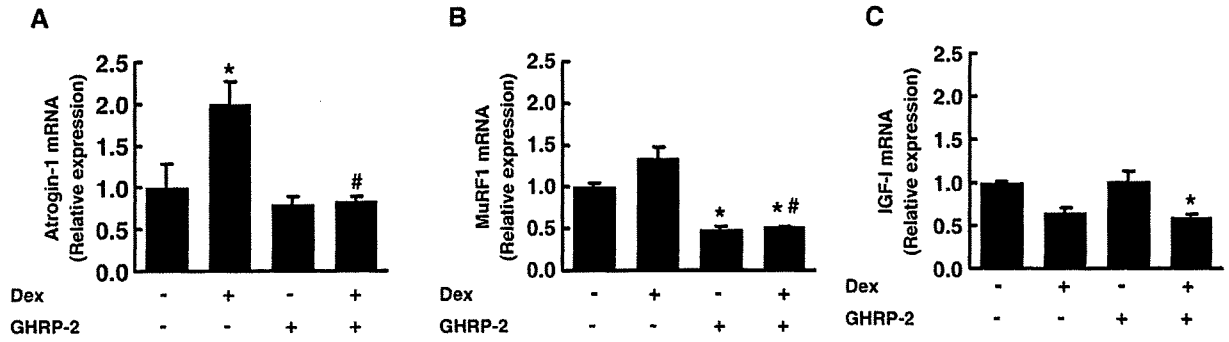

Fig. 2. Subcutaneous administration of GHRP-2 attenuated Atrogin-1 and MuRF1 mRNA levels but not IGF-I in rat soleus muscle. Dexamethasone (Dex, 600 μg/kg) was intraperitoneally injected to 6-week-old male rats once a day for 5 days. GHRP-2 (100 μg/kg) was subcutaneously injected twice a day for 5 days. A; Dex stimulated Atrogin-1 mRNA level in soleus muscle. The Dex-induced Atrogin-1 mRNA level was significantly attenuated by GHRP-2. B; GHRP-2 reduced MuRF1 mRNA level in the presence or absence of dexamethasone. C; IGF-I mRNA level was decreased by dexamethasone but not affected by GHRP-2 in the presence or absence of dexamethasone, * $P<0.05$ vs. control group; #, $P<0.05$ vs. Dex-treated group.

irrespective of the presence of dexamethasone (Fig. 2B). IGF-I mRNA level was reduced by dexamethasone and not affected by GHRP-2 in the presence or absence of dexamethasone (Fig. 2C). Plasma IGF-I levels were not influenced by GHRP-2 whereas dexamethasone reduced the levels (1287.8±53.3 ng/ml in control group, 975.3±90.4 ng/ml in dexamethasone administration group, 1107.9±39.3 ng/ml in GHRP-2 administration group, 887.3±86.0 ng/ml in dexamethasone and GHRP-2 administration group) (Fig. 3).

In vitro experiments

To clarify a direct effect of GHRP-2 on Atrogin-1 and MuRF1 gene expressions, differentiated C2C12 myocytes were used. First, the presence of GHS-R1a, the active ghrelin receptor was examined. PCR analysis showed that GHS-R1a mRNA was present in differentiated C2C12 cells but not in undifferentiated C2C12 cells (Fig. 4). In the differentiated C2C12 cells, 10 μM dexamethasone increased both Atrogin-1 and MuRF1 mRNA levels (Fig. 5A and B). GHRP-2 attenuated the dexamethasone-induced expressions of Atrogin-1 and MuRF1 dose-dependently

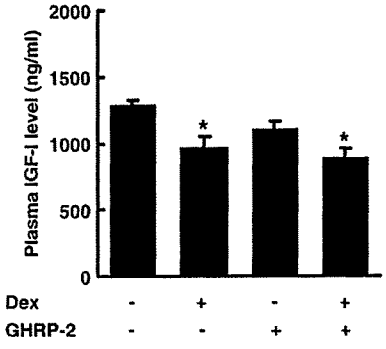

Fig. 3. Effect of the administration of GHRP-2, dexamethasone or both on plasma IGF-I levels in rats. Dexamethasone (Dex, 600 μg/kg) was intraperitoneally injected to 6-week-old male rats once a day for 5 days. GHRP-2 (100 μg/kg) was subcutaneously injected twice a day for 5 days. After 5 days, venous blood was collected under the pentobarbital anesthesia from the rats. Dex decreased plasma IGF-I levels, but GHRP-2 did not affect plasma IGF-I levels. *, $P<0.05$ vs. control group.

(Fig. 5A, B). GHRP-2 decreased basal level of MuRF1 mRNA dose-dependently (Fig. 5B) and showed a tendency to reduce basal level of Atrogin-1 mRNA (Fig. 5A). IGF-I expression in C2C12 cells was not affected by GHRP-2 or dexamethasone (Fig. 5C). To examine whether the effect of GHRP-2 was mediated via GHS-R1a, [D-Lys^3]-GHRP-6, a GHS-R1a antagonist was added to the C2C12 cells before the treatment with dexamethasone, GHRP-2 or both. The suppressive effects of GHRP-2 on Atrogin-1 and MuRF1 mRNA level were partly and completely reversed by [D-Lys^3]-GHRP-6, respectively (Fig. 6).

Discussion

In the present experiment, we found that GHRP-2 attenuated Atrogin-1 mRNA level induced by dexamethasone in rat muscles. Although the mechanism by which dexamethasone causes muscle atrophy is unknown, one possibility is via enhancement of glutamine synthetase activity (Falduto et al., 1992a,b) and the other is via induction of Atrogin-1 expression (Bodine et al., 2001; Lecker et al., 2004).

