Morphological changes to somatotroph cells and *in vitro* individual GH release, in male rats treated with recombinant human GH

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Summary. The effect of in *vivo* chronic administration of recombinant human growth hormone (rhGH) on morphology and individual GH release in somatotroph cells was evaluated in young male Wistar rats. Over an 18-day period, 30-day-old male rats were injected daily with 1.5 IU rhGH/kg (GPG group) or saline (VPG group) by subcutaneous injection. Electron-immunocytochemical, ultrastructural and morphometric studies of somatotroph cells were carried out. Additionally, rat pituitary cells were dispersed and overall and individual GH release was studied by radioimmunoassay and cell immunoblot assay (quantified by image analysis), respectively. The ultrastructure and size of somatotroph cells did not change, but volume density of secretion granules was reduced (p<0.01) by previous in *vivo* GH treatment. At four days, basal GH release of rat pituitary cell monolayer cultures was lower in the GPG group than in the VPG group (p<0.05); after 12 hours of culture, GHRH stimulation of GH release was lower in the GPG group than in the VPG group (p<0.05), and GHRH+SRIH inhibited GH release in the GPG group (p<0.05), but not in the VPG group. The percentage of somatotroph cells was not modified, but the ratio of strongly/weakly GH-immunostained cells had changed; weakly GH-immunostained cells increased from 34% to 55%. Moreover, in *vitro* treatment with GHRH, SRIH, and both, easily changed the strongly/weakly GH-immunostained cell ratio. Individual GH release, however, was not changed by previous in *vivo* GH treatment, although GHRH preferably stimulated a subpopulation of GH cells and SRIH did not inhibit individual GH release. These data suggest that exogenous chronic rhGH treatment down-regulates somatotroph function by modifying the proportion of GH cell subpopulation.

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Introduction

It is widely recognised that growth hormone (GH) administration leads to a decrease in the subsequent response of GH to GH releasing hormone (GHRH) and other stimuli (Nakamoto et al., 1986; Rosenthal et al., 1986; Ross et al., 1987). This appears to be a direct effect of GH and not of IGF-1 (Minami et al., 1997), mediated by an increase in hypothalamic somatostatin secretion (SRIH) (Sheppard et al., 1978; Burton et al., 1992) and a decrease in hypothalamic GHRH release (Conway et al., 1985; Aguila and McCann 1993; Bertherat et al., 1993). The presence of GH-receptor mRNA in pituitary cells, such as somatotropes, lactotropes and gonadotropes (Harvey et al., 1993), suggests that GH acts directly on these cells through paracrine or autocrine mechanisms.

The feedback mechanism that results from the exogenous administration of GH determines a decrease-suppression of GH response to GHRH in a dose-dependent fashion (Clark et al., 1988), lasting for at least 4 hours (Lanzi and Tannenbaum, 1992a). As a result of SRIH inhibition of GH secretion, GH is stored in the cell for a period of time after which the GHRH stimulus produces an even greater GH response from the somatotroph cells (Lanzi and Tannenbaum, 1992b). On the other hand, suppression of hypothalamic GHRH release (Sato and Frohman, 1993; Uchiyama et al., 1994) results in a decrease in the amount of GH that can be released by each somatotroph cell (Matteri et al., 1997). It can therefore be expected that, following administration of exogenous GH, the morphology of the somatotroph cells will be modified.

Kurosumi (1986) described three types of somatotroph cells in the rat, based on their ultrastructural features: type I somatotroph cell containing 350 nm secretion granules; type II somatotroph cell containing

350 nm and 150 nm secretion granules; and type III somatotroph cell with 150 nm secretion granules. Similarly, Takahasi (1991) describes three types of somatotroph cells, in which age, sex and hormonal therapy produce morphological differences. Other authors (Shimokawa et al., 1996; Carretero et al., 1997) also report morphological changes in rat somatotroph cells caused by stimuli, such as calorie-restricted diets or estradiol, respectively. Moreover, rat somatotroph cells may be isolated in basically heterogeneous populations (Snyder et al., 1977) and morphologically characterised as heavily granulated (type II) or sparsely granulated (type I) (Lindstrom and Savendahl, 1996). Frequency, size, ultrastructure and functional heterogeneity of these somatotroph cell subpopulations are all influenced by physiological state, gender and age (Dobado-Berrios et al., 1996a,b).

In rat, chronic (5-day) treatment with recombinant human GH has an inhibitory effect on endogenous pulsatile GH secretion (Lanzi and Tannenbaum, 1992a); in pig, GH secretion *in vitro* is inhibited as a result of a four-weeks *in vivo* GH treatment (Matteri et al., 1997). It remains unclear, however, whether chronic *in vivo* treatment with GH produces the same response in all somatotroph cells and if there is any change in their morphology and functional heterogeneity. Thus, the aim of the present study was to evaluate stimulated basal release in rat somatotroph cells in monolayer cultures, in addition to recording the immunoreactive features of these cells on finalising the culture program. In order to ascertain whether the reduced response of somatotroph cells due to exogenous GH was forced, at least in part and indirectly, by paracrine reactions, the response of individual (basal and post-stimulus) somatotroph cells was measured using a cell immunoblot assay.

