Recombinant Human Growth Hormone Acts on Intermediate-Sized Follicles and Rescues Growing Follicles From Atresia

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Abstract. In order to clarify the role of GH on ovarian function, recombinant human GH (rhGH) was administered to adult spontaneous dwarf rats (SDR) for 14 days. The SDR were sacrificed on the day of estrus and the effects of rhGH on ovarian weight, follicular population and the viability estimated by 5-bromodeoxyuridine (BrdU) incorporation, were studied. RhGH administration caused a significant gain in body weight of the SDR, and the ovarian weight of the GH-injected SDR showed a significant increase in comparison with saline injected SDR. The total population of follicles was significantly greater in the GH-treated group than in the control, and the difference was attributed to the increase in the number of large follicles more than 500 μm . Total uptake of BrdU into granulosa cells was significantly higher in GH-treated rats than in the control, and, in particular, the intermediate-sized follicles (300–500 μm) of the GH-treated rats showed signs of high incorporation of BrdU. These results suggest that hGH acts on intermediate follicles, stimulates their viability and increases the number of growing follicles.

Key words: GH, Ovary, 5-Bromodeoxyuridine (BrdU), Spontaneous dwarf rat

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THERE is increasing evidence that GH plays an important role in follicular growth and pubertal development in mammals [1, 2]. It is reported that suppression of endogenous GH release in female rats reduces ovarian LH receptor content and delays the onset of puberty [3], while GH administration to congenital GH-deficient rats stimulates progesterone secretion and restores the time of the onset of puberty [4]. On the other hand, *in vitro* studies with FSH-stimulated granulosa cell cultures demonstrated that the action of GH on ovarian function is direct and augments LH receptor formation [5, 6]. In addition to its effects on granulosa

cell differentiation, GH treatment also stimulates folliculogenesis. Administration of recombinant bovine GH increases the number of small follicles in cows [7], and treatment with GH augments the folliculogenic response of the human ovary to gonadotropin stimulation [8, 9]. These indicate the importance of GH in the regulation of ovarian function, but the use of GH as a cagonadotropin still remains controversial [10], because the role of GH in the reproductive process is permissive. But interest in the potential use of GH as an adjunctive agent in the context of ovulation induction has been growing. It is therefore necessary to elucidate the *in vivo* role of GH in follicular development, and in particular to morphologically clarify at what stage of follicular development GH evokes its action. In order to elucidate this, we studied the effect of recombinant human GH (rhGH) on follicular development in the congenital GH deficient rats.

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Materials and Methods

Animals

Spontaneous dwarf rats (SDR) produced and supplied by Roussel Morishita Co., Ltd were housed in a temperature-controlled vivarium (at 20 ± 2 °C illuminated from 0700 h to 1700 h). Food and water were available *ad libitum*. Both male and female SDR were reproductively competent, although the litter size was smaller than that of normal parents. A vaginal smear test was carried out before and during the experiments, and it was confirmed that the female SDR had 4 day vaginal cycles.

Experimental procedure

Twenty-one-week-old SDR were divided into two groups, 6 to each group. Subcutaneous injections of recombinant human GH (rhGH) (200 mIU/mL) (Saizen, Ares-Serono Laboratories) were started at 0800 h on the first day and were continued for 14 days in the experimental group. The same amount of saline was administered to the control. Blood sampling was carried out from the cervical vein under light ether anesthesia after 14 days of GH administration (at 0800 h on the estrus day) for radioimmunoassay (RIA) of LH, FSH and inhibin. Blood samples were centrifuged and serum was stored at -20 °C until assayed. Immediately after blood sampling, the ovaries were removed, cleaned of fat, weighed and then fixed in Bouin's solution. One hour before blood sampling, the rats were intraperitoneally injected with 5-bromodeoxyuridine (BrdU) (5 mg/body), a thymidine analog, which is incorporated into DNA in the S-phase of the cell cycle.

Histological analysis

The ovaries fixed in Bouin's solution were embedded in paraplast after dehydration with an ascending ethanol series. Eight-micron-thick serial sections were cut from each ovary, alternate sections were stained with hematoxylin and eosin (HE), and the remainder of the sections was used for the immunohistochemistry to estimate the BrdU incorporation. Every section stained with HE was scanned in a color image analyzer SP-500 (Olym-

pus), and follicles more than 100 μ m in diameter (including theca) showing ovum nuclei were measured. Classification of follicular size into 3 groups (100–300 μ m, 300–500 μ m and more than 500 μ m) was done arbitrarily. BrdU incorporation was visualized in paraplast-embedded material using a monoclonal anti-BrdU antibody to BrdU (Cell proliferation kit RUN.20 Amersham) followed by an indirect peroxidase staining procedure. The BrdU incorporation rate was assessed as the number of BrdU positive nuclei on the representative section (maximal section including oocyte) of each follicle.

