Contents and release of human growth hormone1-43 (hGH1-43) in human pituitary glands and hGH-producing pituitary adenoma cells

Tadashi Shimizu1, Yoshio Murakami1, Masateru Nishik1, Kunio Koshimura1, Yutaka Ok1 and Yuzuru Kato1
1Department of Endocrinology, Metabolism and Hematological Oncology, Shimane University School of Medicine, Izumo 693-8501, Japan and 2Second Division, Department of Medicine, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan
(Received 21 April 2004; and accepted 30 April 2004)

ABSTRACT

Human growth hormone1-43 (hGH1-43) is an amino terminal fragment of 22 kilodalton hGH (hGH1-191). In the present study, we developed a specific sandwich-type enzyme linked immunosorbent assay (ELISA) of hGH1-43. The minimal detectable dose was 3.5 × 10-4 pmol/well. hGH1-191 did not show any crossreactivity to hGH1-43 in the assay up to the dose of 0.25 pmol/well. Using this assay, we investigated the quantitative relationship between hGH1-191 and hGH1-43 in contents of normal human pituitary gland and hGH-producing adenoma cells. We also studied the release of hGH1-191 and hGH1-43 from cultured hGH-producing pituitary adenoma cells. Means (±SD) ratios of hGH1-43/hGH1-191 in the cytosolic fractions were 0.02 ± 0.01% in the normal pituitary and 0.07 ± 0.04% in hGH-producing adenoma cells, respectively. In static experiments, the ratio of hGH1-43/hGH1-191 was 1.21 ± 0.50% in the culture medium of hGH-producing pituitary adenoma cells. hGH1-191 and hGH1-43 were released in parallel in response to TRH, and the hGH1-43/hGH1-191 ratio was 1.37% in perfusion experiments. These observations suggest that the hGH1-43/hGH1-191 ratio in cell content is much lower than previously reported. It was also suggested that the ratio of hGH1-43/hGH1-191 is higher in the releasable pool of hGH in the pituitary and hGH1-43 might be secreted in a similar fashion to hGH1-191.

Human growth hormone (hGH) variants can be identified in pituitary extracts, among which 22 kilodalton (22 k) hGH (hGH1-191) is the most abundant (1). hGH1-43 is a peptide composed of amino terminal 43 amino acids of hGH1-191 (5). hGH1-43 was first isolated from human pituitary glands by Singh et al. in 1983 (11). Thereafter it was detected in human serum by western blotting (4). Functionally, hGH1-43 may have some roles in glucose metabolism, since hGH1-43 potentiated the hypoglycemic effect of insulin in hypophysectomized rats (9), mice (10) and an obese mouse model (2). However, there have been few reports on contents and the release of hGH1-43 from the pituitary possibly due to a lack of reliable assay.

Lopez-Guajardo et al. (6) developed an indirect competitive enzyme linked immunosorbent assay (ELISA) of hGH1-43. However, the assay was not adequate for measuring hGH1-43 samples containing high concentrations of hGH1-191, due to considerable cross-reactivity to hGH1-219. In the present study, we newly developed a specific and sensitive sandwich-type ELISA of hGH1-43. Using the assay, we investigated the quantitative relationship between hGH1-191 and hGH1-43 in the content of normal human pituitary glands and hGH-producing adenomas. Furthermore, we studied the release of hGH1-191 and hGH1-43 from cultured hGH-producing adenoma cells in vitro.
MATERIALS AND METHODS

