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Radioimmunoassay of growth hormone-releasing hormone (GHRH) with a polyclonal antibody against synthetic GHRH(1-29)-Gly_4-Cys-NH_2: method and clinical studies

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Summary

A radioimmunoassay (RIA) for growth hormone-releasing hormone (GHRH) using a polyclonal antibody against synthetic GHRH(1-29)-Gly_4-Cys-NH_2 has been developed. The antiserum (RBM105) showed full cross-reactivity with GHRH-(1-44)NH_2, GHRH-(1-40)OH, GHRH-(1-37)OH and GHRH-(3-44)NH_2, and probably recognized the region of Ala^4 to Lys^{12} of GHRH. Since the sensitivity of the GHRH RIA was 1.5 pg/tube, the lowest detectable plasma level was 5 ng/l when an extract of 0.3 ml of plasma per tube was used. On gelfiltration chromatography, the GHRH immunoreactivity of normal plasma was eluted in the same position as synthetic GHRH. The plasma GHRH concentration in healthy subjects was 20.5 ± 6.5 ng/l (mean ± SD), and in patients with hypothalamic disorders was 17.4 ± 2.0 ng/l. In contrast, the plasma GHRH level in hemodialysis-dependent, chronic renal failure (CRF-HD) patients (38.7 ± 13.1 ng/l) was significantly higher than normal. The acromegalic patients were 24.3 ± 11.9 ng/l, except for one patient with ectopic GHRH syndrome (990 ng/l): his plasma GHRH level reached 7,100 ng/l during operation, and then decreased logarithmically to 70 ng/l after 6 h. Somatostatin at concentrations of 10 and 1,000 nmol/l significantly suppressed

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(GHRH release) from primary culture cells of the GHRH-producing tumor from 17.3 ± 0.92 ng/2×10^5 cells to 9.98 ± 3.61 and 4.32 ± 1.01 ng/2×10^5 cells, respectively after 48 h.

These data indicate that this GHRH RIA is useful for determining the plasma GHRH concentration in normal and diseased states and also for in vitro studies of GHRH release.

Introduction

Since the discovery of growth hormone-releasing hormone (GHRH) [1,2], there have been several reports of preparation of antisera aganist synthetic GHRH to establish a radioimmunoassay (RIA) and immunohistochemical methods for GHRH localization and measurement. However, the results obtained by RIA and in immunohistochemical studies have not been consistent, due to differences in the immunological properties of the anti-GHRH sera [3]. Therefore, it is important to characterize the antisera by determining their specific sites of recognition of the GHRH molecule. Moreover, before RIA determination of plasma GHRH, non-specific substances that interfere with the assay should be removed. In this paper, we describe an RIA for GHRH with a polyclonal antibody against synthetic GHRH(1-29)-Gly_4-Cys-NH_2 recognizing the N-terminal part of the GHRH molecule. We also report plasma GHRH levels in normal subjects and patients with various diseases and the in vitro effect of somatostatin on GHRH release from cultured tumor cells producing GHRH.

Materials and methods

Subjects

Plasma GHRH levels were determined in 34 healthy subjects of 20–40 yr old with no obesity or endocrine disorders, 8 patients with hypothalamic disorders (5 suprasellar germinomas, 2 craniopharygiomas and 1 meningioma), 15 patients with hemodialysis-dependent, chronic renal failure (CRF-HD), and 15 acromegalics. The changes in plasma GHRH and GH concentrations in a patient with ectopic GHRH syndrome [4–6], before, during and after resection of his pancreatic tumor were also measured. Blood samples were drawn from ante-cubital vein into polypropylene tubes containing aprotinin (500 KIU/ml) with EDTA (1.2 mg/ml), promptly placed in an ice bath, and centrifuged at 3,000 rpm for 20 min at 4 °C. The plasma was then stored at −40 °C until assay. For characterization of GHRH immunoreactivity in plasma, samples of 40 ml of plasma were obtained from a healthy subject.