GHRP-2 has an action to stimulate GH secretion from pituitary, which in turn could increase plasma IGF-I levels. Since IGF-I has been reported to be a growth factor causing muscle hypertrophy (Kanda et al., 1999; Schakman et al., 2005), the elevation of plasma IGF-I levels may affect dexamethasone-induced muscle atrophy. Interestingly IGF-I has been already

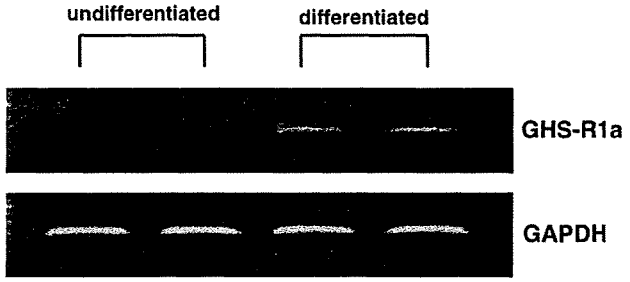

Fig. 4. GHS-R1a mRNA was expressed in differentiated C2C12 myocytes. RT-PCR was performed using total RNA extracted from undifferentiated and differentiated C2C12 myocytes. GHS-R1a mRNA was detected in differentiated C2C12 cells but not in undifferentiated C2C12 cells.

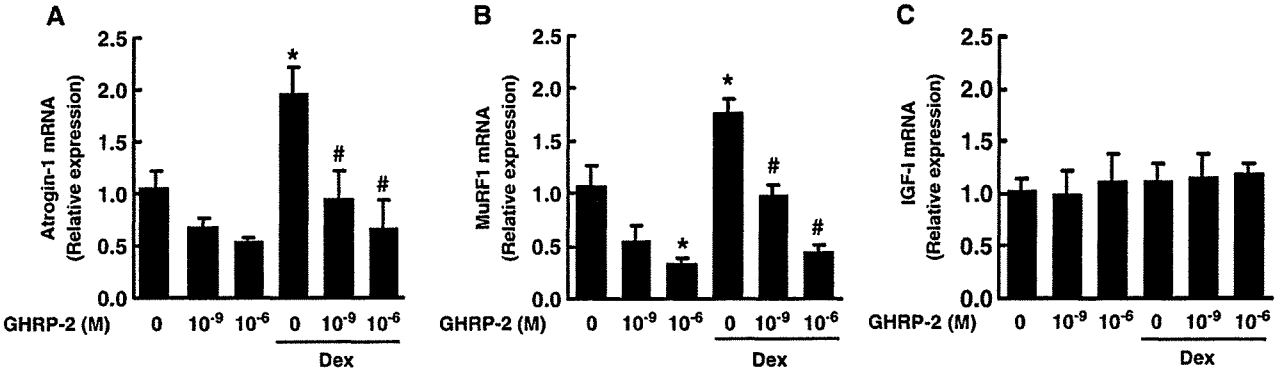

Fig. 5. GHRP-2 attenuated dexamethasone-induced Atrogin-1 and MuRF1 mRNA levels in C2C12 cells. Ten micrometer dexamethasone (Dex) increased both Atrogin-1 (A) and MuRF1 (B) mRNA levels in C2C12 cells. GHRP-2 attenuated the Dex-induced Atrogin-1 and MuRF1 mRNA levels dose-dependently. GHRP-2 decreased basal level of MuRF1 mRNA dose-dependently and showed a tendency to reduce basal level of Atrogin-1 mRNA. C; IGF-I mRNA level in C2C12 cells was not influenced by GHRP-2 or Dex. *, $P<0.05$ vs. control group; #, $P<0.05$ vs. Dex-treated group.

reported to attenuate Atrogin-1 expression in vivo (Sacheck et al., 2004; Stitt et al., 2004). In the present study, however, plasma IGF-I levels were not changed by the treatment with GHRP-2. This finding was consistent with previous reports that GHRP-2 did not increase plasma IGF-I levels in mice (Tschop et al., 2002) and humans (Nijland et al., 1998), suggesting that GHRP-2 does not always increase plasma IGF-I levels. Our data rather suggested that the reduced mRNA levels of Atrogin-1 and MuRF1 in muscle by GHRP-2 was not due to the rise of circulating IGF-I levels. In addition, IGF-I expression in soleus muscles was not affected by GHRP-2 in the present study. Recently, Granado et al. (2005) reported that subcutaneous daily administration of GHRP-2 (100 μg/kg) decreased expression of Atrogin-1 and MuRF1 in atrophic muscle of adjuvant-induced arthritis rats. In their report, plasma IGF-I level was much lower in arthritis rats than in normal control and GHRP-2 did not increase muscle IGF-I mRNA level. Their findings, consistent with our findings, suggested that GHRP-2 decreased Atrogin-1 and MuRF1 mRNA levels through a pathway other than circulating IGF-I and local IGF-I production.

Binding assay using GHS-R ligands has shown specific binding sites in muscle (Papotti et al., 2000) and in vitro application of ghrelin or ghrelin agonists modulated chloride and potassium conductance in rat muscle (Pierno et al., 2003). These findings suggest the presence of GHS-R in skeletal muscle. In this experiment, we found the expression of GHS-R1a in differentiated C2C12 cells. We have already reported that intracellular signal pathways of ghrelin were partly similar to those of insulin and IGF-I (Murata et al., 2002). From the above reasons, we speculated GHRP-2 might work in myocytes to suppress Atrogin-1 and MuRF1 mRNA levels like IGF-I and examined whether GHRP-2 has a direct action on myocytes to inhibit Atrogin-1 and MuRF1 mRNA expressions. GHRP-2 dose-dependently suppressed dexamethasone-induced Atrogin-1 and MuRF1 expressions in C2C12 cells. These findings indicate that GHRP-2 directly acts on myocytes and attenuates the level of Atrogin-1 and MuRF1 mRNA.