RIA

Serum concentrations of FSH and LH were determined in duplicate by a double antibody RIA, with materials supplied by NIDDK. Rat FSH and LH RP-1 were used as a reference standard and the concentration was expressed in μ g/mL. Serum concentrations of inhibin were also determined in duplicate by RIA as previously described [11], and expressed in ng/mL.

Statistics

Comparison of the means for the 2 groups was analyzed by non-parametrical analysis by using the Mann-Whitney method. Results were considered statistically significant if P was <0.05 .

Results

The effects of GH on body weight, ovarian weight and serum hormone levels are shown in Table 1. Significant increases in body weight and ovarian weight were observed in GH-treated rats. The body weight was increased to about 3.4 fold of the control, and the ovarian weight was increased to about 1.5 fold of the control. There were no significant differences between the GH-treated group and the control group in serum FSH concentrations, but serum inhibin levels of GH-treated rats increased significantly ($P<0.05$) compared to the control group. Serum LH was not detected.

Figure 1 demonstrates the effects of GH on follicular development. The total population of follicles was significantly greater in the GH-treated group than in the control, and the difference was attrib-

Table 1. Effect of GH treatment on body weight, ovarian weight and serum hormone levels of SDR

	N	Gain in body weight (g)	Ovarian weight (mg)	Serum FSH (ng/ml)	Serum inhibin (ng/ml)
Control	6	6.3 ± 4.2	24.6 ± 4.1	107 ± 27	2.1 ± 0.3
GH treated	6	21.3 ± 3.0**	36.5 ± 2.4**	118 ± 57	2.8 ± 0.4*

Results are mean ± SD. * $P < 0.05$ and ** $P < 0.01$ vs. control.

Follicle size (diameter: μm)

□ 100~300 ▨ 300~500 □ 500~

Fig. 1. Effect of GH on follicular population in SDR rats. Eight micron-thick serial sections were cut from each ovary. Every section stained with HE was scanned in a color image analyzer SP-500 (Olympus), and follicles more than 100 μm in diameter (including theca) with ovum nuclei were measured. Classification of follicular size into 3 groups (100–300 μm , 300–500 μm and more than 500 μm) was determined arbitrarily. The total number of follicles was significantly greater in GH-treated rats than in the control. The difference was attributed to the increase in the population of large follicles. * $P < 0.01$ vs. control.

intermediate-sized follicles (300–500 μm) of the GH-treated rats showed high incorporation of BrdU.

Discussion

The spontaneous dwarf rats were isolated from a closed colony of Sprague-Dawley rats in 1977 [12]. These rats demonstrated isolated GH deficiency caused by the point mutation in the GH gene, producing an abnormal splicing of GH mRNA [13]. The body weight of SDR is only 30–40% that of normal SD rats at the same age, but increases in response to the administration of normal pituitary extracts [12]. No immunohistochemically positive cells were found when using the antiserum to ovine GH, but other anterior pituitary hormone producing cells, such as PRL, ACTH, TSH, LH, and FSH cells were observed [14, 15]. Both male and female SDR were reproductively competent and are considered to be an excellent experimental model for the study of isolated GH deficiency.

The present study demonstrates that rhGH administration is able to significantly increase ovarian weight, the total number of follicles and immunoreactive inhibin secretion in SDR. These findings are in agreement with previous studies performed on hypophysectomized rats [16] and infertile humans receiving GH and urinary gonadotropins for *in vitro* fertilization [8, 9, 17]. Although recent studies suggest the importance of the role of GH and possibly insulin-like growth factor (IGF)-1 in regulating granulosa cell function [2, 5, 6, 18, 19], it has not been fully elucidated to what extent the action of GH or IGF-1 is prerequisite. Menashe *et al.* [16] reported two women with Laron type dwarfism in whom spontaneous pregnancies occurred and Dor *et al.* [21] demonstrated that it is possible to induce ovulation by human menopausal gonadotropins administration in women with Laron type dwarf-

uted to the increase in the number of follicles larger than 500 μm . Figures 2 and 3 show a comparison of BrdU uptake in the control and the GH-treated SDR. In both groups, BrdU uptake was active in the small to large follicles. Total uptake of BrdU into granulosa cells was significantly higher in GH-treated rats than in the control. Particularly the