Preparation of anti-GHRH serum

Polyclonal antibody was raised in rabbits by serial subcutaneous injections of GHRH conjugates. The GHRH conjugates were prepared by coupling synthetic

GHRH(1-29)-Gly_4-Cys-NH_2(lot no. 12680-58-15, provided by Dr. A.M. Felix, Hoffmann-La Roche Inc., Nutley, NJ, USA) with BSA using maleimidobemzoyl-*N*-hydroxysuccinimide ester (MBS) [7]. The animals were treated with the conjugate at 2- to 3-wk intervals until antibody with a sufficiently high titer was obtained.

Procedure of radioimmunoassay

GHRH(1-44)NH_2 (Peptide Institute, Inc., Osaka, Japan) was radiolabelled with Iodogen by the method of Fraker and Speck [8]. The specific activity of the tracer was estimated by self-displacement [9] as 1,100 μCi/μg. A mixture of 0.4 ml of assay buffer, 0.1 ml of antiserum (final dilution, 1 : 175,000) and 0.1 ml of standard GHRH or plasma extract was incubated at 4 °C for 48 h. Iodinated GHRH-(1-44)NH_2 (about 5,000 cpm/0.1 ml) was added, and incubation was continued at 4 °C for 48 h. Then, 0.1 ml of antirabbit gamma-globulin (1 : 100) were added. After further incubation at 4 °C for 24 h, the mixture was centrifuged at 4 °C for 30 min at 3,000 rpm and the radioactivity of the precipitate was counted in a well-type gamma counter (Aloka, Tokyo, Japan). Duplicate samples were analysed, and a third tube containing the same amount of plasma extract but no anti-GHRH serum was also prepared to determine the non-specific binding of the tracer.

Extraction of GHRH from plasma

GHRH was extracted from plasma samples by adsorption onto hydrophobic C_{18} Sep-Pak cartridges (Waters Associates, Inc, Milford, MA, USA) activated by successive washes with 10 ml volumes of methanol, distilled water, 90% methanol containing 0.1% trifluoroacetic acid (TFA) and distilled water. A sample of 1 ml of plasma was acidified with 80 μl of medium consisting of 80% INHCl, 5% formic acid, 1% TFA and 1% NaCl prepared by a modification of the methods of Bennett et al. [10], and Girgis et al. [11], and applied to the cartridge at a rate of 1 ml/min. The Sep-Pak C_{18} cartridges were washed with 10 ml of 0.1% TFA to remove interfering substances and then GHRH was eluted with 2 ml of 90% methanol containing 0.1% TFA. The remaining methanol was eliminated by evaporation, and the aqueous portion was lyophilized. The residue was dissolved in assay buffer (0.01 mol/l phosphate buffer, pH 7.4, containing 0.14 mol/l of NaCl, 0.05 mol/l of EDTA, 0.01% NaN_3, 0.1% BSA, and 0.1% Triton X-100) and subjected to RIA for GHRH. Plasma GHRH was concentrated 3-fold by this procedure. GHRH was also extracted from cell culture medium by the same method. Recovery was examined by adding synthetic GHRH[1–44]NH_2 at concentrations of 12.5 and 50 ng/l to 1 ml of fresh human plasma.

Gel chromatography of plasma extract

The extract prepared from 40 ml of plasma of a healthy subject was applied to a column (1.6 × 85 cm) of Sephadex G-50 (fine), and eluted with 8 mol/l urea in 1 mol/l acetic acid, at a flow rate of 15 ml/h. The effluent was collected in 3 ml

fractions and applied to a Sep-Pak C_{18} cartridge. The lyophilized materials obtained were dissolved in assay buffer and subjected to RIA for GHRH.

In vitro study

A pancreatic tumor was resected from a patient with the ectopic GHRH syndrome. The tumor tissue was immediately washed several times with Ca^{2+}/Mg^{2+} free 10 mM phosphate buffer-saline (pH 7.4) containing penicillin (100 U/ml) and gentamycin (50 μg/ml) and dispersed tumor cells were obtained as described previously [12]. In vitro studies were started after culture of the cells for 5 days. The cells were incubated for 48 h in the absence or presence of test materials, and then the media were collected by centrifugation at 4 °C, and frozen at −40 °C until assay. At least four replicates cultures were used in each experiment.