To further clarify a direct suppressive effect of GHRP-2 on Atrogin-1 and MuRF1 mRNA levels, [D-Lys^3]-GHRP-6, a GHS-R1a antagonist was used in C2C12 cells. There are two types of ghrelin receptors, GHS-R1a and GHS-R1b (Howard et al., 1996; Mckee et al., 1997). GHS-R1a is an active receptor mediating ghrelin action. GHS-R1b, a splicing variant of GHS-R1a, does not mediate ghrelin signal. We examined the

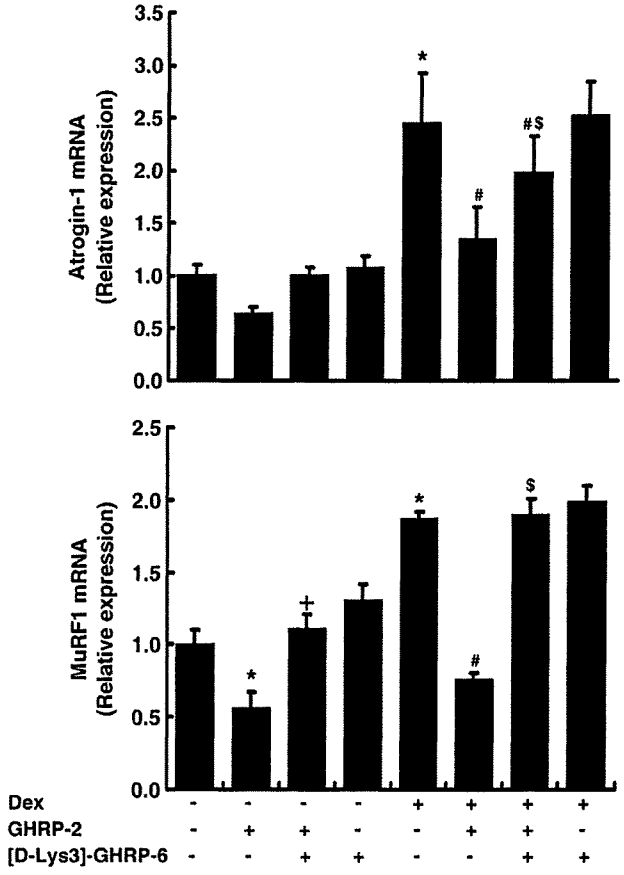

Fig. 6. [D-Lys^3]-GHRP-6, a ghrelin receptor antagonist, reversed the suppressive effects of GHRP-2 on Atrogin-1 and MuRF1 mRNA levels. C2C12 cells were treated with 100 μM [D-Lys^3]-GHRP-6 30 min before the addition of 10 μM dexamethasone (Dex), 1 μM GHRP-2 or both. [D-Lys^3]-GHRP-6 partly and completely reversed the suppressive effects of GHRP-2 on Atrogin-1 and MuRF1 mRNA levels, respectively. *, $P<0.05$ vs. control group; +, $P<0.05$ vs. GHRP-2-treated group; #, $P<0.05$ vs. Dex-treated group; $, $P<0.05$ vs. Dex and GHRP-2-treated group.

specificity of GHRP-2 action using [D-Lys^3]-GHRP-6. We found that [D-Lys^3]-GHRP-6 partly and completely reversed the suppressive effects of GHRP-2 on Atrogin-1 and MuRF1 mRNA levels, respectively. These results suggest that GHRP-2 directly inhibits Atrogin-1 and MuRF1 mRNA level through GHS-R1a.

Since C2C12 cells produce IGF-I (Frost et al., 2003), paracrine or autocrine action of IGF-I may be involved in the suppressive effect of GHRP-2 on Atrogin-1 and MuRF1 mRNA level. To elucidate this possibility, we measured IGF-I mRNA level in C2C12 cells. However, we were not able to find the increase in IGF-I mRNA in C2C12 cells in response to GHRP-2, suggesting that locally produced-IGF-I in C2C12 cells is not involved in the suppressive effect of GHRP-2 on Atrogin-1 and MuRF1 mRNA levels. Dexamethasone also did not influence IGF-I mRNA level in C2C12 cells, although it decreased IGF-I mRNA level in vivo soleus muscle. These results suggest that dexamethasone has an indirect action to reduce IGF-I mRNA level in muscles in in vivo animals. Glucocorticoid is reported to inhibit pulsatile GH secretion (Giustina and Veldhuis, 1998) and reduce GH receptor expression (King and Carter-Su, 1995). As a result, IGF-I mRNA level was thought to decrease in vivo experiment in the present study. Dexamethasone has been reported to reduce the expression in in vivo animals (Gilson et al., 2007), being consistent with our in vivo result.

In summary, GHRP-2 suppressed dexamethasone-induced Atrogin-1 mRNA expressions in in vivo rats without elevating plasma IGF-I and IGF-I mRNA in muscle. Furthermore GHRP-2 decreased dexamethasone-induced Atrogin-1 and MuRF1 expressions in C2C12 myocytes. This effect was blocked by the addition of [D-Lys^3]-GHRP-6, a GHS-R1a antagonist. These findings suggest that a direct action of GHRP-2 through GHS-R1a suppresses Atrogin-1 and MuRF1 mRNA levels in C2C12 cells. GHRP-2 might lead to the protection of muscle atrophy induced by dexamethasone.

Acknowledgements

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Safety and efficacy of growth hormone (GH) during extended treatment of adult Japanese patients with GH deficiency (GHD) ☆

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Abstract

Objectives: To assess the effects of a growth hormone (GH) replacement therapy using a GH dose regimen based on serum insulin-like growth factor (IGF-I) concentrations in Japanese adults with GH deficiency (GHD).
Design: In this multicentre, uncontrolled, open-label study, Japanese adults with GHD who had received either GH replacement therapy (GH–GH group, $n = 35$) or placebo (Placebo–GH group, $n = 36$) in a previous randomised, double-blind, placebo-controlled trial were treated with GH replacement therapy for 48 weeks. GH treatment was started at a dose of 0.003 mg/kg/day administered by subcutaneous injection for the first 8 weeks, after which the dose was adjusted to maintain patients' serum IGF-I levels within the reference range adjusted for age and gender. Body composition, serum lipids, serum IGF-I and IGF binding protein-3 (IGFBP-3) levels were measured throughout study. Symptom and quality of life scores were also determined.
Results: Lean body mass (LBM) was increased compared with baseline (the end of the preceding double-blind trial) at 24 and 48 weeks, with a mean (±SD) increase of 1.3% (±4.2%) at week 48 in the GH–GH group (an increase of 6.6% [±6.0%] from the start of the preceding double-blind trial) and a larger increase of 4.7% (±5.9%) in the Placebo–GH group. Body fat mass (BFM) increased slightly from baseline in the GH–GH group with a mean increase of 2.9 ± 10.6% at week 48 (a decrease from the start of the preceding double-blind trial at 48 weeks of 7.8% [±15.0%]) but decreased by 6.5% (±11.7%) at week 48 in the Placebo–GH group. Serum lipids were unchanged or slightly increased from baseline in the GH–GH group but patients' lipid profiles improved in the Placebo–GH group.

