Endocrine Journal 2000, **47** (5), 563–568

Twenty-Kilodalton Human Growth Hormone (20K hGH) Secretion from Growth Hormone-Secreting Pituitary Adenoma Cells in Vitro

YOSHIO MURAKAMI, TOSHIAKI MORI, KUNIO KOSHIMURA, MASAMICHI KUROSAKI*, ATSUMI TAKENOBU*, YOSHIHIDE HASHIMOTO** AND YUZURU KATO

First Division, Department of Medicine, Shimane Medical University, Izumo 693–8501, Japan
** Department of Neurosurgery, Tottori University Faculty of Medicine, Yonago 683–8504, Japan*
*** Institute of Biological Science, Mitsui Pharmaceuticals Inc., Mobara 297–0017, Japan*

Abstract. Circulating human growth hormone (GH) consists of several molecular isoforms. Increased proportion of circulating non-22K hGH and 20K hGH was reported in active acromegaly. In this study, we studied the release of 20K and 22K hGH from cultured GH-producing human pituitary adenoma cells *in vitro*. Pituitary adenoma cells obtained from 6 acromegalic patients were cultured and submitted to perifusion experiments. Concentrations of 20K and 22K hGH in the serum and the perifusion effluent were determined by specific enzyme-linked immunosorbent assays recently developed. The %20K value varied in a wide range from 3.58 to 8.72% *in vitro* and was lower than in the serum (mean±SD: 6.57±1.88% *vs* 9.08±2.12%, $P<0.05$). There was no correlation between the %20K values *in vitro* and *in vivo* ($r=0.31$, $P>0.05$). The *in vitro* secretions of 20K and 22K hGH were in parallel and strongly correlated ($r=0.953$, $P<0.001$). These findings suggest that different GH-producing pituitary adenoma cells secrete 20K hGH in variable amounts and that the proportion of 20K hGH in the serum might be affected by metabolic clearance of hGH isoforms. It was also suggested that 20K and 22K hGH might be secreted *in toto* from GH-producing human pituitary adenoma cells.

Key words: Growth hormone, Acromegaly, Pituitary adenoma

(*Endocrine Journal* **47**: 563–568, 2000)

CIRCULATING human growth hormone (hGH) consists of several molecular isoforms, among which the 22 kilodalton hGH (22K hGH) is the most abundant [1]. The 20 kilodalton human GH (20K hGH), a naturally occurring isoform of the 22K hGH, arises from the same gene (hGH-N) as the 22K hGH by alternative mRNA splicing [2]. The 20K hGH lacks 15 amino acids at its binding site 1 to hGH receptor [3], resulting in a reduction of its site 1 binding affinity [4]. The 20K hGH differs in some of its metabolic actions. The 20K hGH has weaker diabetogenic and early insulin-like activities than the 22K hGH [5]. Although the 20K hGH is also poorly trapped by hGH-binding protein [4], the plasma survival time of 20K hGH is longer than that of 22K hGH [6].

An increased proportion of circulating non-22K hGH was found in acromegaly [7]. We recently reported that the proportion of 20K hGH, determined by a specific enzyme-linked immunosorbent assay (ELISA), was increased in the serum of patients with active acromegaly [8]. Because the proportion of non-22K hGH was decreased after pituitary surgery, it was postulated that GH-producing adenoma secretes more non-22K GH [7]. However, there have been no *in vitro* studies on the secretion of 20K hGH from GH-producing adenoma.

There has been a growing body of evidence that molecular forms of hGH secreted *in vivo* are non-

Received: January 6, 2000
Accepted: June 30, 2000
Correspondence to: Yoshio MURAKAMI, First Division, Department of Medicine, Shimane Medical University, 89–1 Enya-cho, Izumo 693–8501, Japan

specific for physiological and pharmacological secretory stimuli [8–12]. It is likely that regulatory mechanisms of 20K and 22K hGH secretion are similar, if not identical [8], but there has been no direct *in vitro* evidence.

In the present study, the release of 20K and 22K hGH from cultured GH-producing human pituitary adenoma cells was studied *in vitro*. In addition, the *in vitro* proportion of 20K hGH was evaluated in comparison with *in vivo* proportion of 20K hGH in the serum.