GH RIA

Plasma GH was determined by double-antibody RIA using a GH RIA kit provided by the National Hormone and Pituitary Program, NIAMDD, Bethesda, MD, USA. The sensitivity of this assay was 0.2 ng/ml and the intra- and inter-assay coefficients of variation (CVs) were below 10% [5].

Statistics

All data are expressed as mean ± SD. The results were subjected to analysis of variance, followed by Student's t test, and $P < 0.05$ was regarded as significant.

Results

Characteristics of anti-GHRH antibody

The antibody obtained from 4 rabbits immunized with GHRH conjugate began to show binding to ^{125}I-GHRH(1–44)NH_2 2 mth after the start of injections. In one of these rabbits, the binding titer reached a maximum after 5 months. The polyclonal antibody (RMB105) obtained did not cross-react with PP, VIP, glucagon, secretin, PHI, SRIH, TRH, LHRH, CRF, AVP, NT, ANP, ACTH or GH. The cross-reactivities of various fragments and analogues of GHRH with the antiserum using ^{125}I-GHRH(1-44)NH_2 as a tracer and GHRH-(1–44)NH_2 as standard (Table I), showed that RMB105 recognizes the region of Ala^4 to Lys^{12} of the GHRH molecule, and that its cross-reactivities with GHRH-(1-40)OH, GHRH-(1-37)OH and GHRH-(3-44)NH_2 were 112%, 106%, and 77%, respectively. The association constant was 7.4×10^{10} l/mol, as estimated by Scatchard transformation of the standard curve.

TABLE I

Cross-reactivities of various fragments and analogues of GHRH(1-44)NH_2 with anti-GHRH serum on a molar basis

Peptide	Cross-reactivity (%)	Peptide	Cross-reactivity (%)
GHRH(1–44)NH_2	100	GHRH(13–44)NH_2	0.8
GHRH(1–40)OH	102	GHRH(22–44)NH_2	0.5
GHRH(1–37)OH	114	GHRH(23–44)NH_2	0.03
GHRH(1–29)NH_2	54	GHRH(27–44)NH_2	0.03
GHRH(1–26)OH	57	GHRH(30–44)NH_2	0.02
GHRH(1–20)OH	33	GHRH(3–29)NH_2	58
GHRH(2–44)NH_2	77	GHRH(22–29)NH_2	0.6
GHRH(3–44)NH_2	49	GHRH(1–3)	< 0.01

Standard curve and validity of GHRH RIA

Figure 1 shows a typical standard curve of GHRH RIA. The lowest level of detection of GHRH with 95% confidence was 1.5 pg/tube, and the concentration required to inhibit 50% of the tracer binding (IC_{50}) was about 23 pg/tube. The intra- and inter-assay CVs were determined by replicate determinations ($n = 14$ for intra-assay and $n = 6$ for inter-assay) at 3 concentrations of GHRH (12.5 pg, 25 pg and 50 pg/tube). The CVs of intra- and inter-assay were 5.0–8.4% and

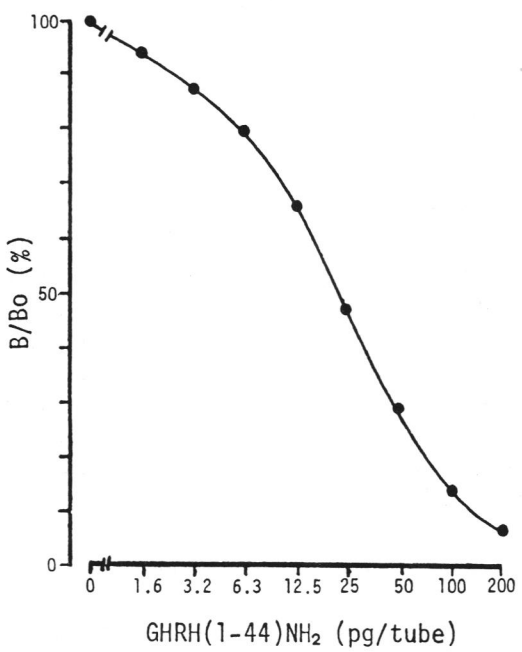

Fig. 1. Standard curve of GHRH radioimmunoassay.

