1. Introduction

Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) [1]. Ghrelin is primarily released from the stomach, but is also secreted from the duodenum and pancreas [1,2]. Peripherally-produced ghrelin influences pituitary hormone secretion, appetite, metabolism, gastrointestinal function, cardiovascular performance, and immune responses. Recently, we characterized ghrelin within the rat hypothalamus [3]; the physiological role(s) of ghrelin secreted from the hypothalamus, however, remains unclear.

Histological analysis indicated that ghrelin receptors localize to a variety of brain regions, including the suprachiasmatic nucleus (SCN) and arcuate nucleus (Arc) within the hypothalamus and the hippocampus [4]. The SCN is important region functioning in the regulation of circadian rhythms, while the Arc plays a primary role in feeding control. The hippocampus has a central role in the regulation of memory. Hypothalamic ghrelin also localizes to the Arc [3]; intracerebroventricular injection of ghrelin induces gene expression of neuropeptide Y and agouti-related peptide and impairs the electrical activity of proopiomelanocortin neurons [5–8]. These results indicate that hypothalamic ghrelin regulates feeding patterns and memory related to feeding.

In this study, we sought to investigate if ghrelin regulates feeding performances by generating ghrelin knockout mice.

2. Materials and methods

2.1. Animal care

All animal protocols were approved by the Ethical Committee for the Research of Life Science of Kurume University. All mice were housed in a 7 a.m. to 7 p.m. light cycle. All experiments were performed with F6 littermate pairs; mice were individually caged during experiments.

2.2. Generation of $ghrl^{-/-}$ mice

These animals will be described in detail in another report. All exons were replaced by a neo cassette. Targeted ES cells and resultant wild-type ($ghrl^{+/+}$), heterozygous ($ghrl^{+/-}$), and homozygous ($ghrl^{-/-}$) pups were genotyped by Southern blot analysis using a 5′-probe, 3′-probe, and exon probe.

2.3. Preparation to tissue and ELISA

To confirm the absence of ghrelin from the stomachs of $ghrl^{-/-}$ mice, tissues were quickly removed after mice were sacrificed. Each sample was diced and boiled for 5 min in a 10-fold volume of water to inactive intrinsic proteases. After cooling, solutions were adjusted to final concentrations of 1 M AcOH and 20 mM HCl. Tissues were homogenized with a Polytron mixer; after centrifugation at 15,000 rpm for 10 min, supernatants were loaded onto Sep-Pak C18 cartridges (Waters, Milford, MA). Cartridges were then washed in 0.9% NaCl and 10% CH_3CN/0.1% TFA before bound peptide was eluted with 60% CH_3CN/0.1% TFA. The eluate was lyophilized and analyzed using an active ghrelin ELISA Kit (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan).

2.4. Immunohistochemistry

Mice was perfused with 4% PFA solution and embedded in paraffin. Immunohistochemical staining of ghrelin was performed using the avidin–biotinylated-enzyme complex (ABC) method in conjunction with a VECTASTAIN ABC-PO kit (Vector Laboratories Inc., Burlingame, CA). Immunostaining was performed as previously described [3,9]. Briefly, sections were deparaffinized in xylene and a graded series of ethanol. Sections were then pretreated with 3% hydrogen peroxide in methanol for 5 min to endogenous peroxidase activity. After rinsing with PBS, sections were treated for 30 min with 1.5% normal goat serum, then incubated with polyclonal rabbit anti-ghrelin antibody (#6-6; diluted 1:80,000) for 16 h at 4 °C. Sections were rinsed in PBS and incubated with biotinylated anti-rabbit IgG for 40 min. After rinsing in PBS, sections were incubated with avidin–biotinylated regents for 1 h. Sections were visualized with DAB solution (DAKO, Kyoto, Japan).

2.5. The analysis of feeding performance and memory

$Ghrl^{-/-}$ mice were housed in a K2-CABIN apparatus (Phenotype analyzing, Nagasaki, Japan) and given a powder diet (CREA, Tokyo, Japan) to estimate feeding patterns. Animals were given free access to feed and water. Using this system, we recorded the amounts of food and water intake every 15 min for 12 days. To reveal the adaptation capability of mice to a negative energy state, we instituted scheduled feedings. Mice were given feeds for a 4 h period only from 9 a.m. to 1 p.m. We then measured the 4-h food intake at 1 p.m. To investigate memory in $ghrl^{-/-}$ mice, mice were housed in the KUROBOX apparatus (Phenotype analyzing, Nagasaki, Japan) [10].

2.6. Statistical analysis

Results are presented as the means±SD for each group. Comparisons between groups were made using ANOVA with a Williams test. $P<0.05$ was accepted as statistically significant.

3. Results

3.1. Target disruption of the ghrl locus

Loss of the *ghrl* allele was confirmed by southern blot and PCR analysis of DNA isolated from $ghrl^{+/+}$, $ghrl^{+/-}$, and $ghrl^{-/-}$ mice (data not shown). Measurement of ghrelin levels by ELISA indicated that the stomachs of $ghrl^{-/-}$ mice did not contain ghrelin. Immunohistochemistry also demonstrated the absence of ghrelin-producing cells in the stomachs of $ghrl^{-/-}$ mice (data not shown). We observed a normal birth ratio of $ghrl^{+/+}$, $ghrl^{+/-}$, and $ghrl^{-/-}$ mice as predicted by Mendelian genetics ($ghrl^{+/+}$: $ghrl^{+/-}$: $ghrl^{-/-}$=243 : 498 : 257). All

$ghrl^{-/-}$ mice appeared grossly normal, undergoing normal development to reach adulthood. Male $ghrl^{+/+}$, $ghrl^{+/-}$, and $ghrl^{-/-}$ mice had similar body weights at all ages (Fig. 1A).

3.2. Feeding pattern of $ghrl^{-/-}$ mice

We hypothesized that ghrelin plays a major role in promoting appetite and regulating feeding patterns. To test this hypothesis, we tested if genetic deletion of ghrelin decreased food intake. When $ghrl^{-/-}$ mice were fed standard chow, we did not observe any significant differences in the cumulative food intake over 10 weeks between male $ghrl^{-/-}$ and $ghrl^{+/+}$ littermates (Fig. 1B). In addition, there were no significant differences in feeding patterns between $ghrl^{+/+}$ and $ghrl^{-/-}$ littermates, despite measurement of food intake every 15 min over 11 days using the K2-CABIN apparatus (Fig. 2A). $Ghrl^{-/-}$ mice ate higher quantities of food in dark phase than in light phase, which was similar to $ghrl^{+/+}$ littermates (Fig. 2B). These results indicate that ghrelin does not have an essential role in feeding patterns.

3.3. Adaptation capability to negative energy states of $ghrl^{-/-}$ mice

If ghrelin is necessary for the feeding behaviors induced by negative energy state, $ghrl^{-/-}$ mice may exhibit abnormal behaviors during scheduled feeding. Within one week, $ghrl^{+/+}$ and $ghrl^{-/-}$ littermates both adapted to scheduled feedings, consuming the same amount of food per day (Fig. 3). There were also no differences in water intake or body weight between the

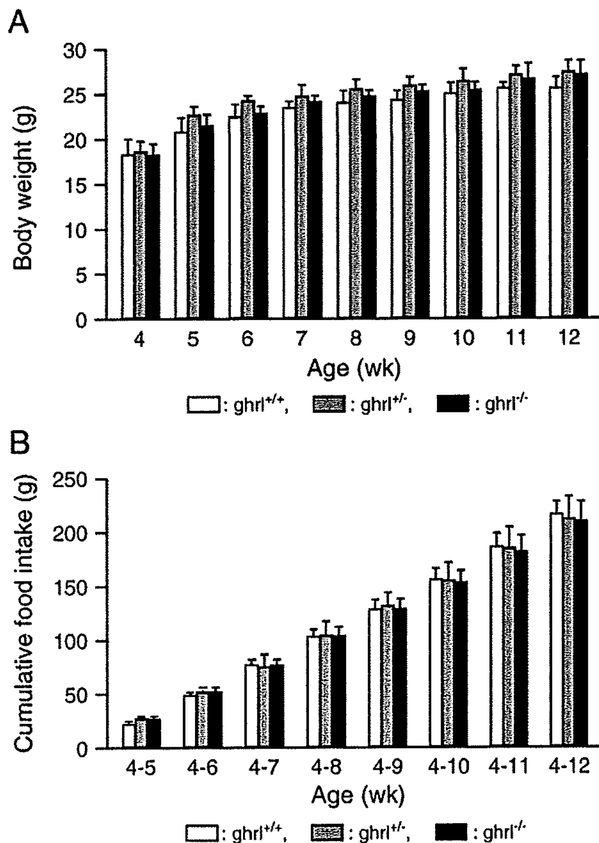

Fig. 1. $Ghrl^{-/-}$ mice exhibit normal growth rates and food intake. (A) Body weight; (B) cumulative food intake. Mice were four weeks old at the beginning of the study ($n=8$, $P>0.05$ [$ghrl^{+/+}$, $ghrl^{+/-}$ versus $ghrl^{-/-}$ mice]).