Materials and Methods

Patients

Six acromegalic patients (4 females and 2 males) were recruited in this study after obtaining informed consent. Their clinical data are summarized in Table 1. All of them had characteristic acromegalic features, high serum levels of hGH and IGF-I, and a pituitary mass on brain MRI. The tumor size was evaluated by measuring the maximal diameters of 3 dimensions on MRI. Plasma hGH levels were not suppressed below 5 μg/L by oral administration of 75 g glucose. Three of them (patients 1, 2 and 3) had associated gonadotropin deficiency, and two patients (patients 2 and 5) were hyperprolactinemic. All the patients had normal thyroid function. Two patients were preoperatively treated with bromocriptine (patient 1) and octreotide (patient 6), respectively.

Serum

Blood samples for serum 20K and 22K hGH determination were obtained by anterocubital vein puncture after overnight fasting less than 1 week before the transsphenoid adenomectomy. Serum was separated by centrifugation and stored at −20°C until assayed.

Cell culture and perifusion experiment

Pituitary adenoma tissues obtained under sterile conditions at surgery were subjected to cell culture and perifusion experiment. Adenoma cells were cultured as previously described [13]. Briefly, the tissues were minced by fine scissors in ice-cold phosphate-buffered saline (PBS) and the cells were dispersed in PBS containing 0.25% trypsin at 37°C for 20 min by gentle stirring by use of a spinner flask. The dispersed cells were collected, filtered through 40-μm nylon mesh, washed three times with Dulbecco's modified Eagle's medium supplemented with 10% FCS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The dispersed cells were cultured in the medium under humidified atmosphere of 5% CO_2-95% air at 37°C. After 2 days, the cells were mechanically harvested. 0.3 to 4.6×10^6 cells were applied onto a small Sephadex G-25 column (diameter; 9 mm, height; 8 mm), and were perifused with Krebs-Ringer bicarbonate buffer, pH 7.4 containing 10 mM glucose and 0.1% bovine serum albumin (KRBG) at a constant flow rate of 0.33 ml/min by use of a peristaltic pump as previously described [13]. KRBG was equilibrated with 95% O_2-5% CO_2 and was kept at 37°C throughout the experiments. Test substances were dissolved in KRBG at 6.35-fold of the final concentration and were infused into the chamber as a 10-min pulse at a rate of 52 μl/min by use of an infusion pump. Human (h) CRH (10^{-6} M), urocortin (hUcn) (10^{-6} M), TRH (10^{-8} M) and GRH (10^{-12}, 10^{-11} and 10^{-10} M) were used for stimulation. We previously reported that urocortin, a ligand to type-2 CRH receptor, stimu-

Table 1. Clinical data in the acromegalic patients examined in this study

Patient	age (yr)	sex	serum IGF-I (ng/mL)	serum PRL (μg/L)	tumor size (mm^3)	hormone deficiency	pretreatment
1	51	F	1280	<0.5	25×38×35	LH, FSH	bromocriptine (22.5 mg/day)
2	51	F	1080	159.3	40×35×30	LH, FSH	
3	47	F	495	18.7	22×17× 8	LH, FSH	
4	26	M	1280	168.6	22×22×56		
5	69	F	410	2.6	15×12×13		
6	54	M	370	3.4	7× 8×11		octreotide (240 μg/day)

lated GH release from adenoma cells [14]. The effluent perfusate was fractionated every 5 min and was stored at −20°C until assayed for 20K and 22K hGH.

Peptides

TRH and hGRH were supplied from Tanabe Pharmaceutical Co., Osaka, Japan and Sumitomo Pharmaceutical Co., Osaka, Japan, respectively. hCRH and hUcn were purchased from Peptide Institute Inc., Minoh, Japan.

20K and 22K hGH ELISA

Concentrations of 20K and 22K hGH in the serum and the perifusion effluent were determined by specific ELISAs as previously described [8]. The minimal detectable concentrations were 5 pg/ml and 50 pg/ml for 20K and 22K hGH, respectively. The intra- and inter-assay coefficients of variation were less than 5% in both assays [8].