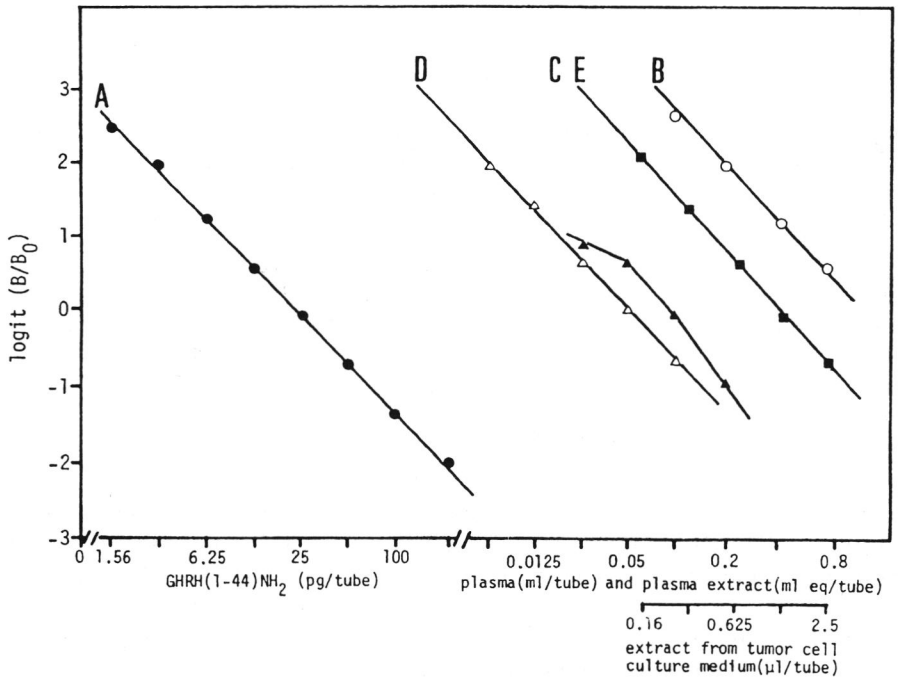

Fig. 2. Inhibition curves of synthetic GHRH(1-44)NH_2(A,●) plasma extract (B,○) from a normal subject normal plasma (C,▲) plasma extracts (D,△) from a patient with ectopic GHRH syndrome and extract from cell culture medium (E,■).

5.3–8.5%, respectively. The recoveries of synthetic GHRH-(1–44)NH_2 added to fresh human plasma at concentrations of 12.5 and 50 ng/l were 83 ± 6.5% ($n = 8$) and 75 ± 2.9% ($n = 8$), respectively.

Characterization of GHRH immunoreactivity in plasma

As shown in Fig. 2, the dose-response curves of plasma extracts from a healthy subject and a patient with ectopic GHRH syndrome and an extract from the cultured medium of pancreatic tumor cells were parallel to the standard curve. In contrast, the dose–response curve of unextracted plasma was not linear. The least detectable plasma level was 5 ng/l of plasma when extracts of 0.3 ml of plasma per tube were used. Gel-filtration chromatography of a plasma extract of a normal subject gave a single peak of GHRH immunoreactivity eluted in the same position as synthetic GHRH-(1-44)NH_2 (Fig. 3).