In patients who received placebo during the double-blind study, individualised GH therapy in this open-label study increased mean LBM at 48 weeks by 6.2 ± 6.8% in patients with CO GHD and by 3.0 ± 4.4% in patients with AO GHD. Changes in mean LBM and mean BFM at week 48 were +4.1 ± 4.5% and −2.4 ± 10.5%, respectively, in females and +5.0 ± 6.7% and −8.9 ± 11.8%, respectively, in males. In patients who received GH treatment during the double-blind study, overall changes in LBM, BFM and IGF-I SD score after 24 weeks and 48 weeks were small, with no significant differences between subgroups.

☆ A multicentre study in Japan coordinated by the Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan.

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While the overall incidence of adverse events was broadly similar in the GH–GH and Placebo–GH groups (97% and 89%, respectively), the incidence of treatment-related events was higher in the GH–GH group (83% *vs* 42% in the Placebo–GH group). Most adverse events in both treatment groups were of mild or moderate severity and not clinically significant. The incidences of oedema and cases of high IGF-I during the IGF-I level-adjusted treatment regimen were lower than those during the preceding fixed dose titration.

Conclusion: Long-term GH replacement therapy was well tolerated in Japanese adults with GHD. GH treatment maintained the improvements in body composition and lipid profiles in the patients previously treated in the double-blind study (GH–GH group) and improved these parameters in previously untreated patients (Placebo–GH group). Individualised GH administration based on IGF-I levels was well-tolerated and effective.

Keywords: Long-term growth hormone treatment; Japanese adults; Safety; Body composition; Serum lipids

1. Introduction

It is recognised that growth hormone (GH) is not only essential for growth in childhood but is also an important component of normal metabolic regulation in adults. Since its introduction into clinical practice more than 10 years ago, GH replacement therapy for adults with hypopituitarism has become a well established treatment modality in Western countries [1–5]. However, there are relatively few data concerning long-term treatment in Japanese or other Oriental populations [6,7]. Like Caucasian adults with GHD, Japanese adults with hypopituitarism appear be at considerably increased risk of cardiovascular outcomes and we have shown that GH replacement therapy has beneficial effects on several cardiovascular risk factors in patients with GHD in a previous double-blind, placebo-controlled clinical trial [8].

In this open-label extension of the double-blind study, patients who had previously received either GH or placebo received 48 weeks of GH replacement therapy, with the dose of GH adjusted for individual patients according to their insulin-like growth factor I (IGF-I) levels. In addition to evaluating the safety of long-term GH replacement therapy, the study assessed the efficacy of treatment in terms of its effects on body composition, serum lipids and quality of life (QoL) measures.

2. Materials and methods

This uncontrolled, open-label study was conducted at 21 study centres in Japan after completion of the preceding 24-week, randomised, double-blind study [8]. Although an open-label design may introduce more bias than a double-blind design, it was not considered ethical to include a placebo group in this long-term study, given the efficacy of GH replacement therapy demonstrated in the preceding double-blind study [8] and in other studies in Caucasian patients [1–5]. In addition, the open-label design is consistent with routine clinical practice. Written informed consent was obtained from all patients.

2.1. Study treatment

In the preceding double-blind study, patients were randomised to 24 weeks of treatment with either recombinant human GH (Genotropin®, Pfizer) or matching placebo. The dose of recombinant human GH in the active treatment group was increased from an initial dose of 0.003 mg/kg/day administered by subcutaneous (s.c.) injection during weeks 0–4 to 0.006 mg/kg/day during weeks 4–8 and finally 0.012 mg/kg/day during weeks 8–24 of the initial study.

There was no washout period between the end of the preceding double-blind study and this open-label study. All patients entering this study (regardless of whether they had previously received active GH replacement therapy or placebo) received recombinant human GH administered at an initial dose of 0.003 mg/kg/day by s.c. injection during weeks 0–8 (Fig. 1). As in the preceding study, treatment was self-administered by the patients each day before bedtime using a pen-style injector. From week 8 to week 48 of this study, the dose of GH replacement therapy was adjusted for each patient so that their serum IGF-I level at the preceding clinic visit fell within the reference range, adjusted for their age and sex, with the maximum permitted dose change being 0.003 mg/kg/day. Dosages of corticosteroids, sex hormones or thyroxine were not changed during the study.

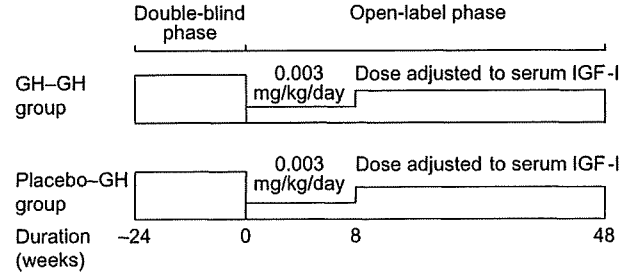

Fig. 1. Study design. This open-label study was preceded by a double-blind study in which patient were randomised to 24 weeks of treatment with either growth hormone (GH) or placebo. IGF-I, insulin-like growth factor I.

All patients attended study clinics for assessment at the end (week 24) of the preceding double-blind study (baseline) when the current study began and at 4, 8, 12, 16, 20, 24, 36 and 48 weeks. Safety was assessed by recording all adverse events reported by patients or investigating physicians during the study period. Events were coded according to the World Health Organization Adverse Reaction Terminology. Laboratory measurements of thyroid hormones, haemoglobin A_{1c} concentrations, total protein, electrolytes, and liver and kidney function were performed centrally. In addition, patients' vital signs (blood pressure, pulse rate) were recorded at each clinic visit.