Statistics

A percent value of 20K hGH in the perifusion sample was calculated as a mean 20K hGH concentration divided by a mean total hGH (20K hGH plus 22K hGH) concentration in the initial 5 fractions (25 min). In vitro responses of 20K and 22K hGH were calculated as total hormone secreted during 30 min after stimulation corrected by the basal secretion during preceding 20 min. Differences between percent values of 20H hGH in the serum and in perifusion sample were evaluated by paired t test. Correlation between variables was estimated by Spearman rank correlation test. $P<0.05$ was considered significant.

Results

Percent values of 20K hGH (%20K) in the perifusion effluent and the serum, and serum total hGH concentrations are summarized in Table 2. Serum total GH levels were not in parallel with IGF-I levels which are known to be affected by nutritional status, age and estrogens. The *in vitro* %20K value varied in a wide range from 3.58% to 8.72%. In 5 of 6 acromegalic patients (except patient 1), the %20K value in the perifusion effluent was lower than that in the serum, and the difference was statistically significant ($P<0.05$), although there was no correlation between the two variables ($r=0.31$, $P>0.05$). The %20K value in the perifusion effluent or in the serum had no apparent relationship between serum total hGH concentration and serum IGF-I level or the tumor size.

Representative profiles of 20K and 22K hGH release from GH-secreting adenoma cells in response to different hypothalamic peptides are shown in Fig. 1 (patient 2) and Fig. 2 (patient 6). As shown, the release of 20K and 22K hGH was in parallel. When %20K and %22K hGH responses to bioactive pep-

Table 2. 20K and 22K hGH levels in the perifusion effluent and the serum in patients with GH-producing pituitary adenoma

Patient	Perifusion effluent		Serum	
	20K/[20K+22K] (%)	[20K+22K] (μg/L)	20K/[20K+22K] (%)	[20K+22K] (μg/L)
1	8.1	98.97	7.0	795.02
2	6.1	140.52	7.6	1048.44
3	8.7	9.46	12.1	53.36
4	7.4	7.70	9.0	26.02
5	3.6	49.68	7.6	40.04
6	5.6	6.29	11.2	11.40
mean	6.6*	52.10	9.1	329.05
SD	1.9	56.41	2.1	466.24

*: $P<0.05$ vs. serum values.

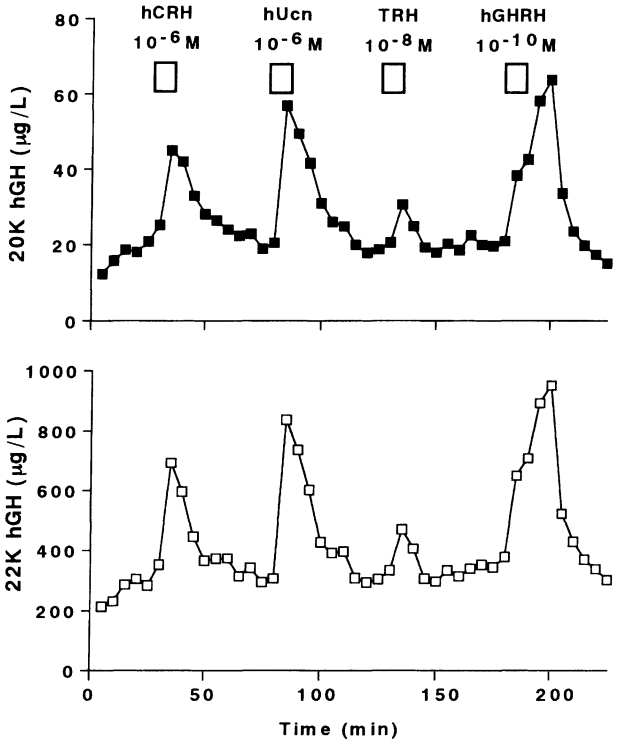

Fig. 1. Representative profiles of 20K (top) and 22K (bottom) hGH release in response to hCRH, hUcn, TRH and hGRH from GH-secreting adenoma cells in patient 2. hCRH (10^{-6} M), hUcn (10^{-6} M), TRH (10^{-8} M) and hGRH (10^{-10} M) were applied as 10-min pulses.