Materials and methods

Animals

30-day-old male Wistar rats were used. The animals were given free access to rat chow (IPM R-20, Letica S.A., Hospitalet, Barcelona, Spain) and tap water. They were housed singly and maintained under conventional conditions (temperature: 22±2 °C 12:12 h light/dark cycle with lights on at 07.00) in the laboratory animal centre of the School of Medicine of Córdoba. The rats were cared for and used in accordance with the European Council guidelines, 86/ 609/ CEE (24/11/1987).

Experimental design

The duration of the experiment was 18 days, and the animals were weighed daily. Rats were divided into two groups (n=6); GH-pretreated group and vehicle-pretreated group. GH-pretreated group (GPG): the animals were injected subcutaneously, daily at 4 pm, with 1.5 IU/kg body weight of recombinant human

growth hormone (rhGH) (Serono Labs., Spain); vehicle-pretreated group (VPG): as the above group, except they received saline serum instead of rhGH.

One day after treatment, the animals were killed by decapitation and the pituitary was removed. The posterior pituitaries were discarded, and the anterior pituitaries were diced into small pieces for morphological studies or for cell dispersion. Another group of 30-day-old male rats (pre-experimental group) was killed for ultrastructural studies.

Immunocytochemistry for electron microscopic, ultrastructural, and morphometric analysis

The small pieces were fixed with a fixative solution (1% glutaraldehyde and 2% formaldehyde in 0.2M cacodylate buffer) for 2 hours, and postfixed with 1% osmium tetroxide for 1 hour. Following dehydration with ethanol they were embedded in Durcupan® ACM. Sections of approximately 300 nm were collected on 300-mesh nickel grids. GH cells were identified on ultrathin sections by immunogold stain (Roth, 1983). After etching with a saturated aqueous solution of sodium metaperiodate, rabbit serum anti-rat GH (UCB Bioproducts, diluted 1:1000, 20h at 4 °C) as the first antiserum, and goat anti-rabbit IgG conjugated with colloidal gold (15 nm in diameter) (Janssen Life Sci, Glen, Belgium; diluted 1:10) was used. For the washes and dilutions of the sera, tris buffer (0.05M, pH 7.4) (TRIS) and 1% albumin bovine serum (BSA) in TRIS were used, respectively. After immunostaining, grids were stained with uranyl acetate and lead citrate. The preparations were examined using a Phillips CM 10 electron microscope and 40 micrographs were taken for each group (20 at x1950 and 20 at x6400). The immunoreaction specificity for rat GH was analysed by omission of the specific antiserum, replacing the antiserum with normal rabbit serum, and adsorption of the specific antiserum with its homologous (rat GH) or heterologous (rat prolactin) hormone.

The morphometric study was performed using the IMAGO software for image analysis. The morphometric procedure was carried out using area analysis (Weibel, 1979) for determined cellular (CeA), cytoplasmic (CyA) and nuclear (NA) areas (in μm^2). The volume density of secretion granules (VvGr; % cytoplasm) and surface density of the rough endoplasmic reticulum (SvRER; cm^{-1}), calculated by the formula of Weibel and Bolender (1973), were selected as stereological parameters.

Cell dispersion and cell culture

The small pieces of anterior pituitary were dispersed in Erlenmeyer flasks with a mixture of 0.02 g/l collagenase (Type V, Sigma Chemical Co.) and 0.01 g/l trypsin 1:250 (Sigma Chemical Co.) in Dulbecco Modified Eagle's Medium (DMEM) (Sigma Chemical Co.). The Erlenmeyer flask was shaken in a water bath at 37 °C for 45 minutes. The cellular suspension obtained

was gently pipetted for ten minutes, washed in DMEM, and centrifuged (100 g for 10 min) twice. The cell pellet was again washed, centrifuged, and resuspended in 10% foetal bovine serum (FBS)-DMEM. Cell yield and viability were checked by the trypan blue exclusion method: cell yields were $6.2 \pm 0.6 \times 10^5$/pituitary, and cell viability was over 90% in all cell dispersions. For tissue culture, the cell suspension was dispensed into 96 wells (2×10^4 cells/well/$200 \mu l$ 10% FBS-DMEM) and incubated in a humidified atmosphere of 5% CO_2 in air, at 37 °C, for 4 days. The monolayer cultures were washed twice with DMEM and fresh serum-free DMEM containing GHRH (10^{-7} M), SRIH (10^{-7} M) or a combination of GHRH and SRIH was added and left for 12 hours. The monolayer cultures were then washed twice with DMEM and fixed with *Bouin's solution* (saturated solution of picric acid, 71.4 ml/100 ml; formaldehyde 35%, 8.8 ml/100 ml; and acetic acid, 4.8 ml/100 ml) for 30 minutes, and washed three times with PBS. Monolayer cultures were immunostained for GH, using the peroxidase-antiperoxidase technique. Anti-rat GH rabbit serum (UCB Bioproducts, 1:1000), swine anti-rabbit serum (Dako, diluted 1:250) and rabbit-PAP complex (Dako, diluted 1:500) was used. Endogenous peroxidase was blocked with H_2O_2 (3%) and non-specific reactions of secondary antibody by incubation in normal swine serum (Dako, diluted 1:40). For the washes and dilutions of the sera, phosphate buffer (PBS) was used. The reaction was developed in freshly prepared 3,3'-diaminobenzidine (Sigma, 0.025% in PBS buffer containing 0.03% H_2O_2). The immunoreaction specificity for rat GH was measured by omission of the specific antiserum, replacing the antiserum with normal rabbit serum, and adsorption of the specific antiserum with its homologous (rat GH) or heterologous (rat prolactin) hormone. The proportion of immunostained GH cells was then calculated in each well.