Body composition (LBM and BFM) was assessed using dual energy X-ray absorptiometry (DEXA) at baseline and at 24 and 48 weeks. All DEXA measurements performed at each of the participating clinical centres were blinded and evaluated centrally at the Department of Radiology, Kawasaki Medical School, Kurashiki City, Okayama, Japan, as previously described [8]. Serum levels of IGF-I and IGFBP-3 were determined at each visit using standard immunoradiometric assays, while serum lipids (total, high-density lipoprotein [HDL-] and low-density lipoprotein [LDL-] cholesterol, triglycerides, phospholipids and non-esterified fatty acids [NEFA]) were determined using enzymatic methods. Biochemical samples were analysed centrally (MBC Inc. Itabashi-ku, Tokyo, Japan).

Subjective and objective symptoms were assessed at each clinic visit using the following seven items: "decreased motor/muscular strength", "inertia/decreased concentration", "depression (a 'down mood')", "emotional instability (unstable temper)", "intemperance (disorder of self-control)", "anxiety" and "sense of alienation (increased sense of alienation in society)". Symptoms were graded as: 0, absent; 1, mild; 2, moderate; or 3, severe. QoL parameters were assessed by Japanese versions of Short Form-36 (SF-36) and Quality of Life Assessment of GH Deficiency in Adults (QoL-AGHDA) at baseline, 24 weeks and 48 weeks. Improvement in QoL was indicated by an increase in SF-36 score or decrease in QoL-AGHDA score.

2.2. Statistics

To assess the safety and tolerability of GH replacement therapy during long-term administration, the incidence rates and severity of all adverse events were recorded for all patients by treatment group and body system. Summary statistics were also calculated for patients' laboratory parameters, height, weight and vital signs. Changes for efficacy parameters except symptom scores and QoL parameters (for which only observed values were assessed) were evaluated using the last observation carried forward method. Differences in body composition and serum lipids between baseline (week 24 at the end of the preceding study when this study began) and week 48 were assessed by paired *t*-tests. All statistical analyses used SAS version 8.2 (SAS Institute, Cary, NC, USA). All statistical tests used a significance level of 5%. SD scores were calculated for serum IGF-I and IGFBP-3 values by comparison with healthy age- and gender-matched Japanese subjects.

The primary safety and efficacy analyses used the intent-to-treat (ITT) population, which comprised all patients who received at least one dose of study treatment and for whom at least one post-dosing assessment was performed.

3. Results

During the preceding double-blind study, 37 patients received GH and 36 patients received placebo. Two patients in the GH treatment group discontinued at the end of this 24-week study because of adverse events. Seventy-one patients who completed the preceding double-blind study then entered the present study. Of these, 35 patients who had received active treatment (GH–GH group) and 36 patients who had received placebo (Placebo–GH group) during the double-blind study phase were assessed for both safety and efficacy during this open-label 48-week GH dose-titrated study phase. Two patients in the GH–GH group and 5 patients in the Placebo–GH group did not complete the full study period.

The baseline demographic characteristics of the patients are summarised in Table 1. There were no differences in demographic parameters between the two treatment groups. Both groups comprised approximately two-thirds male patients and each group had a mean age of approximately 37 years.

Mean baseline serum levels of total cholesterol and LDL-cholesterol were lower and mean levels of HDL-cholesterol were higher in patients in the GH–GH treatment group compared with the Placebo–GH group.

Mean baseline IGF-I values, which had significantly increased over the 24 weeks of GH replacement therapy in the double-blind study, were in the normal range in the GH–GH treatment group and significantly higher than in patients in the Placebo–GH group. Mean baseline IGFBP-3 values were also significantly higher in the GH–GH treatment group than in patients in the Placebo–GH group.

3.1. Efficacy

3.1.1. Body composition

An increase in LBM from baseline was maintained over the 48 weeks of treatment in the patients in the GH–GH treatment group (Table 2). These patients

Table 1
Baseline characteristics of adult Japanese growth hormone (GH) deficient patients by treatment group

		GH–GH ($n = 35$)	Placebo–GH ($n = 36$)
Male/female, *n*		22/13	23/13
Onset of GHD (adult/childhood)		17/18	17/19
Age (years) [range][a]		37.1 ± 12.6 [22–65]	36.7 ± 12.9 [18–64]
Height (cm)[a]		163.7 ± 9.8	161.6 ± 9.9
Weight (kg)[a]		66.9 ± 14.0	62.8 ± 14.7
Body mass index (kg/m^2)[a]		24.8 ± 3.7	23.9 ± 4.4
Lean body mass (kg)	Baseline – 24 weeks[a]	41.7 ± 9.9	39.9 ± 10.3
	Baseline	43.9 ± 10.3	40.4 ± 11.0
Body fat mass (kg)	Baseline – 24 weeks[a]	21.9 ± 7.2	19.9 ± 7.4
	Baseline	19.7 ± 7.3	19.9 ± 7.3
IGF-I (ng/mL)	Baseline – 24 weeks[a]	77.9 ± 61.3	83.3 ± 50.5
	Baseline	249.3 ± 140.7	87.6 ± 54.7
IGF-I SD score	Baseline – 24 weeks[a]	−2.0 ± 1.7	−1.8 ± 1.3
	Baseline	1.0 ± 2.4	−1.7 ± 1.3
IGFBP-3 (μg/mL)	Baseline – 24 weeks[a]	2.0 ± 1.0	2.0 ± 0.8
	Baseline	3.0 ± 1.2	2.2 ± 0.8
IGFBP-3 SD score	Baseline – 24 weeks[a]	−3.9 ± 4.0	−3.2 ± 2.8
	Baseline	−0.8 ± 2.7	−2.7 ± 2.7
Aetiology			
Idiopathic		6 (17.1%)	6 (16.7%)
Germ cell tumour		11 (31.4%)	12 (33.3%)
Craniopharyngioma		8 (22.9%)	4 (11.1%)
Pituitary adenoma (PA)			
Non-functioning PA		4 (11.4%)	5 (13.9%)
Prolactinoma		0	5 (13.9%)
Other		1 (2.9%)	1 (2.8%)
Sheehan's syndrome		2 (5.7%)	1 (2.8%)
Empty sella syndrome		1 (2.9%)	1 (2.8%)
Other		2 (5.7%)	1 (2.8%)

Data shown as mean ± SD for the intent-to-treat population.
GHD, GH deficiency; IGF-I, insulin-like growth factor I; IGFBP-3, IGF binding protein-3.
[a] Start of the randomised, double-blind study preceding the present study.

