed the endogenous episodic secretion of rGH. The differneces in the peripheral levels of exogenous GH that are required to inhibit endogenous GH secretion suggest that GH given acutely by injection or chronically as a transgene product have different modes of action.

The mechanism by which GH regulates its own secretion includes stimulation of somatostatin (SRIF) and inhibition of growth hormone-releasing factor (GRF) releases from the hypothalamus [24, 27, 28]. GH producing cells (somatotorophs) in the pituitary disappear in transgenic mice secreting hGH [20]. It is also possible that low rGH levels in our transgenic rats were due to inhibition of somatotoroph proliferation. GRF stimulates proliferation of somatotorophs, and this mitogenic effect is partially inhibited by somatostatin [29]. It will be worth examining the expression of SRIF and GRF in our transgenic rats to elucidate the precise mechanisms of endogenous rGH suppression.

Both Lines of transgenic female rats had a definite tendency toward precocious puberty. Interestingly, Line 2 transgenic females attained puberty at lower body weights than normal littermates. Thus, hGH could induce precocious puberty without changing growth rate in this Line. Under normal laboratory conditions, the age at vaginal opening is known to correlate nicely with body weight [30], suggesting that some metabolic factor signals the onset of puberty [30, 31]. Line 2 transgenic female rats would be a good model for investigating that metabolic factor, if any, because growth promoting and puberty enhancing activities are separated in this animal model.

When transgenic mice carry the metallothionein-I promoter/hGH construct, hGH gene expression stimulates LH and FSH secretion via the hypothalamus [32, 33]. It is well established that the onset of puberty should be associated with and triggered by the development of neural inputs to the lutenizing hormone releasing hormone (LHRH)-secreting neurons [22]. Thus, GH may act through a central GH receptor and alter the activity or developmental rate of LHRH neurons or neurons modifying LHRH neurons. In fact, GH receptors exist in the median eminence of the hypothalamus [34]. The possibility that hGH acts through a PRL receptor cannot be ruled out because hGH can bind with rPRL receptors [35], and hyperprolactinemia in prepubertal rats, induced by a dopaminergic receptor blocker, advances the onset of puberty [36]. Recently, using transgenic mice carrying the metallothionein-I promoter/hGH construct, Tang *et al.* [37] also demonstrated that a hGH gene product elevates the expression of LH-β and FSH-β genes via a route unrelated to LHRH. This mechanism also may be involved during the process of precocious puberty. Further investigation is needed to identify the hGH action site(s) responsible for inducing precocious puberty.

This study indicates that chronically secreted hGH in transgenic rats alters the endogenous rGH secretory pattern. The results of this investigation also provide evidence that hGH induces precocious puberty in the female rat. The relationship between hGH derived from transgenes and endogenous rGH was first reported in the transgenic rat. Transgenic rats secreting hGH into the blood would be a useful model for elucidating the mechanisms of GH action.

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