GH radioimmunoassay

GH concentrations in the culture media were measured in a double-antibody RIA (Aguilar and López, 1988) using kits provided by NIDDK. All samples from each experiment were measured in the same assay and GH values expressed in ng/ml. Using this method, the intra-assay and inter-assay coefficient of variation were 7% and 12% respectively (Aguilar et al., 1989).

Cell immunoblot assay

Pieces (1.5x1.5 cm) of polyvinylidene difluoride transfer membrane (IMMOBILON™ Millipore) were placed in multiwell plates. Freshly dispersed cell suspensions were placed on the membranes (10^3 cells/50 μl DMEM) and preincubated at 37 °C, 95% air-5% CO_2 for 30 minutes. Next, either 50 μl DMEM on its own, or with GHRH (10^{-7}M), SRIH (10^{-7}M) or a combination of GHRH (10^{-7}M) and SRIH (10^{-7}M), was added to the membranes, and left for 12 hours. After incubation, the

transfer membranes were fixed with *Bouin* liquid for 30 minutes, and immediately washed three times with PBS. The transfer membranes were immunostained using the peroxidase-antiperoxidase method described by Kendall and Hymer (1987). Anti-rat GH rabbit serum (UCB Bioproducts, 1:4000), swine anti-rabbit serum (Dako, diluted 1:250) and rabbit-PAP complex (Dako, diluted 1:500) were used. Endogenous peroxidase was blocked with H_2O_2 (3%) and non-specific reactions of secondary antibody by incubation in normal swine serum (Dako, diluted 1:40). For the washes and dilutions of the sera, phosphate buffer (PBS) was used. The reaction was developed in freshly prepared 3,3'-diaminobenzidine (Sigma, 0.025% in PBS buffer containing 0.03% H_2O_2). The specificity of cell immunoblot for rat GH was examined by removing the antiserum, replacing the antiserum with normal rabbit serum, and absorbing the antiserum with rat GH (UCB Bioproducts) at 4 °C for 24 hours. In addition, cross-reactivity of the primary antiserum to prolactin was checked by incubating, in the presence of anti rat GH, Immobilon membranes onto which 1 μl droplets containing 25 ng/μl purified rat prolactin had been added.

Image analysis

GH-immunostained cell blots were measured using a conventional Nikon microscope equipped with a light source stabilised at 5 volts and connected via a Hitachi television camera to an image-analysis system consisting of a computer (486/66 MHz with a digitising card equipped with IMAGO software) (the SIVA Group, University of Córdoba), an additional monitor (Multisync, NEC) and a digitising board (KURTA). In each membrane, measurement was made of the halo secretion area (in μm^2), optical density (OD) and integrated optical density (IOD = OD x halo secretion area; in arbitrary units) of 60 GH-immunostained cell blots.

Statistical analysis

The results were expressed as mean ± SEM. A minimum of six animals were used in each experimental and control group. Experiments were repeated three times with different groups of animals. The statistical significance of differences between groups was determined by ANOVA, and was accepted at p<0.05. The Student-Newman-Keuls test was used after ANOVA. When the normalised test failed, the Kruskal-Wallis one-way analysis of variance, followed by the Mann-Whitney Rank Sum Test, were used.

Results

Electron microscopy and morphometry-sterology

Ultrastructural examination of immunoidentified somatotroph cells showed that these cells were similar in

Table 1. Morphometric and stereological parameters of male rat somatotroph cells. (Mean ± SEM; areas in μm^2; VvGr: % in cytoplasm; SvRER in cm^{-1}.

PARAMETERS	PRE-EXPERIMENTAL GROUP	VPG	GPG
CeA	55.60±2.90	71.28±8.12[a]	76.63±3.39[a]
CyA	38.69±2.75	45.90±6.71	47.87±3.74
NA	16.90±1.88	25.37±2.78[a]	28.75±2.44[a]
VvGr	26.22±2.48	30.10±4.80	19.99±2.50[b]
SvRER	2.14±0.32	1.28±0.23	1.56±0.16

VPG: vehicle-pretreated group. GPG: GH-pretreated group. CeA cellular area; CyA: cytoplasmic area; NA: nuclear area; VvGr: volume density of secretion granules; SvRER: surface density of the rough endoplasmic reticulum. [a]: p<0.05 vs pre-experimental group. [b]: p<0.01 vs VPG.

both the vehicle-pretreated (VPG) and the GH-pretreated (GPG) groups. Based on the size of the secretion granules, two types of GH cells could be seen: cells with large secretory granules (diameter, 250-350 nm) and cells with large and small secretory granules (diameter, 250-350 and 100-150 nm). In both cases, cells were round or oval in shape, with rounded nuclei. GH cells with larger secretion granules were densely granulated (Fig. 1a) while GH cells with smaller secretion granules were sparsely granulated (Fig. 1b). Therefore, GH cells were similar to type I and type II/III cells, respectively, as described by Kurosumi (1986) and Takahashi (1991).