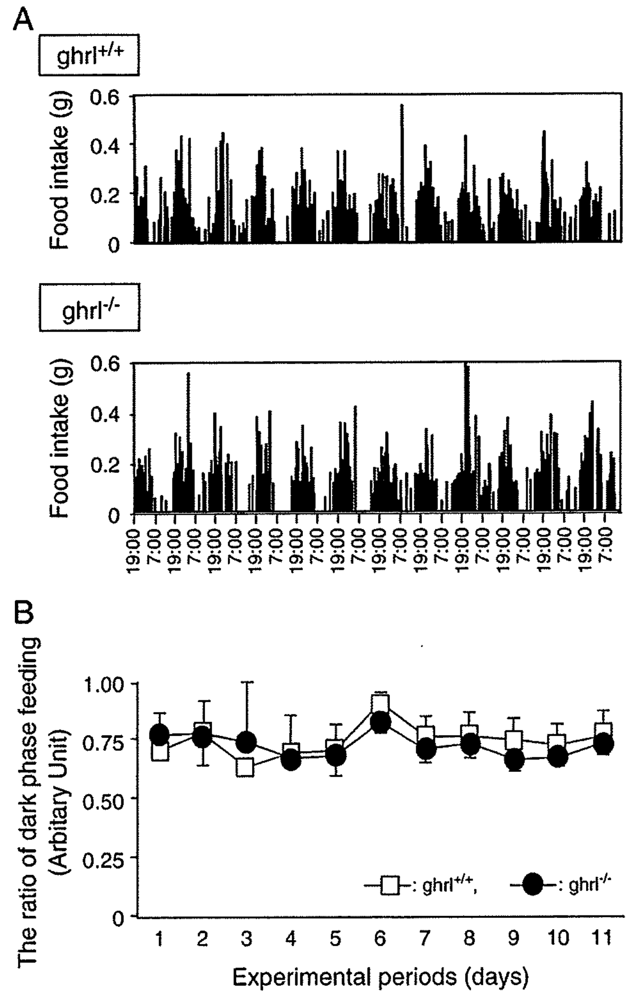

Fig. 2. $Ghrl^{-/-}$ mice display normal feeding patterns. (A) Food intake was recorded every 15 min for 11 days; (B) The ratio of dark phase feeding to total feeds ($n=6$, $P>0.05$ [$ghrl^{+/+}$ versus $ghrl^{-/-}$ mice]).

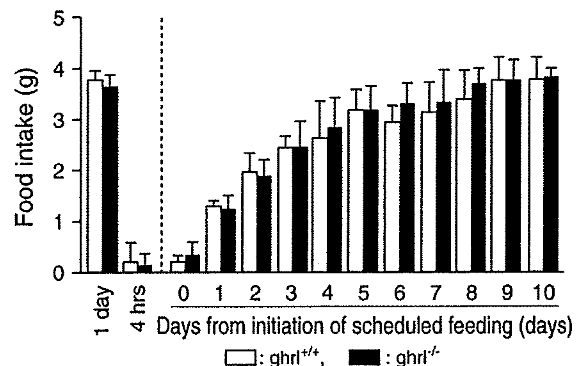

Fig. 3. The adaptation capability of $ghrl^{-/-}$ mice to a negative energy state is also normal ($n=6$, $P>0.05$ [$ghrl^{+/+}$ versus $ghrl^{-/-}$ mice]).

two groups (data not shown). These results demonstrate that *ghrl*$^{-/-}$ mice can adapt to a negative energy state.

3.4. Memory-related feeding performances of *ghrl*$^{-/-}$ mice

To test the physiologic role of ghrelin in feeding memory, we performed a food search test. Low values in *ghrl*$^{-/-}$ mice in comparison to *ghrl*$^{+/+}$ mice would indicate a critical role for ghrelin in this process. To test this assumption, we used a novel apparatus called KUROBOX. This apparatus has four food stations, named regions of interest (ROI), in the four corners of the cage (Fig. 4A). At any one time, however, the mouse can only take powder food from a single station. The correct food station rotated counter-clockwise every 4 h. To analyze the food searching behavior of mice, we used the correct visit ratio. This index is the ratio of visits to the correct ROI to the number of visits to all ROIs. In this experiment, the correct visit ratio was the same for *ghrl*$^{-/-}$ mice as that observed for *ghrl*$^{+/+}$ littermates; this index increased with time in both groups (Fig. 4B). Thus, *ghrl*$^{-/-}$ mice did not exhibit impaired feeding memory.

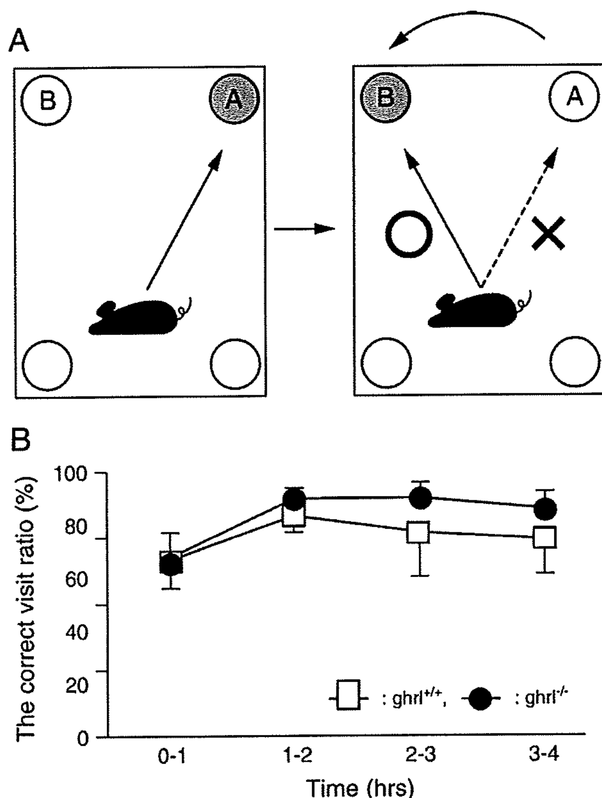

Fig. 4. The memory of feeding in *ghrl*$^{-/-}$ mice is normal. (A) Schematic illustration of the principles of KUROBOX analysis. When feed is first put in ROI A, the mouse always goes to ROI A to eat. Immediately after transferring feeds from ROI A to B, however, the mouse goes to ROI A by mistake at first. Gradually, the mouse goes to the correct ROI B. Investigation of the way of access can help estimate memory tasks in these mice. (B) The correct visit ratio is defined as the ratio of the number of visits to the correct food station to the number of visits to all stations ($n=6$, $P>0.05$ [*ghrl*$^{+/+}$ versus *ghrl*$^{-/-}$ mice]).

4. Discussion

Ghrelin has effects on puberty onset and pregnancy outcomes in rats [11]. Ghrelin also modulates rat testicular function and regulates gonadotropin secretion [12–14]. *Ghrl*$^{-/-}$ mice, however, were fertile and delivered normal litter sizes, indicating that ghrelin is not essential for reproductive function. While a number of reports indicate that ghrelin induces cell proliferation [15–17], there were no gross differences in tissue weights or body weights between *ghrl*$^{+/+}$ *and ghrl*$^{-/-}$ mice. There were no physical or tissue abnormalities identified in *ghrl*$^{-/-}$ mice by our routine analytical protocols. These results suggested that ghrelin is not critical for cell proliferation.

Centrally- or peripherally-injected ghrelin induces acute food intake in rats [18,19]. Ghrelin receptors localize in the SCN and the Arc, important regions in the regulation of circadian rhythms and feeding, respectively [4], suggesting that ghrelin maybe involved in feeding initiation and patterns. *Ghrl*$^{-/-}$ mice, however, have normal feeding patterns, with high food intake in dark phase and low intake in light phase. These results indicate that ghrelin is neither an initiator of feeding nor a regulator of feeding patterns. Ghrelin may act on the SCN to play an unknown role(s) in feeding patterns.

Fasting induces ghrelin secretion from the hypothalamus and stomach in rats [3]. Negative energy states induced by centrally-administered 2-deoxy-D-glucose also stimulates ghrelin secretion from the rat hypothalamus [3]. Plasma ghrelin levels are increased in anorexia nervosa patients and returns to basal levels following weight gain and recovery in these patients [20]. Patients with chronic heart failure (CHF) or chronic obstructive pulmonary disease (COPD) often exhibit a degree of cachexia. Plasma ghrelin levels were significantly higher in CHF patients with cachexia than those without cachexia [21]. Similarly, plasma ghrelin was elevated in underweight patients with COPD, in whom the levels were associated with a cachectic state [22]. Thus, ghrelin secretion is induced by negative energy states, suggesting that ghrelin is necessary for adaptation to negative energy states. In both *ghrl*$^{+/+}$ and *ghrl*$^{-/-}$ mice, however, the capacity to adapt to scheduled feeding was normal. Mice in both groups required approximately one week to eat the same amount of food during over one day. Thus, the absence of ghrelin does not physiologically change the scheduled feedings of mice.

Recently, ghrelin was shown to control hippocampal spine synapse density and memory performance [23]. Therefore, we investigated the memory-related feeding performance of *ghrl*$^{-/-}$ mice using the KUROBOX apparatus. This powerful tool allows us to analyze the memory of feeding. We could not, however, observe any differences in memory-related feeding performance between *ghrl*$^{+/+}$ and *ghrl*$^{-/-}$ mice. In both groups, the correct visit ratio increased with time after transfer of feedings. This result indicates that ghrelin does not control the food searching behaviors or the memory of feeding.

In summary, we did not observe any changes in the feeding performances of *ghrl*$^{-/-}$ mice in this study. Multiple previous reports have demonstrated that ghrelin has an important role in feeding regulation. Therefore, we cannot exclude the possibility that compensatory mechanisms may work to regulate feeding in

$ghrl^{-/-}$ mice. Although we also investigated the gene expression of a subset of orexigenic and anorexigenic peptides, we did not observe any differences between the two groups. It remains possible that an unknown mechanism regulates feeding. Thus, this study demonstrates that ghrelin is not critically required for feeding performance. Further studies will be needed to reveal the essential role(s) of ghrelin in these animals.