ELISA of hGH_{1,4}. hGH_{1,4} and 5Cys-hGH_{1,4} were synthesized by a solid phase method. A white rabbit was immunized by repetitive subcutaneous injections of 5Cys-hGH_{1,4} conjugated with keyhole limpet hemocyanin. Serum anti-hGH_{1,4} antibody was purified with a protein A affinity chromatography. Rabbit antiserum was loaded on a Protein A-cellulose column equilibrated with binding buffer (1.5 M glycine, 3 M NaCl, pH 8.9) and nonabsorbent was washed out by binding buffer. IgG fragment was eluted with 0.1 M citric (pH 3.0) and was ultrafiltrated by a centric YM-50 to replace the elution buffer with phosphate buffered saline (PBS). Anti-hGH_{1,4} IgG (3 mg/mL) was mixed with biotin reagent (1 mg/0.6 mL PBS) and incubated for 2 h on ice. Then the biotinylated anti hGH_{1,4} antibody was ultrafiltrated by a centric YM-50 to remove unreacted biotin reagent and diluted with 1 mL PBS. Anti-hGH_{1,4} IgG was diluted to a concentration of 10 μg/mL in 0.1 M NaHCO_{3}, and 100 μL of the antibody was poured to each well of 96-well microplates and kept at 4°C overnight to fix antibodies.

Assay procedure. Wells were washed with washing solution (154 mM NaCl, 0.05% Tween 20). Two hundred μL assay buffer (10 mM phosphate buffer, pH 7.4, 1.3% BSA, 0.05% Tween 20) and 25 μL standard solution or samples were added to each well and kept at 4°C overnight. The solutions were aspirated and wells were washed 4 times with washing solutions. One hundred μL biotinylated anti-hGH_{1,4} (0.5 ng/mL assay buffer) was added and incubated at room temperature for 120 min with continuous shaking followed by 4 washes. Then 100 μL streptavidin-horseradish peroxidase was added and further incubated at room temperature for 120 min with continuous shaking. After 4 washes, 100 μL o-phenylenediamine (0.83 mg/mL phosphate-citrate buffer, pH 5.0) containing 0.015% H₂O₂ was added to each well and incubated at room temperature for 30 min. The reaction was terminated by adding 100 μL 2 M H₂SO₄. Optical density at 492 nm (reference: 620 nm) was determined using a platereader. All standard solutions and samples were assayed in triplicate. Mean values were used for statistical evaluation.

Assay of hGH_{1,25} and protein. hGH_{1,25} concentrations were determined in duplicate by immunofunctional hGH ELISA kit (Diagnostic Systems Laboratories, Inc, Texas, USA) according to manufacturer's recommendations. The assay utilizes anti-hGH monoclonal antibody and biotinylated recombinant hGH_{1,19}, binding protein (hGHBp), and detects hGH molecules possessing both site 1 and site 2, which could bind to hGH receptors. Protein levels were measured using a Bio-Rad protein assay kit (Bio-Rad Lab, Tokyo, Japan) with bovine serum albumin as a standard.

Preparation of cytosolic fractions. The cytosolic fractions of normal pituitary were prepared as previously described (8). The pituitary tissues were obtained from control patients without endocrine disorders at autopsy within 10 h after death, and immediately frozen at −80°C until used. The pooled pituitary tissues were homogenized using a polytron homogenizer (5000 rpm, 1 min) in ice-cold 0.1 M PBS containing protease inhibitors (5 mg/L aprotinin, 6 μM leupeptin, 4 μM pepstatin A, 1 mM phenylmethylsulphonylfluoride and 1 mM ethylenediamine tetraacetate). The homogenates were centrifuged at 4,000 g for 20 min at 4°C. The supernatant was further centrifuged at 100,000 g for 60 min at 4°C to obtain cytosolic fractions. hGH-producing adenoma tissues were obtained at surgery from three acromegalic patients (cases A, B and C) and processed as same as described above.

Cell culture. Pituitary adenoma tissues were obtained from three other patients with acromegaly (cases D, E and F) under sterile conditions at surgery. They were immediately subjected to cell culture as previously described (7). Briefly, the tissues were minced by fine scissors in ice-cold PBS, and the cells were dispersed in PBS containing 0.25% trypsin at 37°C for 20 min by gentle stirring using a spinner flask. The dispersed cells were collected, filtered through 40 μm nylon mesh, washed three times with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 IU/mL) and streptomycin (100 μg/mL) (culture medium). The dispersed cells (10⁶ cells/mL) were plated on a 24-well dish for static experiments and a 100 mm dish for perfusion experiments, and cultured for two days in culture medium under humidified atmosphere of 5% CO₂-95% air at 37°C.