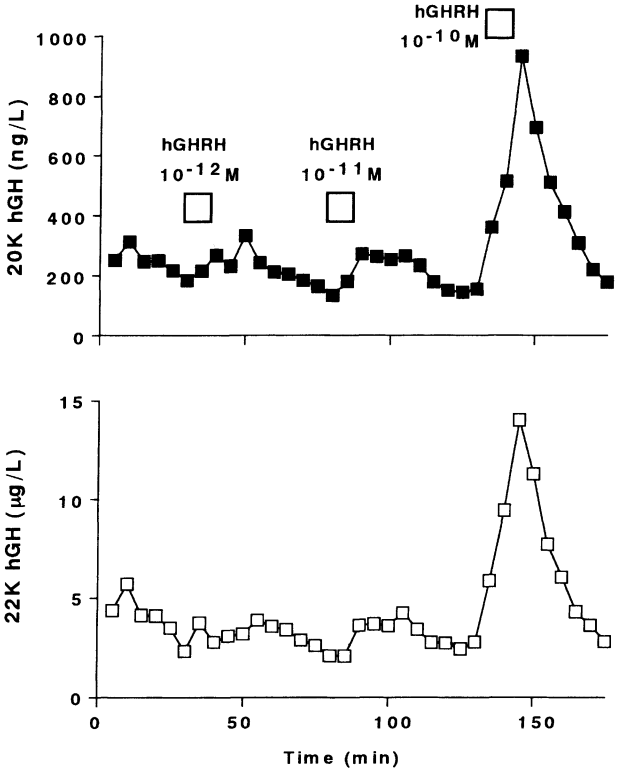

Fig. 2. Representative profiles of 20K (top) and 22K (bottom) hGH release in response to hGRH from GH-secreting adenoma cells in patient 6. hGRH (10^{-12}, 10^{-11} and 10^{-10} M) was applied as a 10-min pulse.

tides in 6 adenomas were evaluated as a whole, there was a strong positive correlation between them ($r=0.953$, $P<0.001$).

Discussion

Boguszewski *et al.* [7, 15] first showed that acromegalics have an increased proportion of circulating non-22K hGH isoforms as determined by 22K GH exclusion assay. Using specific ELISAs for 20K and 22K hGH, we reported that the proportion of 20K hGH was increased in the serum of patients with active acromegaly [8]. In this study, the mean ratio of 20K hGH to total hGH (20K plus 22K hGH) in the serum of 6 acromegalic patients was 9.08± 2.12%. This value was similar to that previously reported by Tsushima *et al.* [8].

The amount of 20K hGH was estimated to be 5%

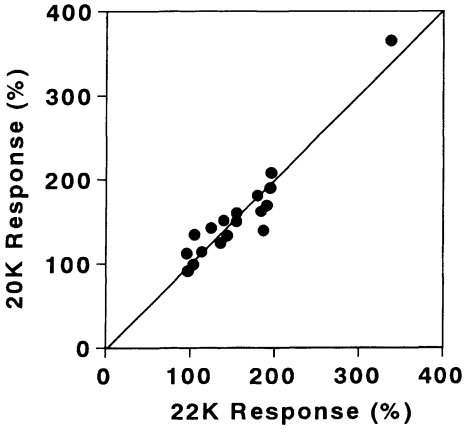

Fig. 3. Correlation between 20K hGH and 22K hGH release in response to hCRH, hUcn, TRH and hGRH from GH-secreting adenoma cells in 6 acromegalic patients. The percent 20K and 22K hGH response was calculated as described in the Materials and Methods. Note the strong positive correlation ($r=0.953$, $P<0.001$).

to 10% of the GH in pituitary extracts [15]. In the report of Sinha *et al.* [17], relative proportion of 20K hGH was as high as 14.8% in extracts of normal pituitary gland. In contrast, the proportion of 20K hGH secreted from cultured human pituitaries was less than 5% [18]. Thus it is likely that some difference might exist between thee relative amount of hGH isoforms in the tissue content and in the serum. To clarify the relative amount of 20K hGH secreted from GH-producing adenoma cells for comparison with serum 20K hGH proportion, we employed the cell culture and perifusion experiment rather than determine the hormone levels in extracts of adenoma tissues. The mean %20K hGH value in the perifusion effluent varied in a wide range from 3.58% to 8.72%. This variability indicates that different GH-producing pituitary adenoma cells secrete 20K hGH in variable amounts. It is difficult to conclude from the present study whether or not GH-producing adenomas secrete a larger proportion of 20K hGH than normal pituitary glands, data for which are not currently available.