Acknowledgments

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Growth Hormone Reverses Nonalcoholic Steatohepatitis in a Patient With Adult Growth Hormone Deficiency

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Background & Aims: **Nonalcoholic steatohepatitis (NASH) is an emerging progressive hepatic disease and demonstrates steatosis, inflammation, and fibrosis. Insulin resistance is a common feature in the development of NASH. Molecular pathogenesis of NASH consists of 2 steps: triglyceride accumulation in hepatocytes with insulin resistance and an enhanced oxidative stress caused by reactive oxygen species. Interestingly, NASH demonstrates a striking similarity to the pathologic conditions observed in adult growth hormone deficiency (AGHD). AGHD is characterized by decreased lean body mass, increased visceral adiposity, abnormal lipid profile, and insulin resistance. Moreover, liver dysfunctions with hyperlipidemia and nonalcoholic fatty liver disease (NAFLD) are frequently observed in patients with AGHD, and it is accompanied by metabolic syndrome.** ***Methods:*** **We studied a case diagnosed as NASH with hyperlipidemia in AGHD. The effect of GH-replacement therapy on the patient was analyzed.** ***Results:*** **Six months of GH-replacement therapy in the patient drastically ameliorated NASH and the abnormal lipid profile concomitant with a marked reduction in oxidative stress.** ***Conclusions:*** **These results suggest that GH plays an essential role in the metabolic and redox regulation in the liver.**

With the increasing prevalence of obesity, diabetes, and metabolic syndrome, nonalcoholic fatty liver disease (NAFLD) has become a common cause of chronic liver disease.[1] NAFLD includes both nonalcoholic fatty liver and nonalcoholic steatohepatitis (NASH). The diagnosis is based on the histologic examination of liver biopsy specimens. NASH is characterized by, in addition to steatosis that is seen in NAFLD, mixed inflammatory cell infiltration, hepatocyte ballooning, and fibrosis.[2] NASH is a serious condition because it progresses to end-stage liver disease. Obesity, metabolic syndrome, type 2 diabetes, and hyperlipidemia are frequently associated with NAFLD including NASH. Insulin resistance is the most common feature in the development of NAFLD/NASH.[3] The cause of NASH appears multifactorial; however, it has been speculated that the molecular pathogenesis of NASH consists of 2 steps: first, insulin resistance status with an accumulation of fat within hepatocytes, and, second, mitochondrial reactive oxygen species causes lipid peroxidation, cytokine induction, and inflammation.[2] These characteristics show a striking similarity with the pathologic conditions observed in adult growth hormone deficiency (AGHD).

AGHD is an established clinical entity characterized by decreased lean body mass and bone mineral density, increased visceral adiposity, abnormal lipid profile, decreased muscle strength and exercise endurance, and diminished quality of life.[4] Recent studies have emphasized the increased morbidity and mortality of patients with hypopituitarism,[5,6] which is closely related to GH deficiency. In a cohort study, AGHD subjects without GH-replacement therapy showed increased incidences of myocardial infarction, cereberovascular diseases, malignancy, and death as compared with the general population.[6] In contrast, the GH-treated subjects demonstrated an overall malignancy and mortality rate comparable with that of the general population. Moreover, liver dysfunction with hyperlipidemia[7] and NAFLD accompanied with the metabolic syndrome manifestation were frequently observed in AGHD patients.[8] In a previous report, one of the AGHD patients was diagnosed with NASH by liver biopsy specimens.[8] Furthermore, it was reported that patients with hypothalamic and pituitary

Abbreviations used in this paper: AGHD, adult growth hormone deficiency; GH, growth hormone; hsCRP, high sensitive C-reactive protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; 8OHdG, 8-hydroxydeoxyguanosine; rhGH, recombinant human GH; ROS, reactive oxygen species.

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dysfunction revealed risk of excessive weight gain, impaired glucose tolerance, and dyslipidemia with subsequent development of NAFLD/NASH with a high prevalence of cirrhosis, increasing their risk for liver-related death.[9] Here, we describe a case of AGHD accompanied by NASH and hyperlipidemia. GH-replacement therapy drastically reversed NASH and hyperlipidemia in the patient. We also analyzed the oxidative stress states in the serum and liver because they were essentially related to the pathogenesis of NASH and metabolic syndrome.

Case Report

A 31-year-old man was referred to our hospital for investigation of hyperlipidemia and liver dysfunction. He was delivered in a breech position and with asphyxia. At birth, his body length and weight had been recorded as 47 cm and 2490 g, respectively. At 7.5 years of age, he was referred for an evaluation of growth delay, and, at that time, his height was 104.8 cm (−3.0 SD below the mean according to the growth chart for Japanese boys), and his weight was 17.6 kg (−1.7 SD). Routine laboratory analysis ruled out hematologic, liver, and renal diseases. The GH responses to provocative tests with the use of insulin and arginine were extremely low (<0.5 ng/mL [undetectable levels]). He was diagnosed as growth hormone, adrenocorticotropic hormone, thyroid-stimulating hormone, leutinizing hormone, and follicle-stimulating hormone deficient and subsequently treated with recombinant human GH (rhGH), hydrocortisone, and thyroxine from the age of 7 years. At 13 years, gonadotropin-replacement therapy was started, and, at the age of 18 years, GH therapy was stopped, and administration of hydrocortisone, thyroxine, and gonadotropin therapy was continued.

The patient's physical examination when he was referred to our hospital at the age of 31 years revealed the following: height, 165 cm; weight, 60 kg; BMI, 22.0; blood pressure, 110/80 mm Hg. He had never consumed alcohol. Laboratory findings demonstrated mild liver dysfunction with type V hyperlipidemia (Table 1, left); hypertriglyceridemia was especially dominant. The serum GH peak levels after insulin and arginine tests were both <0.15 ng/mL (undetectable levels), respectively. Magnetic resonance imaging of the pituitary gland demonstrated a transection in the pituitary stalk, an atrophy of the anterior pituitary gland, and a presence of pseudoposterior pituitary. These findings suggested that the patient had an injury in the pituitary stalk during the delivery in the breech position. An abdominal ultrasonography revealed a typical bright liver, indicating the presence of fatty liver. No hepatitis virus markers were detectable. Liver biopsy was performed to determine the cause of liver dysfunction and hyperlipidemia. The histologic analysis revealed marked steatosis (33.5%) with inflammatory infiltrates and hepatocyte ballooning (Figure 1*A* and 1*B*), and Masson trichrome staining demonstrated perisinusoidal and pericellular fibrosis in acinar zone 3 (Figure 1*C* and 1*D*). These findings indicated a pathologic diagnosis of NASH. According to the classification by Brunt et al,[10] NASH was classified as stage 1 and grade 1. To treat the AGHD status and to determine whether GH-replacement therapy reverses NASH, rhGH was administered daily according to the general protocol[11] after informed consent had been obtained from the patient.

Table 1. Laboratory Studies

	Baseline	After GH treatment
Glucose	93	83 mg/dL (60–110)
Insulin	15	4 IU/mL
AST	88[a]	24 IU/L (13–31)
ALT	85[a]	19 IU/L (8–34)
LDH	309[a]	168 IU/L (115–217)
LAP	60	60 IU/L (35–76)
γGTP	76[a]	17 IU/L (9–57)
ALP	183	121 IU/L (103–321)
ChE	543[a]	414 IU/L (187–453)
LDL cholesterol	143[a]	144[a] mg/dL (70–139)
HDL	31[a]	42 mg/dL (40–60)
TG	712[a]	139 mg/dL (28–149)
Lp(a)	3.8	8.7 mg/dL (0–30)
L-CAT	180.8[a]	75.6 IU/L (67.3–108.2)
Apo AI	130	106 mg/dL (123–194)
AII	38	27 mg/dL (25–44)
B	129[a]	90 mg/dL (52–105)
CII	13.3[a]	4.3 mg/dL (1.5–4.8)
CIII	20.0[a]	7.6 mg/dL (4.6–11.1)
E	11.6[a]	4.1 mg/dL (1.7–6.3)
IGF-I	16.5[a]	147.0 ng/mL (67–318)
HOMA IR	3.4[a]	0.82 (<2.5)
Insulinogenic index	12.2[a]	3.2 (>0.4)
Oral glucose-tolerance test		
Insulin level, IU/mL		
Baseline	15	4
Peak	234	196
120 min	17	11
Glucose level, mg/dL		
Baseline	93	83
Peak	111	142
120 min	72	92

NOTE. The insulin resistance index, index of homeostasis model assessment, is calculated as (the fasting glucose level × the fasting insulin level)/405. (The normal range is less than 2.5.) The insulin secretion index, insulinogenic index, is calculated as the ratio between increases in insulin plasma concentrations during the first 30 minutes following an oral glucose load and increases in glucose (G) plasma concentrations over the same period ($\Delta I30/\Delta G30$). The normal range is more than 0.4.

[a]Indicates abnormal values and the parentheses indicate the normal range.