The culture medium was aspirated and substituted with serum-free culture medium containing 0.1% BSA. The cells were further cultured for 24 h until static experiments. The cells (10⁶ cell/2 cm²) were incubated in fresh serum-free medium for 120 min in the presence of TRH (10⁻⁸ M and 10⁻⁵ M) and hu-
Content and release of hGH_{43}

human GHRH (10^{-7} M). The reactions were terminated by aspirating the incubation medium. The collected samples were stored at -20°C until assayed.

Perfusion experiments were performed as previously described (7). Briefly, the cells were mechanically harvested from a 100 mm dish. 5 × 10^6 cells were applied onto a small Sephadex G-25 column (diameter: 9 mm, height: 8 mm), and perfused with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 10 mM glucose and 0.1% BSA (KRBG) at a constant flow rate of 0.33 mL/min by a use of a peristaltic pump. KRBG was equilibrated with 95% O_2-5% CO_2 and was kept at 37°C throughout the experiments. TRH was dissolved in KRBG at a concentration of 6.35 × 10^{-4} M and infused into the chamber as a 10-min pulse at a rate of 52 µL/min using an infusion pump. The effluent perfusate was fractionated every 5 min and stored at -20°C until assayed.

Statistics. All the experimental date were expressed as mean ± SD. Statistical differences were evaluated by Mann-Whitney's U test. P < 0.05 was considered significant.

RESULTS

A typical standard curve of hGH_{43} ELISA is shown in Fig. 1. The minimal detectable concentration was 3.5 × 10^{-4} pmol/well. The intra- and inter-assay coefficients of variation were 2.8 and 6.6%, respectively. A dilution curve of human pituitary extract was parallel to the standard curve of hGH_{43}. hGH_{43/19} did not show any cross reactivity in the assay at concentrations up to 0.25 pmol/well.

Contents of hGH_{43/19} and hGH_{43} in the cytosolic fractions of normal human pituitary glands and hGH-producing adenoma tissues are summarized in Table 1. The mean ratio of hGH_{43/19}/hGH_{43} was 0.02% in normal human pituitary glands and 0.07% in hGH-producing adenoma tissues. The values were not statistically different.

![Fig. 1 A standard curve of hGH_{43} ELISA (○). Dilution curves of hGH_{43/19} (△) and cytosolic fraction of human pituitary (□) are shown.](image)

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Contents of hGH_{43/19} and hGH_{43} in the cytosolic fractions of normal human pituitary gland and GH-producing pituitary adenoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal human pituitary gland</td>
<td></td>
</tr>
<tr>
<td>Pooled sample</td>
<td>hGH_{43/19} (pmol/mg protein)</td>
</tr>
<tr>
<td>1</td>
<td>10.7</td>
</tr>
<tr>
<td>2</td>
<td>50.1</td>
</tr>
<tr>
<td>3</td>
<td>5.6</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>22.1 ± 24.3</td>
</tr>
</tbody>
</table>

| hGH-producing pituitary adenoma | | |
| Sample | hGH_{43/19} (pmol/mg protein) | hGH_{43} (pmol/mg protein) | hGH_{43/19}/hGH_{43} (%) |
| A | 665 | 0.62 | 0.09 |
| B | 2737 | 0.72 | 0.02 |
| C | 58 | 0.06 | 0.10 |
| Mean ± SD | 1153 ± 1405 | 0.47 ± 0.36 | 0.07 ± 0.04 |
In static experiments, both hGH_{1-191} and hGH_{1-43} were released from hGH-producing adenoma cells as shown in Table 2. The mean (± SD) ratio of hGH_{1-43}/hGH_{1-191} was 1.21 ± 0.50%. As shown in Fig. 2, both TRH (10^{-8} M and 10^{-7} M) and GHRH (10^{-7} M) increased medium concentrations of both hGH_{1-191} and hGH_{1-43} in static experiments. The magnitude of the response was similar between hGH_{1-191} and hGH_{1-43}. In perfusion experiments, the profile of hGH_{1-191} and hGH_{1-43} release in response to TRH from hGH-producing adenoma cells is shown in Fig. 3. hGH_{1-191} and hGH_{1-43} showed a parallel response to TRH. The peak concentrations of hGH_{1-191} and hGH_{1-43} were 21.1 and 0.29 pmol/mL, respectively, indicating the ratio of hGH_{1-43}/hGH_{1-191} was 1.37%. This value was similar to that observed in the static experiments.