Plasma GHRH levels in normal and diseased states

The basal levels of plasma GHRH in healthy adults and patients with various disorders are shown in Fig. 4. The mean plasma level of GHRH in 34 healthy

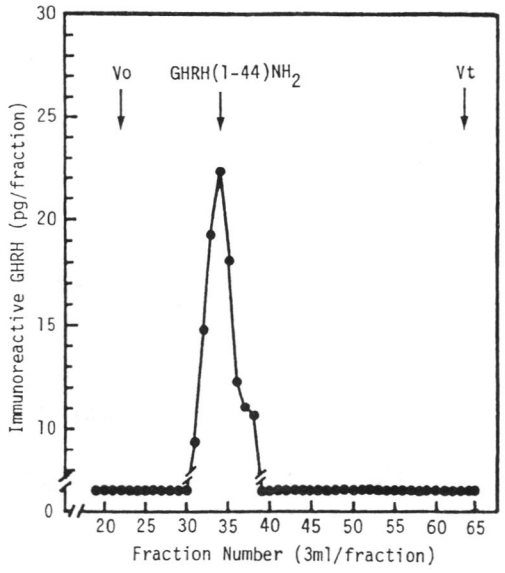

Fig. 3. Distribution of immunoreactive GHRH in fractions of plasma extract from a normal subject on a Sephadex G-50 fine column (1.6×85 cm) eluted with 1 mol/l acetic acid/8 mol/l urea.

adults was 20.5 ± 6.5 ng/l (10.4–36.0 ng/l). The mean level in 8 patients with hypothalamic disorders was 17.4 ± 2.0 ng/l (14.3–20.0 ng/nl). The level in 15 patients with CRF-HD was 38.7 ± 13.1 ng/l (22.0–65.1 ng/l), which was significantly higher than normal ($P < 0.01$). The plasma GHRH levels in 14 patients with acromegaly was 24.3 ± 11.9 ng/l (10.6–50.7 ng/l). A patient with the ectopic GHRH syndrome had a level of 990 ng/l.

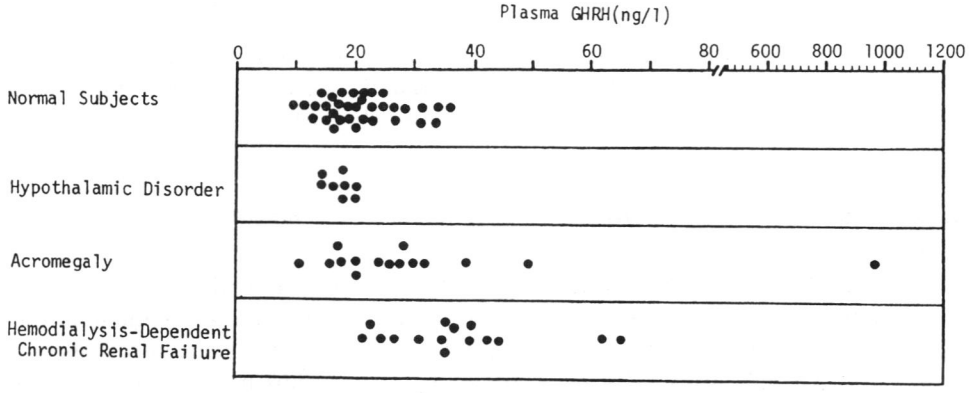

Fig. 4. Plasma GHRH concentrations in healthy subjects and patients with various diseases.