Materials and Methods

Biochemical Analysis and Hormone Assays

Serum glucose, insulin, liver function, high sensitive C-reactive protein (hsCRP), cholesterol, and triglyceride levels were determined using standard methods with the use of automated equipment (Hitachi, Tokyo, Japan) at Kobe University Hospital. Serum levels of GH and insulin-like growth factor-I were determined by means of an immunoenzymetric assay (TOSO, Tokyo,

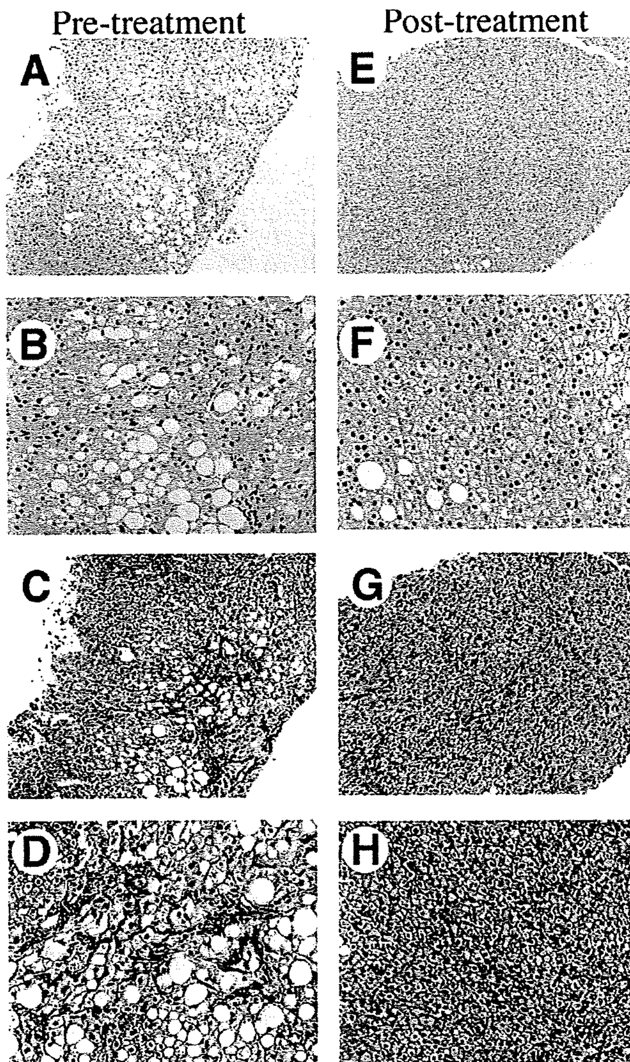

Figure 1. Pretreatment liver biopsy samples (*A–D*) and posttreatment liver biopsy samples (*E–H*). Before treatment, typical NASH characteristics were observed. These include marked steatosis (33.5%) with an inflammatory infiltrate and hepatocyte ballooning (H&E staining; original magnification, *A* and *E* ×100; *B* and *F* ×400) and perisinusoidal and pericellular fibrosis in acinar zone 3 demonstrated by Masson trichrome staining (original magnification, C and *G* ×100; *F* and *H* ×400). In contrast, steatosis was reduced to 7.2% after treatment, inflammatory infiltrate and hepatocyte ballooning disappeared, and fibrosis showed a marked decrease.

Japan) and an immunoradiometric assay (Daiichi Radioisotope Laboratories, Tokyo, Japan), respectively. Serum levels of tumor necrosis factor (TNF)-α and urine-8-hydroxydeoxyguanosine (urine-8OHdG) were determined by a chemiluminescent immunoassay (Funakoshi, Tokyo, Japan) and an enzyme-linked immunoassay (NihonYushi, Tokyo, Japan), respectively.

Histologic Analysis

Written informed consent was obtained from the patient for the liver biopsies. Biopsy specimens were fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into 4-μm-thick sections, and evaluated by H&E staining and Masson trichrome staining before and after GH-replacement therapy.

Immunohistochemistry

The sections were prepared as described above and incubated with monoclonal anti-8-hydroxy-2′-deoxyguanosine/8-hydroxyguanosine (8OHdG/8OHG; 1:100; JaICA, Japan) and antinitrotyrosine monoclonal antibody (MAB5404; 1:10; Chemicon), visualized by using standard immunohistochemical methods, and counterstained by hematoxylin.

Results

RhGH was subcutaneously administered at a daily dose of 3.3 μg/kg at bedtime; subsequently, the dose was gradually increased to 13.3 μg/kg (Figure 2). During this treatment, no additional therapy for liver dysfunction and hyperlipidemia was provided, and the doses of hydrocortisone, thyroxine, and gonadotropins were not changed. No adverse effect was observed except for mild edema in the leg, which disappeared spontaneously. Serum insulin-like growth factor-I levels increased from 47.3 ng/mL in the baseline to 103.0 ng/mL (normal range, 67–318 ng/mL) after treatment, suggesting that the dose of replaced GH was at the physiologic level. As shown in Figure 2, liver dysfunction was rapidly improved during the treatment; 6 weeks later, the values of aspartate transaminase, alanine transaminase, and γ-glutamyl transpeptidase were in the normal range. Re-

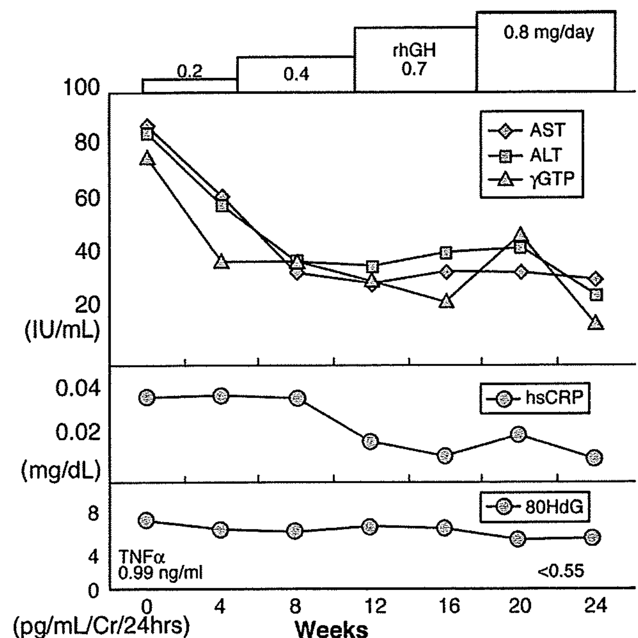

Figure 2. Clinical course during GH-replacement therapy. The daily dose of rhGH was increased gradually. During the treatment, liver dysfunction improved to the normal range. Inflammation and oxidative stress markers such as serum levels of hsCRP and TNF-α, and urine levels of 8OHdG were substantially decreased.

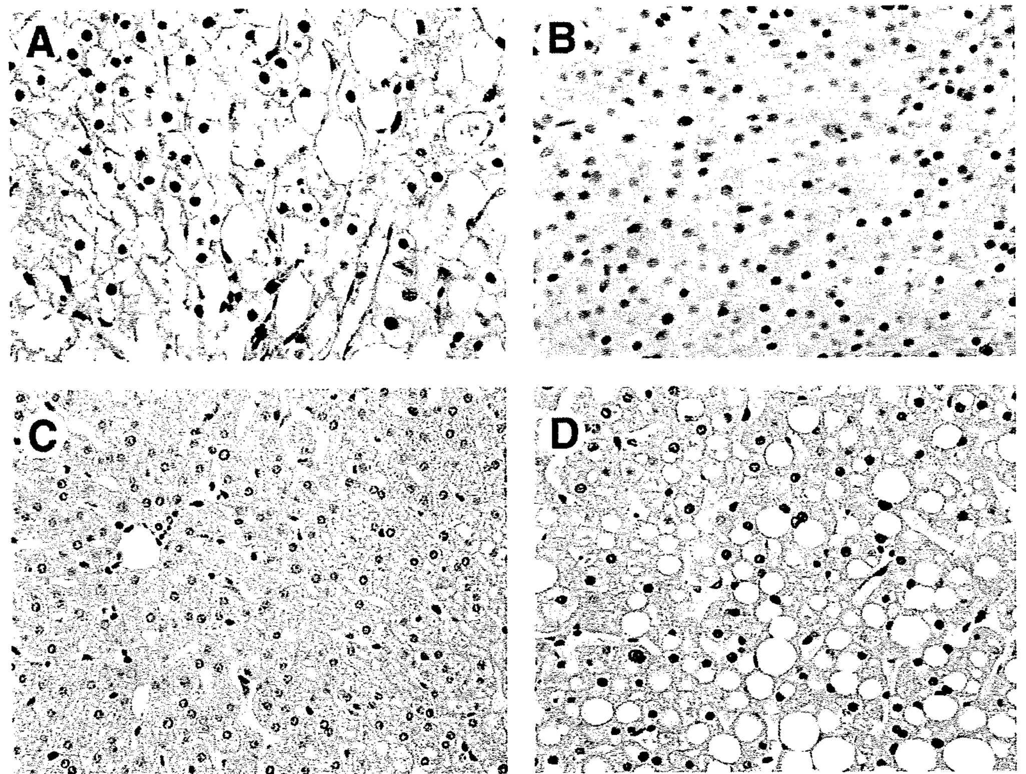

Figure 3. An oxidative stress marker, 8OHdG staining, demonstrated that, in pretreatment liver, most of the hepatocytes were positive for the oxidative stress marker (*A*); however, after treatment, 8OHdG-stained hepatocytes were drastically reduced (*B*). (*C*) A negative control. In normal liver, most of the hepatocytes were negative for 8OHdG staining. (*D*) A positive control. In NASH liver, most of the hepatocytes were positive for 8OHdG staining.

markably, after 6 months of treatment, the markers for liver function and hyperlipidemia values were restored to the normal range, but low-density lipoprotein cholesterol values were not changed (Table 1). Although the total body fat content obtained by dual x-ray absorptiometry did not change (data not shown), visceral obesity decreased after treatment (area of visceral adipose tissue was estimated by using computed tomography: before treatment, 131.6 cm^2 and after treatment, 124.0 cm^2, ie, a decrease of 5.7% was observed). After treatment, a second liver biopsy was performed to assess the histologic changes in the liver. Surprisingly, a drastic improvement in NASH was observed as compared with that observed in the previous biopsy (Figure 1*E–H*). After treatment, steatosis in the liver substantially reduced from 33.5% to 7.2% (Figure 1*A* and 1*E*). The inflammatory infiltrate and hepatocyte ballooning completely disappeared (Figure 1*B* and 1*F*). The perisinusoidal and pericellular fibrosis in acinar zone 3 also markedly improved to a trace surrounding the central vein (Figure 1*C*, 1*D*, 1*G*, and 1*H*). Additionally, we analyzed the changes in inflammation and oxidative status markers during the treatment. Serum hsCRP has been shown to be a useful marker for coronary disease and inflammation status in obesity,[12,13] and urine-8OHdG level is one of the representative markers for the oxidative status in vivo.[14,15] As shown in Figure 2, hsCRP and 8OHdG levels were both decreased during the treatment. Serum TNF-α levels also decreased to an undetectable level after treatment (Figure 2). Immunohistochemical analysis using anti-8OHdG antibody revealed a large number of 8OHdG-positive hepatocytes; this indicates enhanced oxidative stress (Figure 3*A*) in contrast to the normal liver (Figure 3*C*). Notably, after treatment, the 8OHdG-positive hepatocytes showed a marked decrease, and the number was comparable with that present in the normal liver (Figure 3*B* and 3*C*). Another method for the detection of markers for oxidized protein, nitrotyrosine staining, also demonstrated similar results (data not shown).