**DISCUSSION**

Singh *et al.* (11) have first demonstrated hGH_{1-43} in human pituitary glands using DEAE-cellulose

<table>
<thead>
<tr>
<th>Sample</th>
<th>medium hGH_{1-191} (pmol/mL)</th>
<th>medium hGH_{1-43} (pmol/mL)</th>
<th>hGH_{1-43}/hGH_{1-191} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>28.4</td>
<td>0.42</td>
<td>1.55</td>
</tr>
<tr>
<td>E</td>
<td>15.2</td>
<td>0.23</td>
<td>1.49</td>
</tr>
<tr>
<td>F</td>
<td>3.8</td>
<td>0.024</td>
<td>0.65</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>15.8 ± 12.3</td>
<td>0.225 ± 0.198</td>
<td>1.21 ± 0.50</td>
</tr>
</tbody>
</table>

**Table 2** Release of hGH_{1-191} and hGH_{1-43} from hGH-producing pituitary adenoma cells in static experiments

![Graphs showing release of hGH_{1-191} and hGH_{1-43}](image_url)

**Fig. 2** Effect of TRH and GHRH on hGH_{1-191} (top) and hGH_{1-43} (bottom) release from hGH-producing adenoma cells in static examination. The cells (cases E and F) were incubated in DMEM with 0.1% BSA for 120 min in the presence of TRH (10^{-8} M and 10^{-7} M) and GHRH (10^{-7} M). Each value represents mean ± SD pmol/mL of 6 samples. #: P < 0.05, *: P < 0.01 vs. control.
in human pituitary extracts (1, 11). We found in the present study that the ratio of hGH1-43/hGH1-191 was 0.01 to 0.03%, which were much lower than previously reported. The difference may be attributed to different methodology used. In the previous reports, hGH1-43 was detected by DEAE-cellulose chromatography as 5 K hGH, which might contain immunoreactive hGH molecules other than hGH1-43. We also found that the ratio of hGH1-43/hGH1-191 in the cytosolic fractions were not different between normal pituitary glands and hGH-producing adenoma tissues, suggesting that generation of hGH1-43 in tumorous tissues is similar to that of normal pituitary gland.

An interesting and new finding in the present study is that the ratio of hGH1-43/hGH1-191 in the incubation medium was considerably greater than that in the content in hGH-producing pituitary adenomas. It was not plausible that secreted hGH was degraded into hGH1-43 during the incubation period, since a similar ratio was observed in the perfusion experiment in which fractionated perfusate was serially frozen. Thus the ratio of hGH1-43/hGH1-191 might be much higher in the releasable pool compared to the content in hGH-producing adenoma cells.

Mechanisms involved in hGH1-43 generation in hGH-producing cells remain to be further elucidated. Several reports have indicated that hGH1-191 was proteolytically degraded into hGH1-43 by peripheral tissue homogenates in vitro (3, 12, 13). It is possible, therefore, that hGH1-43 is proteolytically processed during posttranslational modification of hGH1-191. In the present study, we found that hGH1-191 and hGH1-43 were released in parallel in response to TRH and GHRH. These finding indicate that hGH1-43 could be simultaneously secreted by stimuli from hGH-producing cells.

In conclusion, we developed a specific ELISA of hGH1-43 which was not interfered by hGH1-191. The ratio of hGH1-43/hGH1-191 in cell content was lower than previously reported. It was suggested that hGH1-43 was generated during posttranslational modification of hGH and that hGH1-43 might be secreted in a similar fashion to hGH1-191.

REFERENCES