In this study, we found that the ratio of 20K hGH secretion *in vitro* was lower than that in the serum. Sinha *et al.* [17] reported that relative proportion of 20K hGH was 14.8% in extracts of normal pituitary gland, while they found only traces of a 20K hGH band in Western blotting in the serum. Taken together, these findings are consistent with the fact that plasma survival time of 20K hGH was longer than 22K hGH [6]. 20K hGH, due to its poor receptor binding, could be cleared at a slower rate than 22K hGH [6]. It was also postulated that 20K hGH tended to dimerize [19] and might escape from glomerular filtration and subsequent degradation in the proximal tubule [6]. The absence of a positive correlation between the *in vitro* and the *in vivo* proportion of hGH indicates a variety of 20K and 22K hGH clearance rate among patients. It was also possible that the proportion of 20K hGH in the serum did not solely reflect relative tumor secretion of 20K hGH but might be affected by metabolic clearance of hGH isoforms.

The serum 20K hGH ratio had no relationship either to serum IGF-I level, the tumor size or preoperative pharmacotherapy in this study of a small number of patients. Using the same ELISAs we previously found that serum 20K hGH proportion and serum IGF-I level were positively correlated when both active inactive (postoperative) acromegalics were combined, while the correlation was not significant in the active acromegalics [8]. Considering the variability of relative 20K hGH secretion among different adenomas and the fact that 20K hGH proportion in the serum could be affected by metabolic clearance, it is not surprising that the serum 20K hGH did not show any correlation to the tumor size. Boguszewski *et al.* [7] suggested that adenomas secreting both GH and PRL seemed to secrete more non-22K hGH isoforms. In our series, however, two patients (patient 2 and 4) whose serum PRL levels exceeded 100 μg/L, exhibited comparable serum 20K hGH proportion with the other patients. This discrepancy could arise from the nature of the different adenomas examined. An alternative explanation is that hGH isoforms other than 20K hGH contributed to the abundant non-22K hGH detected in the former study.

There have been several reports regarding circulating hGH forms after secretory stimulation *in vivo* [9–12]. Stolar *et al.* [12] demonstrated that polyacrylamide gel electrophoresis profiles after GRH stimulation were similar to those obtained after L-DOPA stimulation in normal men. Baumann *et al.* [11] reported that the release of proportions of hGH molecule was not specific for secretory stimuli both in normal and acromegalic subjects. In this study, we clearly demonstrated the *in vitro* 20K and 22K hGH release in response to different secretory stimuli were correlated with each other. This is the first line of evidence for a parallel release of 20K and 22K hGH from GH-producing adenoma cells and supports a notion of *in toto* release [11] of these two moieties of hGH in acromegaly.

In conclusion, our present findings suggest that 20K and 22K hGH might be co-secreted from GH-producing human pituitary adenoma cells. It was also suggested that the high 20K hGH ratio in the serum of patients with active acromegaly could be attributed both to variable 20K hGH production by the adenoma cells and to a slow metabolic clearance rate of this moiety.