In the pre-experimental group, the nuclear (NA) and cellular (CeA) areas of somatotroph cells were smaller than VPG and GPG (p<0.05) (Table 1), but not the cytoplasmic area (CyA). Volume density of secretion granules (VvGr) in GPG was less (p<0.01) than in VPG, while surface density of RER of somatotroph cells was not different between groups (Table 1).

Monolayer culture

At four days, GH release in rat pituitary cell monolayer cultures was higher in VPG than in GPG

(Fig. 2) (33.715±1.455 vs 29.985±0.897 ng/ml, p<0.05). After 4 days, it was observed that in the 12-hour rat pituitary cell monolayer cultures (Fig. 3) GHRH stimulated GH release both in VPG (2.67 times; 18232±1388 vs 6838±958 ng/ml. P<0.001) and in GPG (2.12 times; 12546±1170 vs 5907±632 ng/ml. P<0.0001). This release was greater in VPG than in GPG (P<0.05). SRIH greatly inhibited GH release in both groups (VPG: 928±108 vs 6838±958 ng/ml; GPG: 998±125 vs 5907±632 ng/ml P<0.0001). GHRH+SRIH did not inhibit GH release in either group, although release was greater in VPG than in GPG (P<0.05).

Analysis by immunocytochemistry revealed somatotroph cells as either strongly (type I-like GH cells of Kurosumi) or weakly (type II/III-like GH cells of Kurosumi) immunostained (Fig. 4). The proportion of immunostained somatotroph cells (Fig. 5) was virtually constant in both groups (VPG and GPG), as well as in the control culture and under treatment with GHRH, SRIH and GHRH+SRIH. However, the proportion of strongly and weakly immunostained somatotroph cells, which is not modified in VPG, undergoes notable changes in GPG. The percentage of weakly/strongly immunostained GH cells in control cultures was 34/66 in VPG and 55/45 in GPG. In GPG, GHRH increased the percentage of strongly immunostained GH cells and GHRH+SRIH increased the percentage of weakly immunostained GH cells.

Cell immunoblot assay

The analysis of integrated optical density (IOD; in arbitrary units) (Table 2) showed that individual GH release was the same for the control cultures of both groups (Fig. 6a). In VPG, treatment with GHRH resulted in a 1.5-fold increase in individual GH release (p<0.05), whereas in GPG (Fig. 6b), it produced a 3.4-fold increase (p<0.001). SRIH inhibited individual GH release in VPG (p<0.05) but not in GPG. GHRH+SRIH did not modify individual GH release of somatotroph cells in either group.
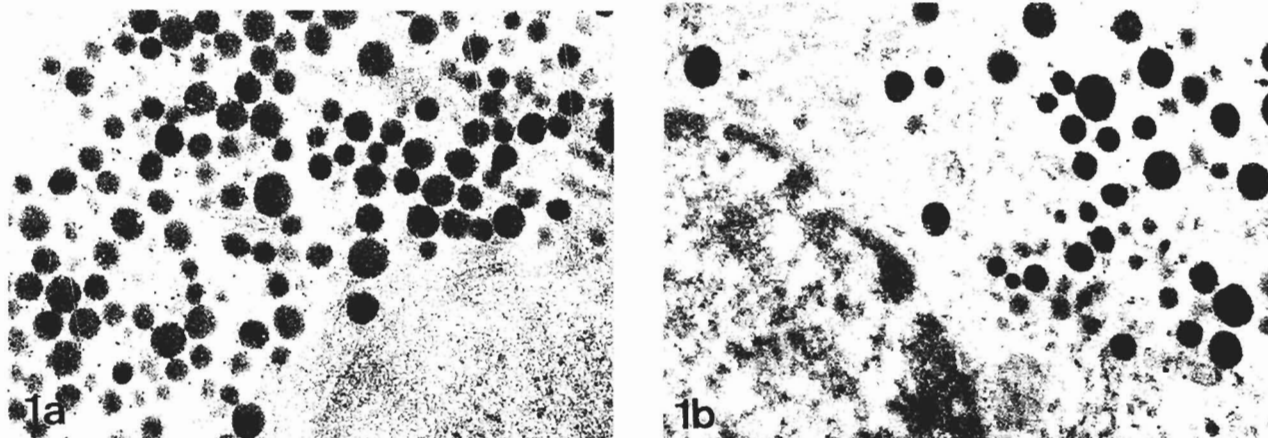

**Fig. 1. a.** Kurosumi's type I GH-immunostained cell. **b.** Kurosumi's type II GH-immunostained cell. x 19,600

Table 2. Optical density, area, and integrated optical density of GH-immunostained cell blots (Mean ± SEM; optical density and integrated optical density in arbitrary units; areas in μm^2).

