RAPID COMMUNICATION

Detection of Exogenous Growth Hormone (GH) Administration by Monitoring Ratio of 20kDa- and 22kDa-GH in Serum and Urine

SHINICHI MOMOMURA, YOSHIHIDE HASHIMOTO*, YUKIO SHIMAZAKI* AND MINORU IRIE**

Toranomon Hospital, 2-2-2 Toranomon, Minato-Ku, Tokyo 105-0001, Japan

**Mitsui Pharmaceuticals, Inc., 1900-1 Togo, Mobara, Chiba 297-0017, Japan*

***Toho University, 5-21-16 Ohmorinishi, Ohta-ku, Tokyo 143-8540, Japan*

Abstract. We previously demonstrated that individual subjects have fairly constant ratios of serum concentrations of 20 kDa- (20K) and 22 kDa-GH (22K). The aim of this study is to demonstrate the possibility of utilizing the changes in the ratio of 20K/22K for detecting the exogenous administration of 22K. A male patient with idiopathic dilated cardiomyopathy (age 51) received 22K (4 U, s.c.) every other day. The concentrations of 20K and 22K in serum and urine were measured using enzyme-linked immunosorbent assays before and after administration. The administration of 22K increased total GH concentration, and markedly decreased the ratio of 20K/22K in serum, especially 2–10 h after the administration. From calculations, it became clear that the concentration of exogenous 22K reached a peak between 2–4 h after the administration and decreased to a negligible level after 24 h. The ratio of 20K/22K in the 0–24 h urine was 5 times lower than that in the 24–48 h urine. These data suggest that, by monitoring the ratio of 20K/22K in serum or urine, it is possible to determine whether or not GH has been externally administered and to calculate the serum GH that has been administered.

Key words: 22kDa-GH, 20kDa-GH, Doping, ELISA

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GROWTH hormone (GH) therapy has been indicated for the treatment of GH deficient patients, and for some other indications and purposes [1, 2]. In addition, GH has been a subject to be tested for doping as one of prohibited drugs in sports [3, 4]. Two different kinds of GHs, 22 kDa- (22K) and 20 kDa-GH (20K), are known to be produced through alternative splicing of mRNA [5]. The 20K is a naturally occurring isoform lacking residues 32–46 of 22K [6] and comprises approximately 5–20% of circulating GH [7–11]. Recently, we have constructed two enzyme-linked immunosorbent assays (ELISAs), which

specifically detect 20K or 22K in blood [9, 10]. The level of circulating 20K was highly correlated to that of 22K in both normal subjects and patients, and the proportion of 20K in each individual subject was fairly constant even after pharmacological and physiological stimuli. Currently, we conducted a pharmacokinetic study of recombinant 20K in human subjects [11], and found that the 24-h profile of serum 20K level in the placebo group is parallel to that of 22K. In contrast, administration of 20K resulted not only in remarkable decrease in the ratio of serum concentrations of 20K and 22K, but also apparent suppression of GH secretion. These results strongly suggest that, by monitoring the ratio of 20K/22K either in serum or urine, it may be possible to determine whether or not GH has been externally administered and to calculate the concentration of serum GH that has been administered. The present

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Correspondence to: Yoshihide HASHIMOTO, Ph.D., Institute of Biological Science, Mitsui Pharmaceuticals, Inc., 1900-1 Togo, Mobara, Chiba 297-0017, Japan.

study is an attempt to detect the external administration of 22K by measuring 20K and 22K in serum and urine and to calculate the concentration of 22K administered externally. We also report the establishment of specific ELISAs for urinary concentration of 20K and 22K.