Discussion

We demonstrated that GH-replacement therapy led to dramatic improvement in NASH and dyslipidemia in an AGHD patient. It is well-known that, as a case of metabolic syndrome, dyslipidemia with elevated low-density lipoprotein level, decreased high-density lipoprotein level, and elevated triglyceride level are frequently associated with AGHD,[5,16] and GH-replacement therapy reverses these abnormalities.[16,17] Although the morbidity of NASH in AGHD has not been clarified, liver dysfunction with hyperlipidemia[7] and NAFLD are more frequently observed in AGHD patients than in the patient without AGHD.[8] Furthermore, in patients with hypothalamic and pituitary dysfunction, the NAFLD development was relatively rapid with a high prevalence of cirrhosis. This places the patients at risk for liver-related death,[9] suggesting that NAFLD, especially NASH is a serious complication in AGHD patients with panhypopituitarism. Thus far, there has been no useful surrogate marker

identified as a substitute for liver biopsy for diagnosing NASH, and this situation hampers an accurate diagnosis of NASH in NAFLD.

Although our study was not randomized and it is difficult to completely exclude the possibility that the results were obtained because of sampling error, the improved liver status appeared to be due to GH-replacement therapy rather than the natural course of recovery, considering the poor progressive prognosis of NASH.[18] Furthermore, in the present case, the effect of GH on NASH suggested that the condition in AGHD was essentially related to the onset of NASH.

With regard to the pathogenesis in NASH, the presence of metabolic syndrome with insulin resistance,[11] elevated levels of inflammatory markers,[19,20] and oxidative stress[21,22] in AGHD patients fulfill the pathogenic conditions for the onset of NASH. In particular, enhancement of oxidative stress is one of the essential factors in the development of NASH from NAFLD as a second hit.[23] Our data demonstrated that, in the AGHD patient with NASH, oxidative stress increased in the serum and liver. It was shown to decrease drastically after GH-replacement therapy, which is concomitant with the histologic improvement. Although it is not yet clear whether GH replacement exerts a direct or an indirect action on the reduction in oxidative stress in the liver, previous reports[21,22] showed that GH exerted an antioxidative stress effect in vivo.

It has been reported that GH-replacement therapy decreased the serum levels of hsCRP[24] and TNF-α[25] in AGHD patients. TNF-α plays a key role in causing inflammation and insulin resistance in the pathogenesis of NASH.[26] In the present case, it is speculated that the reduction in serum levels of TNF-α during the replacement therapy exerted a positive effect on the liver status.

It is important to elucidate the morbidity of NASH in AGHD because of the poor prognosis of NASH as compared with that of NAFLD. Interestingly, decreased plasma levels of GH in NAFLD patients have been reported,[27] suggesting that reduced GH levels could cause the onset of general NASH/NAFLD. Although the results may not be applicable to most NAFLD patients who are not GH deficient, considering the physiologic role of GH, for example, having a lipolytic effect in adulthood, GH treatment could be an option for the treatment of some NAFLD/NASH patients.

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CLINICAL STUDY

A simple diagnostic test using GH-releasing peptide-2 in adult GH deficiency

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Abstract

Objective: The international, first-line diagnostic test for adult GH deficiency is the insulin tolerance test (ITT), which is contraindicated in some patients due to severe adverse events. Alternatives such as GH-releasing hormone combined with arginine or GH-releasing peptides (GHRP) have been proposed. We validated the use of GHRP-2 for diagnosing adult GH deficiency (GHD).

Methods: Seventy-seven healthy subjects and 58 patients with peak GH < 3 μg/l by ITT were enrolled. After overnight fasting, a 100 μg dose of GHRP-2 was administered intravenously; blood samples were taken during the subsequent 2 h and GH measured by immunoradiometric assay.

Results: Serum GH peak occurred within 60 min after GHRP-2 administration in all subjects. GH responses to GHRP-2 were not affected by gender, but were slightly lower in elderly subjects and those with adiposity, although these did not influence diagnosis of GHD. Repeated tests showed favourable reproducibility. Peak GH concentrations after GHRP-2 were significantly ($P < 0.001$) lower in patients (1.36 ± 2.60 μg/l) than the healthy group (84.6 ± 60.9 μg/l) with no difference between hypothalamic and pituitary diseases. Serum GH concentration at the point where sensitivity of response crossed with specificity ranged from 15 to 20 μg/l. A cut-off value of 15 μg/l for diagnosing GHD with GHRP-2 corresponded to the diagnostic value of 3 μg/l in the ITT.

Conclusions: The GHRP-2 provocative test showed favourable reproducibility and was mildly influenced by age and adiposity. Severe GH deficiency could be diagnosed with high reliability using a 15 μg/l (9 μg/l when GH calibrated with recombinant World Health Organization 98/574 standard) cut-off for peak GH concentration.

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Introduction

With the increasing worldwide recognition that growth hormone (GH) therapy is required at all stages of life for patients with GH deficiency (GHD), accurate diagnosis is required before commencing long-term treatment. GHD, in both adults and children, is diagnosed biochemically by provocative tests of GH secretory reserve (1); the insulin tolerance test (ITT) was accepted as the international, first-line diagnostic test of choice according to the consensus guidelines for the diagnosis and treatment of adults with GHD (2). The diagnostic criterion for GHD severe enough to warrant therapy was defined as a peak serum GH concentration (C_{max}) less than the arbitrary cut-off of 3 μg/l with the ITT (2).

An advantage of the ITT is that it allows the evaluation of the complete hypothalamic–pituitary–somatotroph axis, making it useful in patients with both hypothalamic and pituitary disease. However, there are a number of disadvantages to this test. GH secretion is induced by hypoglycaemia, so there is a potential for hypoglycaemia-related adverse reactions (3). It is contraindicated in patients with coronary or cerebrovascular disease, a history of ischaemic heart disease or any convulsive disease and particular attention is needed for the elderly (3). Furthermore, it was reported that the reproducibility of the GH response in an ITT was not sufficient (4–6); a false-negative result, suggestive of insufficient GH secretion, was sometimes obtained even in normal subjects and this was particularly influenced by age and adiposity (7). ITT needs multiple blood sampling and requires close monitoring of patients for several hours in a specialised investigation unit. Thus, there is a need for a simple effective test to establish GHD.

Many other GH provocative tests are employed in clinical practice. The combination of arginine plus GH-releasing hormone (GHRH) has been proposed as a

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useful, reproducible, alternative test to the ITT (8, 9). It is not affected by age but by gender and adiposity (9, 10). Patients with primary hypothalamic disease, such as radiation-induced GHD, may exhibit false-positive responses to the arginine plus GHRH test (11, 12).

Use of a group of peptides called GH-releasing peptides (GHRP) has also been investigated, either alone or in combination with GHRH (13–15). GHRPs are synthetic secretagogues (16) that elicit a dose-dependent and specific GH release by binding to a specific receptor, for which ghrelin has been shown to be the natural ligand (17). The combined GHRH plus GHRP test has been shown to be well tolerated and sensitive in diagnosing GHD (13–15). The influences of age and adiposity have been investigated (18, 19), but more studies are needed to confirm these findings. GHRP-2 has the structure: D-alanyl-3-(2-naphthyl)-D-alanyl-L-alanyl-L-tryptophyl-D-phenylalanyl-L-lysinamide dihydrochloride and is one of the most potent GHRPs (20). Pre-clinical studies demonstrated that its GH-stimulating effect was attenuated when the hypothalamic/pituitary gland connection was impaired (21, 22). In the present communication, we report clinical studies in GH-deficient patients, as well as healthy adult subjects, to validate the use of a single dose of GHRP-2 as a diagnostic agent for GHD resulting from hypothalamic or pituitary disease.

Subjects and methods

Subjects

A total of 135 subjects were enrolled in this study, comprising 58 patients previously diagnosed as having severe GHD, from a peak serum GH value of <3 μg/l in ITT, and 77 healthy adult subjects enrolled as a control group. Patients were excluded from the study for the following reasons: accompanying hypothyroidism or central diabetes insipidus not treated with adequate substitution therapy, receiving a GH preparation, presence of a chromosome abnormality or a serious complication and pregnancy or possible pregnancy. Efforts were made to recruit patients with hypothalamic and pituitary stalk diseases in the study to validate the efficacy of GHRP-2. Prior to starting the study, approval was obtained from the Institutional Review Board of each medical institution and written informed consent was obtained from each subject.

The 77 healthy subjects included 53 males and 24 females, with an age range from 20 to 76 years (median: 26 years) and a body mass index (BMI) ranging from 13.9 to 36.2 kg/m^2 (median: 22.0 kg/m^2). The subjects included six males, who were classified as overweight (obesity grade 1 by Japanese criteria (23)), with a BMI$\geq$25 kg/m^2 and $<$30 kg/m^2 (median: 25.7 kg/m^2), and six males classified as obese (obesity grade 2 by Japanese criteria, with a BMI $\geq$30 kg/m^2 (median: 33.3 kg/m^2)).

The 58 patients with severe GHD comprised 37 males and 21 females, with an age range from 17 to 64 years (median: 39 years) and a BMI ranging from 19.5 to 30.3 kg/m^2 (median: 24.4 kg/m^2). There were no statistically significant differences in gender or age between the healthy control subjects and the GH-deficient patients; however, the BMI was significantly higher in the patient group ($P=0.001$). Table 1 shows the pertinent clinical data of the patients with GHD; serum IGF-1 S.D. score was calculated according to the recently established normative data from 1110 healthy Japanese adult (24). The most common tumour was germinoma ($n=18$) reflecting the relatively high prevalence of this tumour type in Japan (25). Two out of the thirteen patients with craniopharyngioma and all 18 with germinoma had previous cranial irradiation.