	OPTICAL DENSITY		AREA		INTEGRATED OPTICAL DENSITY	
	VPG	GPG	VPG	GPG	VPG	GPG
Control	0.0513±0.0044	0.0825±0.0057*	1359.77±171,91	618.91±132.72[a]	70.52±12.04	50.61±10.32
GHRH	0.0512±0.0040	0.1164±0.0096*[1]	1693.82±268,40	1394.71±337.03[c]	108.04±13.94[2]	174.61±43.82[3]
SRIH	0.0485±0.0050	0.7090±0.0066*	799.23±69,572	574.69±66.940[b]	43.55±7.212	50.46±8.87
GHRH+SRIH	0.0527±0.0039	0.1101±0.0088*[1]	986.93±80,319	458.44±75.669[a]	91.39±18.70	64.83±8.77

VPG: vehicle-pretreated group; GPG: GH-pretreated group; *: significantly different vs VPG (p<0.0001); [a], [b]: significantly different vs VPG (p<0.0001 and p<0.001, respectively); [c]: p<0.01 vs control GPG; [1]: p<0.001 vs control GPG; [2]: p<0.05 vs control VPG; [3]: p<0.0001 vs control GPG.
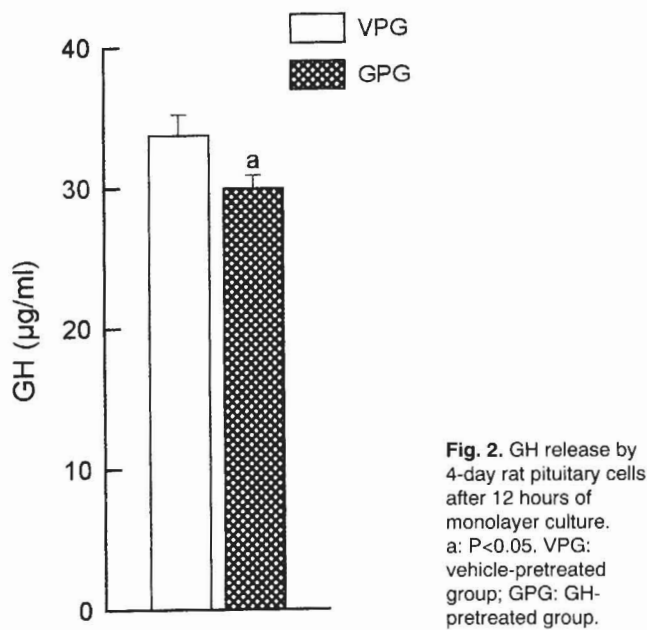

Fig. 2. GH release by 4-day rat pituitary cells after 12 hours of monolayer culture. a: P<0.05. VPG: vehicle-pretreated group; GPG: GH-pretreated group.
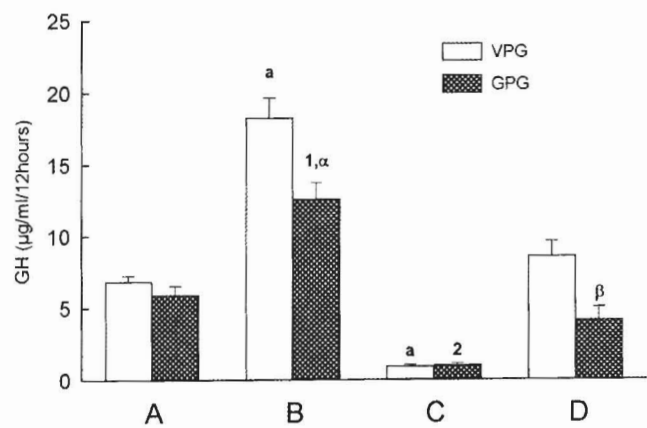
Fig. 3. GH release by 4-day rat pituitary cells after 12 hours of monolayer culture. VPG: Vehicle-pretreated group; GPG: GH-pretreated group. A: control culture; B: GHRH treatment; C: SRIH treatment; D: GHRH+SRIH treatment. a: P<0.0001 vs control of VPG; 1: P<0.01 vs control of GPG; 2: P<0.0001 vs control of GPG; α: P<0.05 vs GHRH of VPG; β: P< 0.05 vs GHRH+SRIH of VPG.

In VPG, the optical density (OD) of GH-immuno-stained cell blots was not significantly different between cultures (Table 2), whilst in GPG the OD of all membrane cultures was greater than in VPG (Table 2, p<0.0001) and after GHRH and GHRH+SRIH treatment (p<0.001). However, the areas of GH-immunostained cell blots of control and GHRH+SRIH (p<0.0001) and GHRH (p<0.001) in GPG were smaller (Table 2) than in VPG. In GPG, only GHRH treatment increased the area of GH-immunostained cell blots (p<0.0 1).