Subject and Methods

Subject and protocol

The protocol for this study was approved by the Toranomon Hospital (Tokyo, Japan) Institutional Review Board. A 51-yr-old male patient with idiopathic dilated cardiomyopathy and no signs and symptoms of hypopituitarism was found in this patient. Because of his physical condition, provocative tests for GH secretion were not performed. It was decided to administer 22K for the purpose of the improvement of his condition. After obtaining his written informed consent, the patient was subcutaneously administered 4 U of 22K (Genotropin, Pharmacia-Upjohn, Tokyo, Japan) every other day, according to the method of Fazio *et al.* [12]. Blood samples were collected on day 2 and 4 at -9, 2, 4, 10, 24 and 39 h after each administration. Serum samples were kept at -20°C until assayed. Urine samples were collected during 0-24 and 24-48 h after administration, and kept at -20°C after the addition of 1 g/L bovine serum albumin (BSA, Bayer, Kankakee, IL) until the time of centrifugal ultrafiltration. After thawing and initial centrifugation to remove debris, the supernatant urine was concentrated 10-20 fold in an ultrafiltration apparatus (Ultrafree-15 centrifugal filter device, Millipore, Bedford, MA) by centrifuging at $2,000 \times g$ at 4°C for 60 min.

Assays

Concentrations of 20K and 22K in sera were measured by specific ELISAs, as described previously [9]. Concentrations of 20K and 22K in urine were measured by the same ELISAs for serum samples, with minor modifications. Briefly, 0.1 mL of assay buffer (PBS supplemented with 10 mg/L heterophilic blocking reagent (Scantibodies Laboratory, Santee, CA), and 10g/L BSA, pH 7.4) and 0.1 mL of stan-

dard or urine samples were added to each well of a microtiter plate which was precoated with monoclonal antibody. After incubation and washing, peroxidase-labeled monoclonal antibody (POD-D14, 0.5 mg/L, Mitsui Pharmaceuticals Inc., Tokyo, Japan) was applied, followed by the addition of 3,3',5,5'-tetramethyl-benzidine (Sigma, St. Louis, MO) substrate. The reaction was terminated with sulfuric acid and absorbance was determined at 450 nm. In 20K ELISA, the microtiter plates were coated with monoclonal anti-20K antibody (D05, Mitsui Pharmaceuticals Inc.), and in 22K ELISA with monoclonal anti-GH antibody (A36020047P, BiosPacific, Inc., Emeryville, CA) which is specific to 22K.

Calculation of concentrations of endogenous and exogenous GH

Assuming that the endogenous ratio of 20K/22K (referred to as ER) in a human subject is constant and not influenced by GH administrations, the concentrations of exogenous and endogenous GH were calculated as follows;

The ER before administration of 22K:

$$ER = [\text{measured 20K conc.}] / [\text{measured 22K conc.}] \times 100 (\%)$$

After administration of 22K, endogenous and exogenous 22K concentrations were given by the following equations.

$$[\text{endogenous 22K conc.}] = [\text{measured 20K conc.}] \times [100/ER]$$

$$[\text{exogenous 22K conc.}] = [\text{measured 22K conc.}] - [\text{endogenous 22K conc.}]$$

Results

Validity of the 20K and 22K ELISAs in urine

The cutoff values were 5 pg/mL for both 20K and 22K in urine. Within- and between-assay coefficients of variations were less than 10% in both ELISAs. Serial dilution of human urine gave linear results down to the limit of detection in both ELISAs. The recovery of added 20K (20 and 100 pg/mL) into human urine was 91.7-97.3% in the 20K ELISA, and that of added 22K (20 and 100 pg/mL) was 107-110% in the 22K ELISA.

Effects of 22K administration on the concentrations of 20K and 22K in serum

The 22K levels in serum reached a peak between 2 and 4 h, and subsequently decreased at 39-h after administration (Table 1 and Fig. 1). The 20K levels tended to decrease especially at 10 h after administration. The endogenous ratio of 20K/22K (ER) in this subject was calculated from the serum concentrations at 22:00 on day 1 (10.2%). Marked decrease in the ratio of 20K/22K was observed at 2–10 h after administration and almost 10–25 fold reductions were seen in comparison to the ER level. During the subsequent 24-h observation period, the ratio gradually returned to the ER level. From the calculation described in *Subject and Methods*, the concentration of exogenous 22K reached a peak between 2 and 4 h after administration and decreased to a negligible level after 24 h.