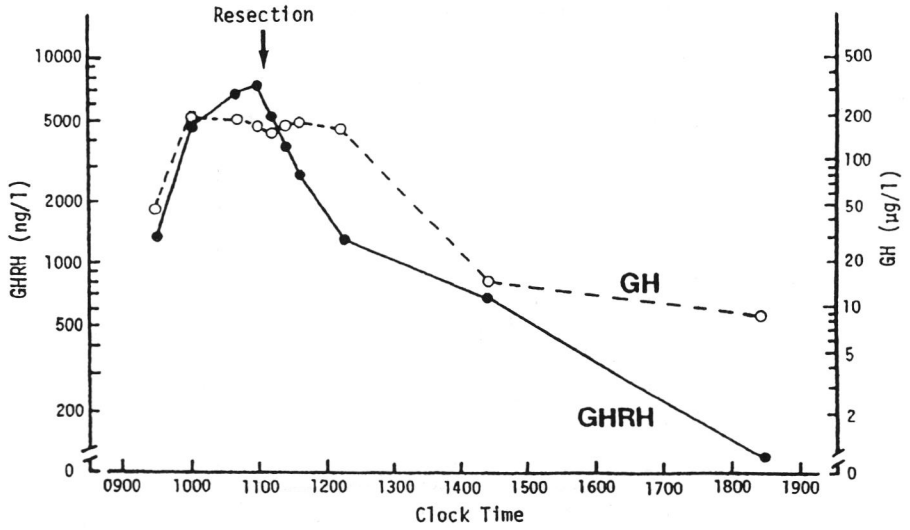

Fig. 5. Changes in plasma concentrations of GHRH and GH before, during and after resection of the GHRH-producing pancreatic tumor.

Changes in plasma GHRH and GH concentrations before, during and after resection of a GHRH-producing pancreatic tumor

Figure 5 shows the changes in plasma GHRH and GH concentrations in the patient with the ectopic GHRH syndrome before, during and after resection of his pancreatic tumor. The plasma GHRH level reached a maximum of 7,000 ng/l during the operation, but decreased 6 h after resection of the tumor to 70 ng/l. The plasma GH level decreased in parallel with the plasma GHRH concentration.

GHRH release from cultured pancreatic tumor cells into the incubation medium

GHRH release from cultured tumor cells into the medium was examined during culture for one month. The GHRH release gradually decreased from 17 ng/2-days to 4 ng/2-days during the first 20 days of culture (Fig. 6).

Effect of somatostatin on GHRH release from cultured tumor cells

Cultured tumor cells were incubated in the presence of synthetic somatostatin at concentrations of 0.1, 10 and 1,000 nmol/l. GHRH release into the medium was reduced significantly and dose-dependently by somatostatin at concentrations of 0 nmol/l (17.3 ± 0.92 ng/2×10^5 cells, 10 nmol/l (9.98 ± 3.61 ng/2×10^5 cells) and 1,000 nmol/l (4.52 ± 1.01 ng/2×10^5 cells) (Fig. 7).

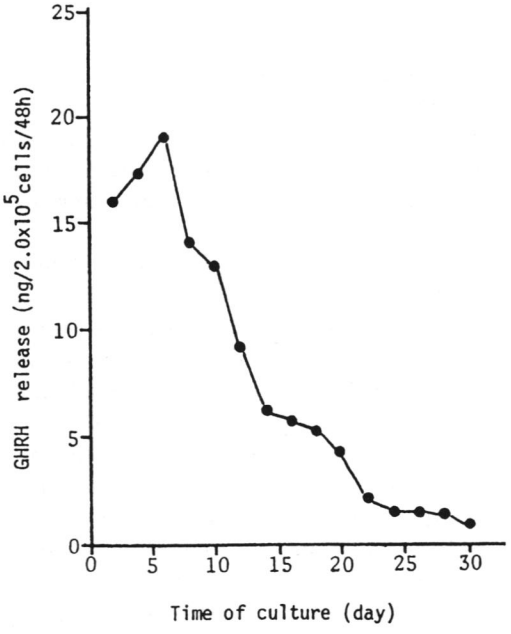

Fig. 6. GHRH release from cultured GHRH-producing pancreatic tumor cells into the medium.