Table 1 Clinical data of the patients with severe GH deficiency.

						Hormonal replacement[a]			
Etiology	**Number**	**Gender** (M/F)	**Age median** (range)	**BMI mean±S.D.**	**IGF-1 S.D. score median** (range)	1 H	2 H	3 H	DDAVP
Primary pituitary diseases									
Adenoma[b]	10	8/2	47 (31:60)	24.8±2.7	−2.06 (−7.15:−0.53)	2	2	5	2
Sheehan syndrome	4	0/4	(40:62)	22.7±3.8	(−6.88:−1.24)	0	3	1	0
Others[c]	5	4/1	41 (31:63)	26.0±3.5	−4.00 (−6.48:−0.65)	0	1	3	2
Primary hypothalamic diseases									
Craniopharyngioma[d]	13	6/7	31 (17:64)	22.8±2.4	−3.68 (−8.97:−0.16)	1	3	8	7
Germinoma[d]	18	11/7	32 (22:47)	25.3±3.1	−3.06 (−11.81:0.23)	2	6	7	14
Pituitary stalk transection	5	5/0	28 (20:41)	23.0±2.1	−3.74 (−5.33:−2.29)	0	1	4	1
Others[e]	3	3/0	(48:60)	26.8±1.5	(−3.93:−0.99)	0	3	0	3

[a]1 H/2 H/3 H-number of pituitary hormone replacements (thyroid hormone, glucocorticoid, sex steroids).
[b]Included eight non-functioning adenoma, one Cushing's disease and one prolactinoma.
[c]Included two idiopathic, two trauma and one Rathke's cleft cyst.
[d]Two craniopharyngioma and all 18 germinoma patients received cranial irradiation.
[e]Included two Langerhans cell histiocytosis and one hypertrophic pachymeningitis.

GH provocative tests

ITT All 58 patients with severe GHD were diagnosed by ITT. After overnight fasting, regular insulin (0.05–0.1 U/kg) was administered via an antecubital vein, blood samples were drawn before and 30, 45, 60, 90 and 120 min after insulin injection and serum GH concentrations were determined. All patients reached the required levels of hypoglycaemia (< 2.2 nmol/l) and had clinical signs of hypoglycaemia.

GHRP-2 test A single dose of GHRP-2 (KP-102, Kaken Pharmaceuticals, Tokyo, Japan) was given to each of the subjects under fasting conditions. The GHRP-2 was administered intravenously at a dose of 100 μg. In order to investigate the reproducibility of GH secretion induced by GHRP-2, three doses (25, 50 and 100 μg) were re-administered to 21 healthy subjects with 4-week washout periods between testing.

Blood samples were taken before and 15, 30, 45, 60, 90 and 120 min after GHRP-2 administration and the serum GH concentrations were determined. For evaluation of safety of GHRP-2, blood pressure and heart rate were measured and laboratory tests of haematology and blood chemistry were performed before and after administration.

GH determination

GH concentrations were measured using an IRMA kit (Daiichi Radio Isotope Research Institute, Tokyo, Japan) calibrated with GH standard WHO IRP 66/217. The peak values of serum GH concentration (C_{max}) were standardised with the formula established by the Foundation for Growth Science in Japan to adjust for the inter-assay kit variations (26). However, recent changes have resulted in the use of a new standard, WHO 98/574, in Japan, whereby vales are to be revised by multiplying by 0.6 (27). Values below the detection limit (< 0.05 μg/l) were handled as 0.05 μg/l.

Data analysis

The data were expressed as mean ± S.D. if normally distributed or as median and ranges if the data were skewed. Differences between groups were examined by χ^2 or *t*-tests. Kruskal–Wallis one-way ANOVA was used to compare normally distributed multiple independent groups, whereas ANOVA on ranks was used if the data were skewed. A *P* value < 0.05 was taken as significant. The receiver-operating characteristics (ROC) and sensitivity–specificity curves were investigated to establish the standard C_{max} value for diagnosis of GH deficiency with GHRP-2. For evaluation of two-variable conformance, the figure obtained by plotting the difference between C_{max} values (logarithm) of serum GH obtained with GHRP-2 and insulin against each mean C_{max} value (logarithm) (Bland–Altman plot; (28)) was used for the comparison of response intensity between GHRP-2 and insulin.

Results

Figure 1 shows the serum GH C_{max} value in each subject after the administration of GHRP-2. In the control group, the C_{max} was 87.4 ± 62.9 μg/l (range: 15.9–345.1 μg/l; median: 69.9 μg/l). In the patient group, the C_{max} for serum GH was 1.36 ± 2.60 μg/l (range: 0.05–14.8 μg/l; median: 0.38 μg/l). The C_{max} value was significantly higher in the control group than in the patient group ($P < 0.001$) and was not more than 15 μg/l in any of the 58 patients, but was not < 15 μg/l in any of the 77 subjects in the control group.

The influences of age, gender and adiposity on GH response to GHRP-2 were analysed for all 77 control subjects and the results are shown in Table 2. There were no significant differences in GH C_{max} between males and females in the age range 20–59 years, and between pre- and post-menopausal females in the age range 40–59 years. A small differences in the C_{max} of serum GH was observed between males and females aged over 60 years ($P = 0.030$). Among the three age groups assessed, significant differences were observed; the GH C_{max} was higher in the age group of 20–39 years than in the age group of 40–59 years ($P < 0.001$) and over 60 years ($P < 0.001$). A significant difference was also observed between overweight (BMI ≥ 25 kg/m^2 and < 30 kg/m^2; $P < 0.001$) or obese (BMI ≥ 30 kg/m^2; $P = 0.019$) subjects and lean (BMI < 25 kg/m^2) subjects in the age group 20–39 years. In the age group > 40 years, BMI affected the C_{max} of serum GH even within lean (BMI < 25 kg/m^2)

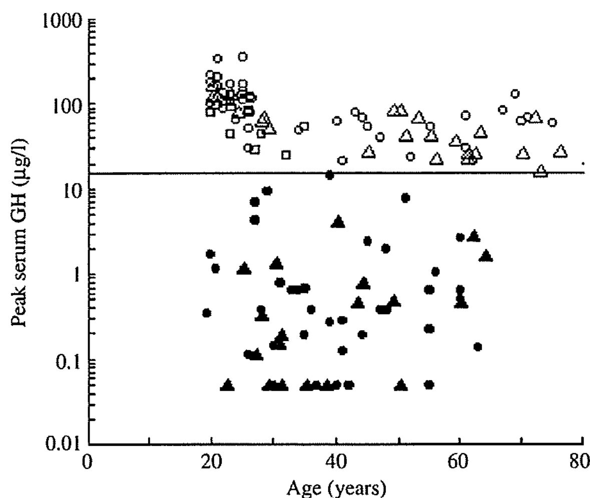

Figure 1 Individual peak GH concentrations after GHRP-2 administration in control subjects and patients with severe GHD defined from an ITT; the horizontal dotted line shows the value of 15 μg/l.

Table 2 Effects of age, gender and menopause, and age and body mass index (BMI), on peak serum growth hormone (GH) concentration (C_{max}) following GH-releasing peptides-2 administration to healthy control subjects.

	n	C_{max} (μg/l)	**Median** (range)	
Age, gender and menopause				
20–39 years	45	114.3±67.5	104.9 (24.2–345.1)	
Male	37	117.0±72.2	104.9 (24.2–345.1)	
Female	8	101.5±40.6	97.8 (51.6–167.9)	
40–59 years	16	50.3±22.0	47.5 (20.9–85.6)	$P<0.001$ vs 20–39 years
Male	8	49.2±20.4	52.1 (20.9–78.8)	$P<0.001$ vs 20–39 years male
Female	8	51.5±24.9	43.2 (22.8–85.6)	$P=0.010$ vs 20–39 years female
Pre-menopause	4	60.2±29.6	(27.4–85.6)	
Post-menopause	4	42.9±19.1	(22.8–68.8)	
60–76 years	16	48.7±30.6	38.1 (15.9–130.5)	$P<0.001$ vs 20–39 years
Male	8	64.8±33.4	63.8 (21.4–130.5)	$P=0.005$ vs 20–39 years male
Female	8	32.6±17.3	26.4 (15.9–69.3)	$P=0.002$ vs 20–39 years female $P=0.030$ vs 60–76 years male
Age and BMI				
20–39 years				
≤21.9 kg/m^2	22	121.2±59.3	115.4 (29.9–329.4)	
22–24.9 kg/m^2	14	138.1±81.0	124.5 (48.6–345.1)	
≥25 kg/m^2	9	60.2±27.8	52.3 (24.2–101.2)	$P<0.001$ vs ≤21.9 kg/m^2, 22–24.9 kg/m^2
≥30 kg/m^2	6	58.1±27.0	48.2 (28.1–101.2)	$P=0.019$ vs ≤21.9 kg/m^2, 22–24.9 kg/m^2
40–76 years				
≤21.9 kg/m^2	10	67.4±28.0	64.9 (29.2–130.5)	
22–24.9 kg/m^2	19	41.9±21.2	37.0 (15.9–85.6)	$P=0.0106$ vs ≤21.9 kg/m^2
≥25 kg/m^2	3	37.9±27.2	(21.4–69.3)	

subjects. Analysis of the area under the curve (AUC) values for serum GH concentration against time after administration (data not shown) showed similar trends to the analysis of C_{max} values.

In 21 male control subjects, aged 20–29 years, various doses of GHRP-2 (25, 50 and 100 μg) were administered twice to investigate the reproducibility of GH response. The correlation between peak GH values from the first and second injections are shown in Fig. 2. Marked inter-individual variations of GH responses to GHRP-2 appeared to blunt the dose-related increase in GH secretion. However, the Spearman correlation coefficient for C_{max} was 0.89, indicating favourable reproducibility.