Effects of 22K administration on the concentration of 20K and 22K in urine

The 20K level in the urine collected after 0–24 h the administration was not changed compared to the urine collected after 24–48 h (Table 2). In contrast, the 22K level in the 0–24 h urine was 7 times higher

Fig. 1. Changes in serum concentrations of 20K (●) and 22K (○) and the ratio of 20K/22K (bar) after administration of 22K. The arrows represent the time of 22K administration.

Table 2. Changes in urinary concentrations of 20K and 22K and the ratio of 20K/22K after administration of 22K.

Post-dose (h)	Urinary		
	20K (pg/mL)	22K (pg/mL)	20K/22K (%)
0–24	0.94	111	0.8
24–48	0.66	16.2	4.1

Table 1. Changes in serum concentrations of 20 kDa- (20K) and 22kDa-GH (22K) and the ratio of 20K/22K after administration of 22K. The patient was subcutaneously administered 4 U of 22K at 7:00 a.m. on day 2 and 4.

Day of study	Clock Time	Post-dose (h)	Serum ^{a)}			Endogenous ^{b)}		Exogenous ^{c)}
			20K (ng/mL)	22K (ng/mL)	20K/22K (%)	22K (ng/mL)	22K (ng/mL)	
1	22:00	–9	0.113	1.11	10.2 ^{d)}	1.11	—	
2	9:00	2	0.123	17.22	0.7	1.21	16.01	
	11:00	4	0.068	18.43	0.4	0.67	17.76	
	17:00	10	0.026	6.25	0.4	0.25	6.00	
3	7:00	24	0.143	1.49	9.6	1.40	0.09	
	22:00	39(–9)	0.107	0.93	11.5	1.05	–0.12	
4	9:00	2	0.732	17.26	4.2	7.19	10.07	
	11:00	4	0.287	14.21	2.0	2.82	11.39	
	17:00	10	0.060	6.58	0.9	0.59	5.99	
5	7:00	24	0.476	5.41	8.8	4.68	0.73	
	22:00	39	0.097	1.04	9.3	0.96	0.08	

^a measured by specific ELISAs

^b [Endogenous 22K conc.] = [measured 20K conc.] × (1.11/0.113)

^c [Exogenous 22K conc.] = [measured 22K conc.] – [Endogenous 22K conc.]

^d defined as endogenous ratio (ER)

than that in the 24–48 h urine. The ratio of 20K/22K in the 0–24 h urine was 5 times lower than that in the 24–48 h urine.

Discussion

Considering the fact that the total GH level varies according to physiological conditions, it cannot be concluded that 22K has been externally administered only by the observation of increase in total GH level. In this study, the 22K administration not only increased the total GH level, but also markedly decreased the ratio of 20K/22K in the serum, especially at 2 to 10 h after administration. Since the ratio of 20K/22K is not affected by change of the total GH level or stimuli of pituitary gland [9–11], the decrease in the ratio of 20K/22K would appear to be a good criterion to detect the exogenous administration of 22K. Therefore, the monitoring of the ratio of 20K/22K in serum can be reasonably applied as a method of checking for GH doping.

Currently, Wu *et al.* [13] demonstrated a method of checking GH doping using the ratio of 22K/total GH in serum. However, it is not possible to distinguish endogenous and exogenous GH after administration of GH. By contrast, the present calculation method using the ratio of 20K/22K makes it possible to estimate the concentration of exogenous GH,

hence it can be utilized to determine the change of exogenous GH which is not affected by the intermittently secreted, endogenous GH.

The ELISAs developed in this study cannot directly detect the low concentration of 20K in urine. By concentrating the urine sample, however, the ELISAs are sensitive and accurate enough to determine the ratio of 20K/22K in urine. Thus, it becomes possible to monitor the ratio, by which the external administration of 22K can be detected. Collection of urine is much easier compared to blood sampling, by which it is not always possible to check GH doping in certain cases. In this connection, this method of determining the ratio of 20K/22K in urine is a potential method for checking GH doping.

In conclusion, we have demonstrated the possibility of utilizing changes in the ratio of 20K/22K for detecting GH doping, although the data were based on samples collected from a single subject. Since the assay methods are well established, advanced studies, which are now under way, are needed to confirm the possibility presented in this study and to establish the most suitable way of detecting GH abuse.

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