Figure 7 shows % IOD frequency distribution of the individual GH release of somatotroph cells shown in Table 2. In VPG, the control culture revealed 59.87% of GH-immunostained cell blots with IOD values of less than 50, and 15.38% below 100. Treatment with GHRH inverted these values. Treatment with SRIH produced IOD values under 50 in 74.39% of the GH-immuno-stained cell blots, and treatment with GHRH+SRIH resulted in a small proportion of the somatotroph cells (5.12%) releasing a great deal of GH, with IOD values over 350. In GPG, the control culture recorded 68.42% of GH-immunostained cell blots with IOD values below
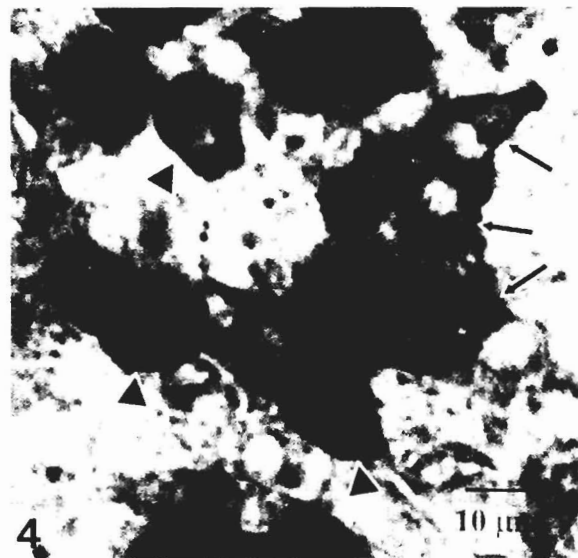

Fig. 4. GH-immunostained cells in rat pituitary cell monolayer cultures. The GH immunostain was strong (head arrows) or weak (arrows). x 850
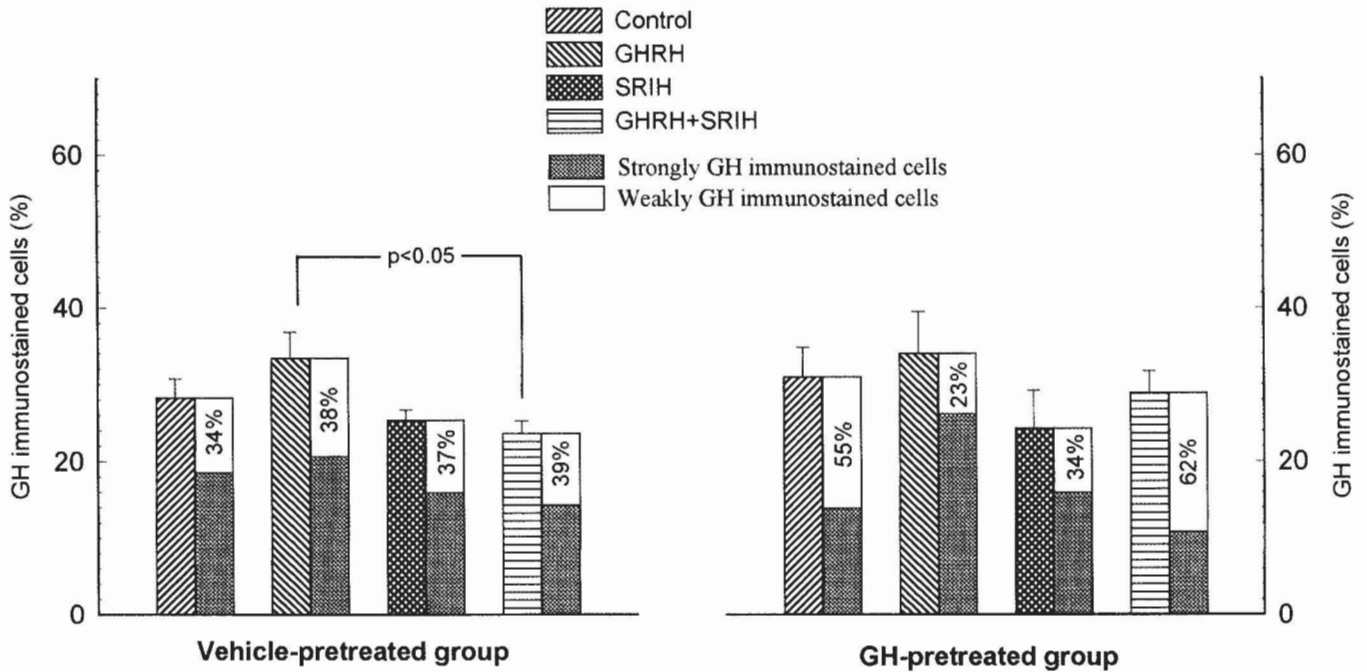
Fig. 5. GH-immunostained cells (% total) of rat pituitary cell monolayer cultures. After four days the monolayer cultures were treated for 12 hours with GHRH, SRIH or both. The stacked bars show the proportion of GH weakly/strongly-immunostained cells of each respective bar.
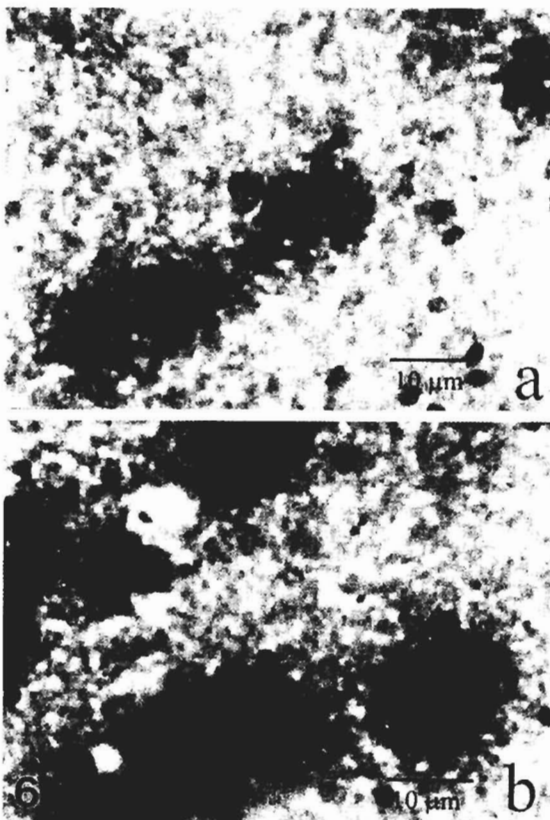
Fig. 6. GH-immunostained cell blots, **a.** Control culture. **b.** GHRH treatment of GH-pretreated group. x 1,100
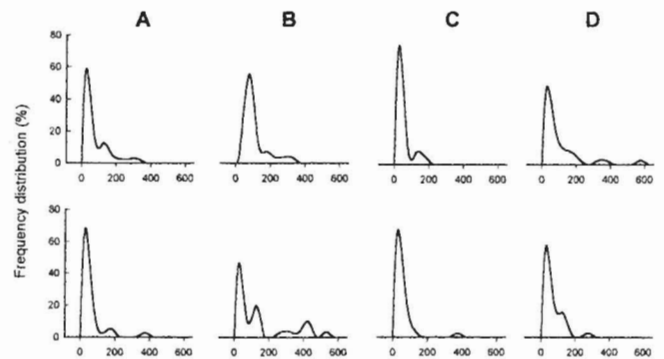
Fig. 7. Frequency distribution of integrated optical density (% IOD) of individual GH release of somatotroph cells, in vehicle-pretreated group (upper panel) and GH-pretreated group (lower panel). A: control culture; B: GHRH treatment; C: SRIH treatment; D: GHRH+SRIH treatment.