Figure 3 shows a scatter diagram in which the 58 patients with a peak serum GH value <3 μg/l in the ITT are plotted on the horizontal axis, in the increasing order of C_{max}, with the corresponding serum GH C_{max} values after administration of GHRP-2. Most of the patients, whose C_{max} value was less than the detection limit in the ITT showed almost no response to GHRP-2 and seven of the patients, whose C_{max} was higher than 0.5 μg/l in the ITT showed a C_{max} value higher than 3 μg/l in response to GHRP-2.

GHD patients were divided by underlying primary causes according to the presumed hypothalamic or pituitary origin. Presumed hypothalamic causes were craniopharyngioma, germinoma, pituitary stalk transection and other hypothalamic tumour such as Langerhans cell histiocytosis; presumed pituitary causes included pituitary adenoma, Sheehan syndrome and other causes. Figure 4 summarises the results of C_{max} in control subjects and in patients with presumed primary hypothalamic and pituitary diseases according to the age group of 17–39 years or ≥40 years. Both hypothalamic and pituitary diseases showed equally attenuated GH responses to GHRP-2 irrespective of the age.

Table 3 shows the results for peak serum GH in patients with GHD and in controls stratified by age and BMI because C_{max} was dependent on both parameters in healthy subjects. There was no overlap between peak GH values in the patient group and the corresponding control group. Table 4 shows the time of peak serum GH

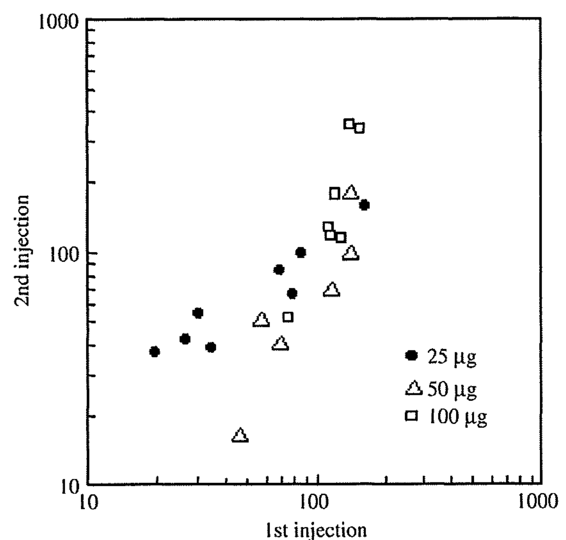

Figure 2 Correlation between peak GH values after the first and second injections of GHRP-2, at doses of 25, 50 and 100 μg, in healthy control subjects.

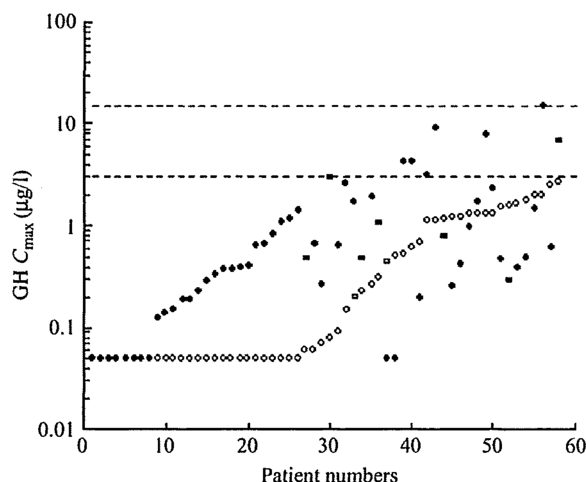

Figure 3 Comparison of peak GH values during an ITT (open symbols) and after GHRP-2 administration (closed symbols), in patients with severe GHD; horizontal dotted lines show 15 and 3 μg/l.

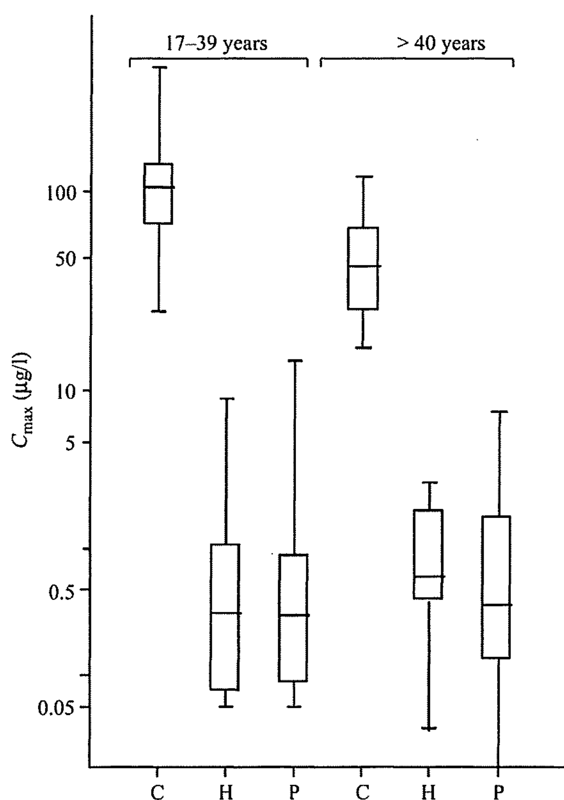

Figure 4 Box and whisker plots representing peak GH responses (C_{max}) to GHRP-2 in control subjects (C) and GHD patients with presumed primary hypothalamic (H) and pituitary (P) diseases; the horizontal line in the box indicates the median, the lower and upper boundaries of the box indicate the 25th and 75th percentiles and error bars above and below the box indicate the 97.5th and 2.5th percentiles.

concentration (T_{max}) after the administration of GHRP-2. The peak value was seen within 15 min after administration in 74% (43/58) of the GH-deficient patients and within 30 min in 88% (68/77) of the control subjects. The peak value was seen within 60 min after the administration in all 132 subjects, except for three of the control group who showed GH peak at 60 min after GHRP-2 administration. When the serum GH concentration at 30 min ($C_{30\ min}$) was compared with the peak serum GH concentration (C_{max}) during the GHRP-2 test, both values were almost of the same magnitude (Fig. 5) with no overlap for $C_{30\ min}$ between the patient and control group.

In order to set a cut-off value for diagnosis of GHD with GHRP-2, the sensitivity and specificity on changing the assumed cut-off value were evaluated as an ROC curve and as a sensitivity–specificity curve. The AUC of the ROC curve was close to 1 (Fig. 6), confirming that GHRP-2 showed high precision as a diagnostic agent for GH secretory capacity. In the sensitivity–specificity curve (Fig. 6), the serum GH concentration at the point where the sensitivity crossed with the specificity, i.e. the borderline value showing both high sensitivity and high specificity, ranged from 15 to 20 μg/l, if all patients were included. This was consistent with the results of the Bland–Altman analysis (data not shown) performed to compare the GH secretory capacity of GHRP-2 with that of insulin. The mean difference in C_{max} of serum GH between GHRP-2 and insulin was 0.702; in retro-conversion, this means that the GH secretion capacity of GHRP-2 was 5.03 times higher than that of insulin. Based on the above results, it was considered reasonable to set the cut-off value for diagnosis of severe GHD with GHRP-2 at 15 μg/l, corresponding to the cut-off value of 3 μg/l for the diagnosis by ITT.

The adverse reactions noted after the administration of GHRP-2 are shown in Table 5. Adverse reactions were reported by 31 of the 58 subjects (53.4%, 45 episodes) in the patient group and 23 of the 77 subjects (29.9%, 34 episodes) in the control group, i.e. in 54 of the 135 subjects (40.0%, 79 episodes) in total. The major adverse reactions were hot flush, borborygmus and white blood cell count increase; all adverse events were mild, except one episode of sweating (moderate) in a healthy control subject, and all were transient and resolved without any treatment.

Discussion

The present findings show that a single dose of GHRP-2 elicited reproducible GH secretion in control subjects and that GH response is not influenced by gender but is affected by age and adiposity to some extent. Severe GHD can be diagnosed with high reliability using a cut-off of 15 μg/l for peak serum GH concentration in patients with both hypothalamic and pituitary diseases.

Table 3 Peak GH concentrations (C_{max}) in response to GH-releasing peptides-2 in patients with GH deficiency compared with control subjects, stratified by age and body mass index.

Age (years)	BMI (kg/m^2)	n	GHD patient C_{max} (μg/l) Mean±S.D.	GHD patient C_{max} (μg/l) Median (range)	n	Control subject C_{max} (μg/l) Mean±S.D.	Control subject C_{max} (μg/l) Median (range)
17–39	≤21.9	10	1.7±2.9	0.35 (0.05–9.1)	22	121.2±59.3	115.4 (29.9–329.4)
	22–24.9	12	1.6±4.2	0.17 (0.05–14.8)	14	138.1±81.0	124.5 (48.6–345.1)
	≥25	9	1.1±2.2	0.19 (0.05–6.8)	9	60.2±27.8	52.3 (24.2–101.2)
40+	≤21.9	5	0.63±0.63	0.48 (0.05–1.7)	10	67.4±28.0	64.9 (29.2–130.5)
	22–24.9	4	2.0±1.8	(0.48–4.2)	19	41.9±21.2	37.0 (15.9–85.6)
	≥25	18	1.2±1.9	0.43 (0.05–7.6)	3	37.9±27.2	(21.4–69.3)

The GHRP-2 test met the criteria reported to be necessary for the validation of a GH provocative test (29), which are: i) to be potent and reproducible, with reproducibility assessed in normal subjects, ii) the influence of gender, age and adiposity should be validated in control subjects, iii) effectiveness needs to be assessed in controls and patients, evaluated by ROC curve analysis, and iv) the cut-off point for the test should be established by ROC curve analysis and is the value that provides the best pairing of sensitivity and specificity.