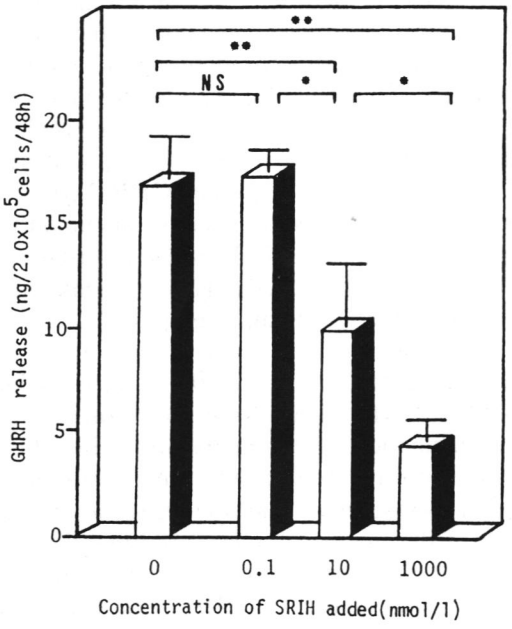

Fig. 7. Effects of somatostatin (SRIH) on GHRH release from cultured GHRH-producing pancreatic tumor cells. Values represent the mean $\pm$ SE of 4. * $P < 0.05$, ** $P < 0.01$. NS, not significant.

Discussion

The antiserum described here cross-reacted with GHRH-(3-44)NH_2, which is considered to be a major metabolite of GHRH produced by dipeptidylaminopeptidase in the blood [13]. On gelfiltration chromatography of the plasma extract, GHRH immunoreactivity was eluted as a single peak in the same position as synthetic GHRH, indicating that the RIA detected immunoreactive GHRH in plasma.

In humans, GHRH is mainly located in the hypothalamus and pituitary stalk, with only very small amounts in other organs such as the digestive tract, pancreas and adrenal gland [14]. Episodic release of GHRH into the peripheral blood can be detected during slow-wave sleep [15], and oral administration of L-dopa stimulates the release of GHRH and GH in healthy subjects [16–18], but not in patients with hypothalamic disorders [17]. The GHRH level in the peripheral blood appears to reflect the release of GHRH from the hypothalamus into the hypophyseal-portal vein resulting in GH release from the pituitary. This conclusion is supported by animal experiments [19]. In patients with hypothalamic disorders, the plasma GHRH level ranged from 14.3 to 20.0 ng/l, and within normal range (10.4–36.0 ng/l). The plasma GHRH concentration in patients with CRF-HD (38.7 ± 3.5 ng/l) was higher than that in healthy subjects, suggesting that GHRH and its metabolites in the plasma are removed, at least in part, by the kidney. The plasma GHRH concentration in patients with acromegaly, except for one patient with ectopic GHRH syndrome, ranged from 10.6 to 50.7 ng/l.

A patient with the ectopic GHRH syndrome had a high level of plasma GHRH (990 ng/l), consistent with results reviewed by Sano et al. [20]. The values determined by the present GHRH RIA in this patient was slightly higher than these that we determined previously [21]. This discrepancy seemed to be due to differences in the antisera and extraction methods used. We monitored the changes in the plasma GHRH levels before, during and after resection of the pancreatic tumor. During operation, the plasma GHRH concentration increased to 7 times of the basal level, probably as the result of manipulation. After removal of the pancreatic tumor, the plasma GHRH level decreased to 70 ng/l after 6 h. From this finding, the $t_{1/2}$ of plasma GHRH was calculated as 30 min, which is similar to the value reported by Frohman et al. [22].

Cells from this tumor obtained at surgery were cultured, and the effect of somatostatin on the release of GHRH into the culture medium was investigated using this GHRH RIA. GHRH release from the cultured tumor cells was significantly suppressed by incubation with somatostatin. This effect is reported to be mediated by an somatostatin receptor on the tumor cells as reported by Reubi et al. [23]. Scatchard analysis showed that the dissociation constant (K_d) of the somatostatin receptor of the tumor cells was 0.58 nmol/l and its maximal binding capacity (B_{max}) was 3.5×10^{-10} mol/g (unpubl data). These data indicate that a somatostatin analogue could suppress GHRH release from a GHRH-producing tumor. In 1984, von Werder et al. first reported use of an somatostatin analogue (octretide) in treatment of a patient with a GHRH-producing metastatic foregut

carcinoid with acromegaly [24]. Since then, 4 acromegalic patients with GHRH-producing metastatic tumors have been treated with octretide [25–27], with beneficial effects on acromegaly.

Acknowledgements

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