Table 2
Changes in mean (±SD) lean body mass (LBM) and body fat mass (BFM) at 24 and 48 weeks in Japanese adults treated with open-label growth hormone (GH) replacement therapy (intent-to-treat population)

	GH–GH ($n = 35$)				Placebo–GH ($n = 36$)			
	Study baseline – 24 weeks[a]	Baseline	Week 24 (% change from baseline)	Week 48 (% change from baseline)	Study baseline – 24 weeks[a]	Baseline	Week 24 (% change from baseline)	Week 48 (% change from baseline)
LBM	41.7 ± 9.9	43.9 ± 10.3	44.1 ± 10.4 (0.6 ± 4.2)	44.4 ± 10.4 (1.3 ± 4.2)	39.9 ± 10.3	40.4 ± 11.0	41.9 ± 11.0 (4.0 ± 5.8) [$p = 0.0002$][b]	42.1 ± 11.0 (4.7 ± 5.9) [$p < 0.0001$][b]
BFM	21.9 ± 7.2	19.7 ± 7.3	20.3 ± 7.4 (3.7 ± 11.0)	20.2 ± 7.5 (2.9 ± 10.6)	19.9 ± 7.4	19.9 ± 7.3	19.0 ± 7.5 (−4.6 ± 12.3) [$p = 0.0291$][b]	18.6 ± 7.3 (−6.5 ± 11.7) [$p = 0.0019$][b]

[a] Start of the randomised, double-blind study preceding the present study.
[b] *t*-Test for within-group comparison of % change from baseline.

had already shown a significant ($p = 0.0003$ *vs* placebo) 4.7% increase in mean LBM over the course of the preceding double-blind study [8]. A steady increase in LBM was observed in patients in the Placebo–GH group, who had not previously received GH replacement therapy in the double-blind study. The observed increase in mean LBM in this group (4.0% at week 24, 4.7% at week 48; Table 2) is broadly comparable with that observed with active treatment in the GH–GH group in the preceding double-blind study.

Although slight increases from baseline in mean BFM were observed at weeks 24 and 48 in patients in the GH–GH treatment group (in which mean BFM had been reduced by 9.3% from baseline at the end of the double-blind study, $p = 0.0004$ *vs* placebo), mean BFM remained at a level comparable with the start of

the double-blind study (Table 2). In contrast, a steady decrease in mean BFM was observed in the Placebo–GH group over the course of this study (Table 2). These patients had previously shown a small increase in BFM when receiving placebo during the preceding double-blind study. There were no significant changes in patients' mean weight from baseline to week 48 in either treatment group.

3.1.2. Serum IGF-I and GH dose

Although mean serum IGF-I levels at baseline were markedly higher in the GH–GH group (who had received active GH replacement therapy in the preceding 24 weeks of the double-blind study) compared with patients in the Placebo–GH group, mean values at weeks 24 and 48 were broadly similar in the two groups, reflecting a decrease in the mean IGF-I level in the GH–GH group and a marked increase in this parameter in the Placebo–GH group (Fig. 2). The decrease in the mean IGF-I level in the GH–GH group reflects the study dosing guidelines which, following the initial 8-week period of administration of a fixed dose of GH replacement therapy, required that the dose of GH be adjusted as required to maintain serum IGF-I levels within the reference range for each patient. As a result, the mean of the serum IGF-I SD score moved to the middle of the reference range (−1.96 to 1.96 SD score) in both treatment groups. Following the initial 8-week period, during which all patients received GH at a dose of 0.003 mg/kg/day, the mean GH dose in the subsequent dose-adjustment period increased until week 16–20 and then stabilized through to week 48. The mean doses of GH replacement therapy at weeks 36–48 of 0.0063 ± 0.0037 mg/kg/day in the GH–GH group and 0.0057 ± 0.0027 mg/kg/day in the Placebo–GH group were lower than the final maintenance dose administered to the GH–GH group in the forced, incremental dose regimen used in the preceding double-blind study phase.

The effect of GH replacement therapy on serum IGF-I SD scores in the two treatment groups is illustrated in Fig. 2. While IGF-I SD scores for 18 of 35 patients in the GH–GH group were outside the normal reference range (−1.96 to 1.96 SD score) at baseline, 28 of 35 patients (80%) at week 24 and 28 of 34 patients (82%) at week 48 had values within the reference range due to dose adjustment. Similarly, while 22 of 36 patients in the Placebo–GH group had IGF-I SD scores below the lower limit of the reference range at baseline, 28 of 33 patients (85%) at week 24 and 27 of 32 patients (84%) at week 48 had values within the reference range.

3.1.3. Serum lipids

The changes in patients' serum lipid profiles are summarised in Table 3. Among patients in the GH–GH treatment group there was a slight increase or little