The magnitude of GH response to GHRP-2 in control adult subjects was reproducible and consistent with previous studies (30, 31). Rapid GH response to GHRP-2 may reduce the number of blood samples and time required for patients in the investigation unit. In the majority of control subjects, the peak occurred at 30 min and for most GH-deficient patients, it was earlier, at 15 min, but the GH values at 30 min were almost of the same magnitude as the peak values. Therefore, a single 30 min sample would provide similar diagnostic information on peak GH as that reported for the GHRH plus GHRP test (14, 15, 32).

Gender had no significant influence on GH response to GHRP-2, but age- and adiposity-affected response in the present study. Previous studies using GHRP-6, hexarelin or ghrelin had shown that gender did not influence the GH response in control subjects (33, 34). However, in older subjects aged 49–76 years, males were reported to respond better to GHRP-2 than females (35), which is in agreement with our findings in control subjects >60 years.

With regard to age, our findings that GH response to GHRP-2 was reduced in control subjects over 40 years when compared with younger adults are in agreement with previous studies using hexarelin or ghrelin (33, 34). However, GHRP-6 alone or combined with GHRH was not affected by age (13, 18, 36). We could not explain why GHRP-6 was apparently not affected by age but GHRP-2 is.

Recent studies have shown that normative values of GH response to GHRH plus GHRP-6 should be defined according to the BMI (18, 19). In our study, GH response to GHRP-2 was found to be affected by an increase in BMI. GH response was reduced but remained substantially preserved in control subjects of BMI over 25 kg/m^2. Further investigation is needed to define normative data.

In our analysis of the sensitivity–specificity curves, comparing healthy subjects with GH-deficient patients, the serum GH concentration at the point where sensitivity crossed with specificity, i.e. the value showing high sensitivity and high specificity, ranged from 15 to 20 μg/l. The intended use of GHRP-2 is not mass screening but exact diagnosis of subjects highly likely to have the disease. Therefore, it was considered reasonable to set the cut-off value for diagnosis of severe GHD with GHRP-2 at the more stringent level of 15 μg/l and this cut-off value was provided 100% sensitivity and 100% specificity. The value was also consistent with results from tests using combined GHRP-6 and GHRH in lean subjects. GHRP-6 shows a weaker GH stimulating effect than GHRP-2 and requires augmentation with GHRH (37). GHRP and GHRH act through different receptors and have a synergistic effect on GH secretion. Combined administration of GHRH and GHRP-2 has been suggested as a diagnostic test of GH deficiency (16). However, our results may indicate that GHRP-2 alone is sufficient to evoke GH secretion.

The sites of action of GHRP are considered to be both pituitary and hypothalamus. Popovic *et al.* (38) have shown that about 16% of patients, who have had cranial radiotherapy may have hypothalamic–pituitary dysfunction, evident from a poor GH response to ITT, and

Table 4 Time to peak serum GH concentration after GHRP-2 stimulation in control subjects and patients with GH deficiency.

	n	Times (min) 15	30	45	60
Controls	77	13	55	6	3
Patients	58	43	14	1	0

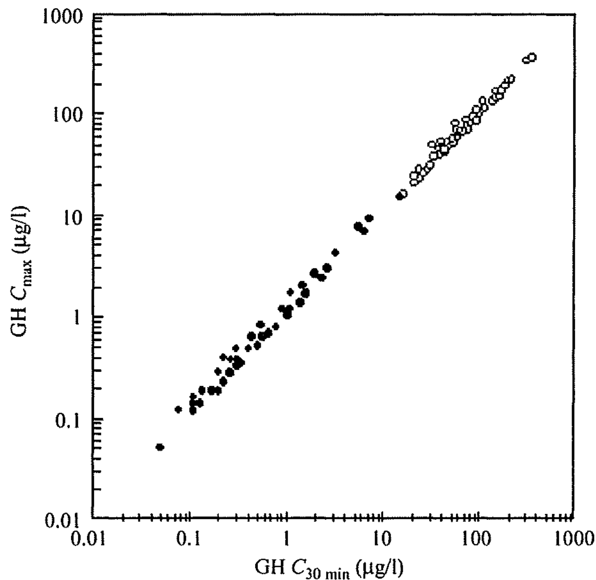

Figure 5 Relationship between serum GH concentration at 30 min ($C_{30\ min}$) and peak concentration (C_{max}) after GHRP-2 administration in patients with GHD (closed symbols) and control subjects (open symbols).

exhibit false-positive responses to the combined administration of GHRH plus GHRP-6. The same phenomena were also observed in the case of combined arginine plus GHRH (11, 12). The comparative analysis of hypothalamic and pituitary diseases in the present study indicated that GHRP-2 could equally diagnose GHD of both types, similar to the ITT. However, more patients with mild GHD due to hypothalamic lesion are needed to clarify the sensitivity and specificity of GHRP-2 tests.

The adverse reactions observed in the GHRP-2 provocative test were flushing, borborygmus and white blood cell count increase. All were transient and disappeared without treatment, indicating good safety profile of GHRP-2. Flushing and sweating were observed frequently in other studies with GHRP (15, 30, 34). The endogenous GH secretagogue, ghrelin, was reported to enhance digestive tract movement (39) and GHRP-2 has similar effects to ghrelin in increasing appetite in humans (40); the borborygmus probably relates to the physiological effects on gastric motility.

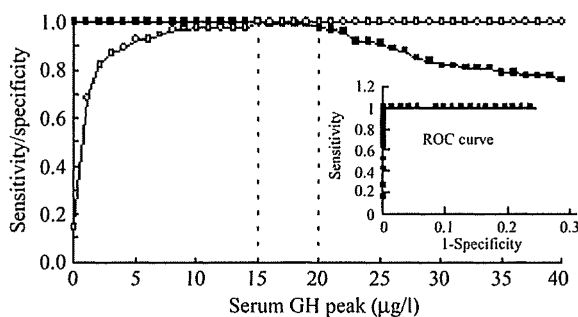

Figure 6 Plot of the variation in sensitivity (open symbols) and specificity (closed symbols) for GH peak following GHRP-2 administration; insert indicates the ROC curve of GH responses to GHRP-2.

Table 5 Adverse reactions following GHRP-2 administration to healthy control subjects and patients with GH deficiency.

	Controls (n=77)	**Patients** (n=58)	**Total** (n=135)
Hot flush	14	10	24
Borborygmus	8	11	19
Increased WBC	5	1	6
Sweating	5	1	6
Nausea	0	2	2
Drowsiness	0	3	3
Taste perversion	2	0	2
Dry mouth	0	2	2
Dizziness	0	1	1
Others	0	14	14
Total adverse reactions (n (%))	23 (29.9)	31 (53.4)	54 (40.0)

In Japan, the guidelines for diagnosis of GH deficiency, issued by the Hypothalamic–Pituitary Dysfunction Study Group of the Ministry of Health, Labour and Welfare, states that at least two kinds of provocative tests are necessary to diagnose GHD. Diagnostic tests are performed using stimulating agents selected from among insulin, arginine, glucagon and L-DOPA for the diagnosis of adult GH deficiency. When the peak serum GH concentration is <3 μg/l in at least two different stimulation tests, severe GH deficiency is diagnosed. In the paediatric field, GH stimulating agents include clonidine in addition to those used in adults; severe GH deficiency is diagnosed when the peak serum GH concentration is <5 μg/l in at least two tests. GHRH has also been used as a diagnostic agent for GH deficiency, but the test is strongly influenced by the GH secretion-suppressing system (somatostatin). It is thought that many false-negative results are seen, that the GH response decreases with aging and the reproducibility of GH secretion is insufficient, with large inter-individual differences. GHRH test is, therefore, not included in the GH provocative tests specified in the Japanese guidelines. From the present result, we decided to include the GHRP-2 test as an alternative for diagnosing severe GHD in adults.

In conclusion, the provocative test with a single dose of GHRP-2 showed favourable reproducibility and was not influenced by gender. The decreased response with aging or adiposity did not affect the discrimination between healthy control subjects and GH-deficient patients, and the diagnostic capacity of GHRP-2 for severe GHD was very high irrespective of the hypothalamic or pituitary causes of diseases. From the results of this study, we would recommend a GHRP-2 dose of 100 μg intravenously and measurement of GH response at 30 min. Maximum specificity and sensitivity was achieved using a diagnostic cut-off value of 15 μg/l (9 μg/l when

calibrated with GH standard WHO 98/574 as reference (27)). Subsequent studies are needed to directly compare the utility of GHRP-2 and the combined GHRH plus arginine or GHRH plus GHRP as an alternative to the ITT. We believe that GHRP-2 is a useful and safe alternative diagnostic agent for severe GHD.

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Purification and characterization of feline ghrelin and its possible role

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Abstract

Ghrelin, a novel 28-amino acid peptide with an *n*-octanoyl modification at Ser[3], has been isolated from rat and human stomach as an endogenous ligand for the growth hormone secretagogue receptor. Here, we purified feline ghrelin and examined its possible physiological role in cats. The major active form of feline ghrelin is a 28-amino acid peptide octanoylated (C8:0) at Ser[3]; except for one amino acid residue replacement, this structure is identical to those of rat and human ghrelins. However, much structural divergence in peptide length and fatty acid modification was observed in feline ghrelin: peptides consisting of 27 or 26 amino acids lacking Gln[14] and/or Arg[28] were found, and the third serine residue was modified by octanoic acid (C8:0), decanoic acid (10:0), or unsaturated fatty acids (C8:1, C10:1 and C10:2). In agreement with the structural divergence, two kinds of cDNA with different lengths were isolated. Administration of synthetic rat ghrelin increased plasma growth hormone levels in cats, with a potency similar to that in rat or human. Plasma levels of ghrelin in cats increased approximately 2.5-fold after fasting. The present study indicates the existence of structural

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