References

1. Baumann G, Stolar MW, Amburn K (1985) Molecular forms of circulating growth hormone during spontaneous secretory episodes and in the basal state. *J Clin Endocrinol Metab* 60: 1216–1220.
2. DeNoto FM, Moor DD, Goodman HM (1981) Human growth hormone DNA sequence and mRNA structure: possible alternative splicing. *Nucleic Acids Res* 9: 3719–3730.
3. Lewis UJ, Bonewald LF, Lewis LJ (1980) The 20,000-dalton variant of human growth hormone: location of the amino acid deletions. *Biochem Biophys Res Commun* 92: 511–516.
4. Wada M, Uchida H, Ikeda M, Tsunekawa B, Naito N, Banba S, Tanaka E, Hashimoto Y, Honjo M (1998) The 20-kilodalton (kDa) human growth hormone (hGH) differs from the 22-kDa hGH in the complex formation with cell surface hGH receptor and hGH-binding protein circulating in human plasma. *Mol Endocrinol* 12: 146–156.
5. Baumann G (1991) Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins. *Endocr Rev* 12: 424–449.
6. Baumann G, Stolar MW, Buchanan TA (1985) Slow metabolic clearance rate of the 20,000-dalton variant of human growth hormone: implications for biological activity. *Endocrinology* 117: 1309–1313.
7. Boguszewski CL, Johannsson G, Bengtsson B-Ç00A, Johansson A, Carlsson B, Carlsson LMS (1997) Circulating non-22-kilodalton growth hormone isoforms in acromegalic men before and after transsphenoidal surgery. *J Clin Endocrinol Metab* 82: 1516–1521.
8. Tsushima T, Katoh Y, Miyachi Y, Chihara K, Teramoto A, Irie M, Hashimoto Y, Study Group of 20K hGH (1999) Serum concentration of 20K human growth hormone (20K hGH) measured by a specific enzyme-linked immunosorbent assay. *J Clin Endocrinol Metab* 84: 317–322.
9. Dore S, Brisson GR, Fournier A, Montepetit R, Perrault H, Boisvert D (1991) Contribution of hGH20K variant to blood hGH response in sauna and exercise. *Eur J Appl Physiol* 62: 130–134.
10. Baumann G, MacCart JG, Amburn K (1983) The molecular nature of circulating growth hormone in normal and acromegalic man: evidence for a principal and minor monomeric forms. *J Clin Endocrinol Metab* 56: 946–952.
11. Baumann G, Stolar MW (1986) Molecular forms of human growth hormone secreted in vivo: Non-specificity of secretory stimuli. *J Clin Endocrinol Metab* 62: 789–790.
12. Stolar MW, Baumann G, Vance ML, Thorner MO (1984) Circulating growth hormone forms after stimulation of pituitary secretion with growth hormone-releasing factor in man. *J Clin Endocrinol Metab* 59: 235–239.
13. Yamauchi K, Murakami Y, Koshimura K, Nishiki M, Tanaka J, Kato Y (1996) Involvement of pituitary adenylate cyclase-activating polypeptide in growth hormone secretion induced by serotoninergic mechanisms in the rat. *Endocrinology* 137: 1693–1697.
14. Murakami Y, Mori T, Koshimura K, Kurosaki M, Hori T, Yanaihara N, Kato Y (1997) Stimulation by urocortin of growth hormone (GH) secretion in GH-producing human pituitary adenoma cells. *Endocrine J* 44: 627–629.
15. Boguszewski CL, Hynsjö L, Johannsson G, Bengtsson BÇ00A, Carlsson LMS (1996) 22-kD growth hormone exclusion assay: a new approach to measurement of non-22kD growth hormone isoforms in human blood. *Eur J Endocrinol* 135: 573–582.
16. Lewis UJ, Dunn JT, Bonewald LF, Seavey BK, Vanderlaan WP (1978) A naturally occurring structural variant of human growth hormone. *J Biol Chem* 253: 2679–2687.
17. Sinha YN, Jacobsen BP (1994) Human growth hormone (hGH)-(44-191), a reportedly diabetogenic fragment of hGH, circulates in human blood: measurement by radioimmunoassay. *J Clin Endocrinol Metab* 78: 1411–1418.
18. Baumann G, MacCart JG (1982) Growth hormone production by human pituitary glands in organ culture: evidence for predominant secretion of the single-chain 22,000 molecular weight form (isohormone B). *J Clin Endocrinol Metab* 55: 611–618.
19. Stolar MW, Amburn K, Baumann G (1984) Plasma "big" and "big-big" growth hormone (GH) in man: an oligomeric series composed of structurally diverse GH monomers. *J Clin Endocrinol Metab* 59: 212–218.