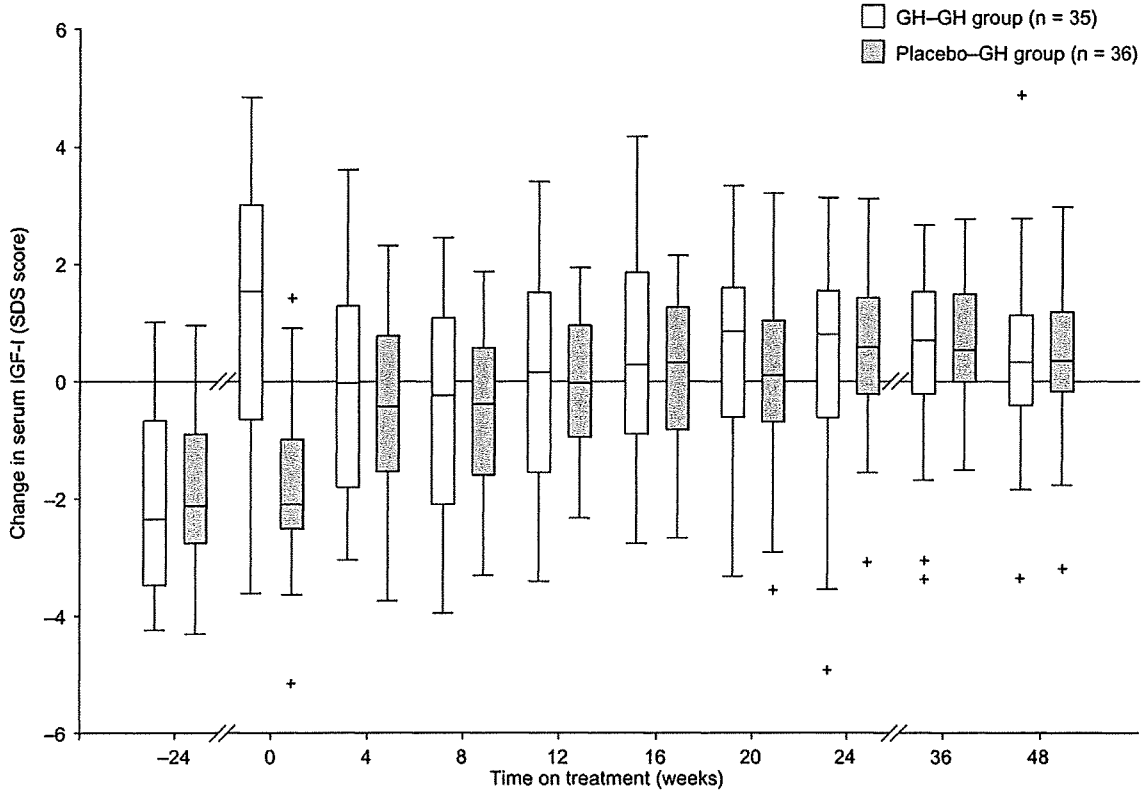

Fig. 2. Changes in mean serum insulin-like growth factor I (IGF-I) SD scores in patients treated with growth hormone (GH) replacement therapy. From week 8 to 48 of the study, the dose of GH was adjusted in each patient to maintain serum IGF-I levels within the reference range.

Table 3
Changes in serum lipid profiles from baseline at 24 and 48 weeks in Japanese adults treated with open-label growth hormone (GH) replacement therapy (intent-to-treat population)

	GH–GH ($n = 35$)				Placebo–GH ($n = 36$)			
	Study baseline – 24 weeks[a]	Baseline	Week 24 (change from baseline)	Week 48 (change from baseline)	Study baseline – 24 weeks[a]	Baseline	Week 24 (change from baseline)	Week 48 (change from baseline)
Total cholesterol (mmol/L)	5.29 ± 1.13	4.98 ± 0.94	5.13 ± 1.06 (0.14 ± 0.62)	5.22 ± 1.02 (0.24 ± 0.58)	5.60 ± 0.90	5.66 ± 1.16	5.38 ± 1.17 (−0.29 ± 0.73) [$p = 0.0246$][b]	5.39 ± 1.05 (−0.27 ± 0.65) [$p = 0.0181$][b]
HDL-cholesterol (mmol/L)	1.32 ± 0.38	1.38 ± 0.40	1.42 ± 0.40 (0.04 ± 0.21)	1.44 ± 0.43 (0.05 ± 0.18)	1.28 ± 0.34	1.30 ± 0.36	1.34 ± 0.33 (0.04 ± 0.26)	1.38 ± 0.39 (0.08 ± 0.26)
LDL-cholesterol (mmol/L)	3.30 ± 0.98	2.94 ± 0.84	2.91 ± 0.79 (−0.03 ± 0.61)	2.97 ± 0.74 (0.02 ± 0.53)	3.64 ± 0.77	3.53 ± 1.02	3.21 ± 0.96 (−0.31 ± 0.70) [$p = 0.012$][b]	3.16 ± 0.83 (−0.37 ± 0.65) [$p = 0.0018$][b]

All values expressed as mean ± SD.
HDL, high-density lipoprotein; LDL, low-density lipoprotein; NEFA, non-esterified fatty acids.
[a] Start of the randomised, double-blind study preceding the present study.
[b] *t*-Test for within-group comparison of % change from baseline.

change from baseline in mean total cholesterol and LDL-cholesterol, and a trend towards a slight increase in HDL-cholesterol. Mean serum levels of total cholesterol and LDL-cholesterol at week 48 remained below, and serum HDL-cholesterol above, the values recorded at the start of the double-blind study. In the Placebo–GH group, mean serum total cholesterol and LDL-cholesterol decreased significantly from baseline, with smaller changes in mean HDL-cholesterol levels.

3.1.4. Symptom scores and QoL parameters

In patients in the GH–GH treatment group, who continued to receive active GH replacement therapy after the double-blind study ended, improvement or maintenance of symptom scores compared with baseline were recorded at weeks 24 and 48. In the Placebo–GH group, the symptom score for decreased motor ability and/or muscle strength, the most frequently reported symptom at baseline, improved with GH replacement therapy from 0.6 ± 0.9 at baseline to 0.1 ± 0.3 at week 48.

In the GH–GH treatment group all QoL parameters of the SF-36 assessment were either unchanged or improved from baseline at weeks 24 and 48. QoL domains showing the most improvement at week 48 compared with baseline were 'physical functioning', 'role/physical', 'vitality' and 'role/emotional'. In addition, further improvement in the mean (±SD) QoL-AGHDA score (7.6 ± 7.6 at the start of the double-blind study, 5.9 ± 7.2 at baseline) was noted at weeks 24 (5.0 ± 6.6) and 48 (5.0 ± 6.2) during this study. In the Placebo–GH group improvements in the SF-36 'physical functioning', 'role/physical', 'bodily pain' and 'general health' domain scores compared with baseline were recorded at week 24. At week 48, all SF-36 domain scores were improved from baseline in this group, with the greatest improvement in the 'vitality/energy' domain. The mean (±SD) QoL-AGHDA score in Placebo–GH group was 8.1 ± 6.4 at the start of the double-blind trial, 6.0 ± 5.6 at baseline in the present study, 5.7 ± 5.8 at week 24 and 5.7 ± 6.4 at week 48.