50, and 2.63% greater than 350. Treatment with GHRH resulted in IOD values greater than 300 in 19.63% of the cells. To summarise, the majority of somatotroph cells in VPG released GH quantities of under 300 IOD units (unimodal release), whilst the majority of somatotroph cells in GPG released quantities of GH under 200 IOD units, while a small group of cells released quantities of GH over 350 IOD units (bimodal release).

Discussion

The exogenous administration of rhGH inhibits GH release (Willoughby et al., 1980) and GHRH-stimulated

GH release for a short period of time (Stachura et al., 1988). These effects appear to be mediated by an increased secretion of hypothalamic SRIH (Aguila and McCann, 1993) and by a decrease in hypothalamic GHRH (Conwey et al., 1985; Bertherat et al., 1993). However, it is not well documented what the morphological changes and individual GH release of somatotroph cells would be after the chronic administration of rhGH.

The present study shows that chronic administration of rhGH to young male rats inhibits basal and stimulated GH release *in vitro*. Moreover, it modifies the proportion of strongly/weakly immunostained GH cells (Kurosumi's type I and type II/III-like GH cells, respectively), although it does not modify the size and total proportion of somatotroph cells. Our results show that *in vivo* administration of rhGH results in untreated, SRIH-treated and GHRH+SRIH-treated pituitary cells, *in vitro*, produced unimodal frequency distributions of GH immunoblot cells, and that GHRH-treated pituitary cells *in vitro* produce bimodal frequency distributions of GH-immunoblot cells, suggestive of a subpopulation of somatotropes preferentially responsive to this secretagogue.

Chronic rhGH administration does not modify the ultrastructure of somatotroph cells in rat, since two cell types, type I and type II/III, similar to those described by Kurosumi (1986) and Takahashi (1991) were found. Nor does it modify the size of rat somatotroph cells. However, the size of the nuclear and cellular areas of VPG and GPG was greater than in the pre-experimental group; this difference appears to be due to an increase in size of rat somatotropes, between the first and second month of life (Takahashi, 1991; Carretero et al., 1997; Castaño et al., 1997).