Fig. 2. Photomicrograms of histological sections of the intermediate-sized follicles from control and GH-treated rats injected with BrdU 1 h before sacrifice. Uptake of BrdU into granulosa cells was higher in GH-treated rats than in the control. Particularly the intermediate-sized follicles (300–500 μm) of the GH-treated rats showed signs of high incorporation of BrdU.

Fig. 3. Comparison of BrdU uptake into follicles of control and GH treated SDR rats. Total uptake of BrdU into granulosa cells was significantly higher in GH-treated rats than in the control. The difference was attributed to the uptake into the intermediate-sized follicles. * $P < 0.01$ vs. control.

ism, suggesting that GH or IGF-1 is not essential but plays a modulatory role in ovarian follicular development. The present findings show the presence of spontaneous follicular development from the small- to large-sized follicles in SDR.

Recombinant human GH was thought to be non-lactogenic [22], but recent studies have shown that GH reacts with lactogenic receptors and rhGH increases the number of terminal end buds and alveolar buds in hypophysectomized rats [23]. It is therefore likely that the stimulatory effects of rhGH on follicular development are mediated through lactogenic receptors. In this study, we demonstrated that SDR treated with rhGH had been showing signs of a normal estrus cycle during rhGH administration, suggesting that the lactogenic effects of rhGH were less manifested. In addition, that GH exerts its effects on follicular development through somatogenic receptors would be supported by following observations: 1) PRL inhibits ovarian weight gain by decreasing the number of follicles in PMS-treated immature rats in a dose related manner [24], whereas a daily dose of 1600 mIU of rhGH significantly increased the weight of the ovaries in PMS-treated immature rats

(our unpublished data), and 2) GH stimulates follicular growth and maturation but PRL does not have such effects in perfused rabbit ovary [25, 26]. Although expression of GH receptor mRNA has been demonstrated in the rat ovary [27], further studies on characterization of the biochemical properties of GH receptors and analysis of interaction between GH and its receptors are required.

As shown in Fig. 2, a significant increase in the number of follicles in the GH treated rats was due to the increase in the number of large follicles but there was no significant difference in the number of small and intermediate follicles. The increase in the number of follicles leads to increased serum inhibin levels, but the serum FSH of rhGH treated rats was unchanged, suggesting that the inhibitory effect of inhibin on FSH secretion was offset by GH treatment by unknown mechanisms. The effect of GH on the follicle population showed remarkable contrast to that of activin A, a dimer of the β -subunit of inhibin, which increased not only large follicles but also small and intermediate follicles [28]. Stahmokinetic analysis of granulosa cells indicates that the large follicles in adult rats on the day of estrus are destined to become atretic during that night or the morning of the next day [29]. Furthermore, it has been shown that these large follicles are seemingly healthy but are not capable of ovulating in response to exogenously administered hCG [30]. If this is the case, the large follicles on the day of the estrus are remnant follicles which were stimulated by endogenous FSH but were not ovulated. The low BrdU incorporation into the large follicles in this study would support the hypothesis that the large follicles have lost viability.

Although small follicles which have a single or double layer of granulosa cells showed signs of

high BrdU incorporation, there were no significant differences between the GH-treated group and the control in BrdU incorporation, suggesting that the effects of GH are less evident in these small follicles at a very early stage of development. High BrdU incorporation was also observed in the intermediate follicles, and in particular the BrdU incorporation into intermediate follicles was significantly higher in GH-treated rats than in the control group, suggesting that GH exerts its action on the intermediate follicles. These intermediate follicles are thought to be driven from the new cohort and ovulate in the subsequent cycle [29], so that viability of the intermediate follicles is of particular importance in defining the number of growing follicles in the subsequent cycle. Our present study was quite compatible with recent findings by Eisenhauser *et al.* [31], which demonstrated that GH prevents follicular apoptosis and acts as an ovarian survival factor.

From these observations, it is concluded that GH is not mandatory for follicular development, but augments follicular growth in the presence of FSH. GH acts on intermediate follicles and increases their viability to protect from atresia and increases the number of growing follicles.

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