GH release is inhibited *in vitro* by the *in vivo* administration of GH (Willoughby et al., 1980; Matteri et al., 1997), and this was confirmed by the present study. Therefore, and in agreement with Castaño et al. (1994), we believe that pituitary cells display a program of dynamic secretory activity *in vitro*, previously predefined *in vivo*. Nevertheless, the total percentage of immunostained GH cells found at the end of the culture does not change as a result of *in vivo* administration of rhGH, although the strongly/weakly GH-immunostained cell proportions are altered. Indeed, for *in vivo* saline-treated rats the proportion of strongly GH-immuno-stained cells (dense GH cells or Kurosumi's and Takahashi's type I cells) is similar to that described above (Kurosumi, 1986; Takahashi, 1991; Dobado-Berrios et al., 1996a,b), but changes from 66% to 45% for *in vivo* GH-treated rats and, in turn, the weakly GH-immunostained cells (light cells or Kurosumi's and Takahashi's type II/III cells) increase from 34% to 55%. The authors believe that the change in strongly/weakly GH-immunostained cell proportions may well account for the decrease in volume density of the secretion granules from somatotropes, as recorded for animals *in vivo* treated with rhGH, since no distinction was made

when measuring this parameter in terms of the different types of GH cells.

In monolayer cultures, the stimulus of GHRH; SRIH o GHRH+SRIH did not change the proportion of strongly/weakly GH-immunostained cells for *in vivo* saline-treated rats, but these same stimuli appreciably modified this proportion for *in vivo* GH-treated rats. The increase in strongly GH-immunostained cells *in vitro* is probably due to an increase in GH secretion, in the case of stimulation with GHRH (Billestrup et al., 1986), and to GH intracellular storage in the case of stimulation with SRIH (Lanzi and Tannenbaum, 1992a,b). However, the authors can offer no explanation as to why *in vitro* GHRH and SRIH interaction increases the weakly GH-immunostained cells obtained from rats receiving rhGH *in vivo* to 62%. Accordingly, following *in vivo* administration of rhGH, the rat somatotrope population, traditionally described as heterogeneous (Snyder et al., 1977; Neill and Frawley, 1983; Frawley and Neill, 1984; Lindstrom and Savendahl, 1996), changes its proportions and presumably also its ability for GH secretion/release. Thus, like the gonadotrope population (Childs, 1995), the somatotrope population appears to be fairly dynamic and convertible in the rat, and a combination of auto-regulatory events involving GH, IGF-I, GHRH, SRIH and possibly others may play a role in modulating expression by a given subpopulation. In agreement with Dobado-Berrios (1996a), we believe that the numerical predominance of a somatotrope subpopulation (weakly GH-immunostained cells) provides a cytological basis for reduced rhGH-mediated GH release.

In vivo rhGH administration did not produce changes in the individual basal GH release of the *in vitro* somatotroph cells. This finding does not, however, mean that *in vitro* GHRH results in increased stimulation of GH release, despite the higher absolute values found in the GH-pretreated group. Indeed, and as confirmed by the frequency distribution of GH immunoblot cells from the GH-pretreated group, GHRH produces an increase in the number of GH cells which release large amounts of hormone (nearly 20%), although the remaining cells release lower amounts of GH (bimodal release). This response points to the existence of a subpopulation of somatotroph cells preferentially responsive to GHRH (Frawley and Neill, 1984; Ho et al., 1986). On the other hand, SRIH *in vitro* did not inhibit individual GH release when rhGH was administered *in vivo*; in any case, GH immunoblot cells formed frequency distributions that were unimodal. Lindstrom and Savendahl (1996) described a greater reduction of GH release in sparsely granulated somatotroph cells in the presence of SRIH. The present authors found a higher proportion of weakly GH-immunostained cells as a result of using rhGH *in vivo*, and so a greater reduction of GH release in the presence of SRIH should be expected; we believe that this contradiction is due to the fact that the results reported by Lindstrom and Savendahl refer to enriched fractions of somatotroph cells.

Using the cell-blotting technique described by

Kendal and Hymer (1986), both the size and the optical density of GH immunoblot cells can be determined, thus obtaining a relative parameter for individual GH release (Dobado-Berrios et al., 1992a,b), as opposed to the reverse haemolytic plaque assay technique, which only allows for the measurement of plaque size. Moreover, use of cell-blotting ensures that paracrine interactions are minimised in two ways (Ramirez et al., 1997). Firstly, it increases the distance between individual cells. Secondly, the membrane used for culture retains a putative paracrine signal of peptide nature in the vicinity of the releasing cells, similar to that described for the hormone secreted by the cells. Thus, our research established that the optical density of the GH immunoblot cells is uniform in the vehicle-pretreated group and variable in the GH-pretreated group, probably because GH release comes from different intracellular pools in both cases (Stachura et al., 1986a,b), or because of the existence of cell subpopulations that are heterogeneous (Stachura and Tyler, 1986) not only morphologically but also functionally.

In summary, our results suggest that *in vivo* chronic rhGH administration to young male rats produces a lower GH release because it increases the proportion of weakly GH-immunostained somatotroph cells (light cells or Kurosumi's type Il/Ill cells), which release a lower amount of GH. Under these conditions, GHRH forces individual GH release to be basically done by a subpopulation of GH cells and SRIH does not inhibit individual GH release. Further studies are needed to explain the mechanisms governing these cell responses and their physiological relevance.

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