3.1.5. Effects of GH therapy in patient subgroups

The responses to GH treatment according to gender and time of onset of GHD are shown in Table 4. Overall, the changes in LBM and IGF-I SD score after 24 weeks and 48 weeks in patients who received GH treatment during the double-blind study were small, with no significant differences between subgroups.

In the Placebo–GH group, there was a greater increase in mean LBM at 48 weeks in patients with CO GHD compared with patients with AO GHD (6.2 ± 6.8% *vs* 3.0 ± 4.4%). The response to GH treatment in patients who received placebo during the double-blind study was lower in females than in males.

Titration of GH dose resulted in administration of broadly similar mean doses of GH in males and females in the Placebo–GH group and although this achieved higher IGF-I SD scores in males than in females (0.6 ± 1.2 *vs* 0.2 ± 1.4 at week 48), the change in SD score from baseline was similar in these two groups. Similarly, individualised treatment resulted in higher IGF-I SD scores at week 48 in patients with AO GHD than in those with CO GHD in the Placebo–GH group (Table 4), despite administration of higher mean GH doses in patients with CO GHD.

The different responses to treatment of patient subgroups was also evident in the GH–GH group, with markedly higher mean doses of GH achieving lower IGF-I SD scores in females compared with males and also in patients with CO GHD compared with those with AO GHD by the end of the study (Table 4).

3.2. Safety

A total of 481 adverse events were reported in 65 of the 71 patients (91.5%) during the 48-week study period,

Table 4
Mean percent change in lean body mass (LBM) and mean change in serum insulin-like growth factor I (IGF-I) SD score from baseline according to gender and time of onset of growth hormone (GH) deficiency (intent-to-treat population)

Patient subgroup	GH–GH					Placebo–GH				
	n	Baseline – 24 weeks[a]	Baseline	Week 24 (% change from baseline)	Week 48 (% change from baseline)	*n*	Baseline – 24 weeks[a]	Baseline	Week 24 (% change from baseline)	Week 48 (% change from baseline)
LBM										
Male	22	45.2 ± 10.0	48.0 ± 10.0	48.3 ± 10.3 (0.6 ± 4.3)	48.8 ± 9.8 (1.8 ± 4.9)	23	45.0 ± 8.6	45.9 ± 9.5	47.6 ± 8.8 (4.5 ± 6.2)	47.9 ± 9.0 (5.0 ± 6.7)
Female	13	35.9 ± 6.4	36.8 ± 6.2	37.0 ± 5.9 (0.7 ± 4.2)	37.0 ± 6.6 (0.4 ± 2.8)	13	30.9 ± 6.0	30.8 ± 5.5	31.8 ± 5.9 (3.1 ± 5.1)	32.0 ± 5.7 (4.1 ± 4.5)
Childhood onset	18	40.8 ± 8.3	42.7 ± 8.4	43.7 ± 9.4 (2.0 ± 4.6)	44.0 ± 9.2 (2.9 ± 3.6)	19	38.1 ± 8.5	39.0 ± 9.8	40.9 ± 9.8 (5.4 ± 5.9)	41.2 ± 9.8 (6.2 ± 6.8)
Adult onset	17	42.7 ± 11.5	45.0 ± 12.1	44.6 ± 11.8 (−0.9 ± 3.1)	44.8 ± 11.9 (−0.5 ± 4.3)	17	42.0 ± 12.0	42.0 ± 12.3	43.0 ± 12.4 (2.5 ± 5.3)	43.2 ± 12.4 (3.0 ± 4.4
IGF-I SD score										
Male	22	−1.9 ± 1.6	1.3 ± 2.2	0.5 ± 1.5 (−0.8 ± 1.6)	0.3 ± 1.5 (−1.0 ± 2.1)	23	−1.5 ± 1.4	−1.5 ± 1.5	0.7 ± 1.4 (2.1 ± 1.1)	0.6 ± 1.2 (2.1 ± 1.9)
Female	13	−2.1 ± 1.8	0.4 ± 2.6	0.2 ± 2.0 (−0.2 ± 1.5)	0.7 ± 1.6 (0.3 ± 1.8)	13	−2.4 ± 0.7	−2.2 ± 0.7	0.3 ± 1.2 (2.5 ± 1.1)	0.2 ± 1.4 (2.4 ± 1.4)
Childhood onset	18	−3.2 ± 0.9	−0.3 ± 1.9	−0.2 ± 1.3 (0.1 ± 1.5)	−0.1 ± 1.2 (0.2 ± 2.0)	19	−2.4 ± 1.0	−2.4 ± 0.9	0.1 ± 1.4 (2.5 ± 1.1)	0.3 ± 1.4 (2.7 ± 2.0)
Adult onset	17	−0.7 ± 1.3	2.3 ± 2.1	1.1 ± 1.8 (−1.3 ± 1.3)	1.1 ± 1.6 (−1.3 ± 1.9)	17	−1.1 ± 1.3	−1.0 ± 1.4	1.0 ± 1.0 (2.1 ± 1.1)	0.6 ± 1.1 (1.6 ± 1.3)

All values expressed as mean ± SD.
[a] Start of the randomised, double-blind study preceding the present study.

with 250 events reported in 33 patients (94.3%) in the GH–GH treatment group and 231 events in 32 patients (88.9%) in the Placebo–GH group. The most common adverse events in the two treatment groups are summarised in Table 5. Overall, the most common events were upper respiratory tract infection, emotional lability, abnormal thinking and psychotic depression. The majority of adverse events in both treatment groups were of mild or moderate severity, with only four severe adverse events reported in three patients. These events were one incident of psychotic depression, one incident of colonic diverticulitis and one incident of abnormal thinking in the GH–GH group, and one incident of secondary carcinoma (recurrent cervical cord tumour) in the Placebo–GH group. Of these events, the case of colonic diverticulitis was not judged to be related to study treatment.

Oedema was less frequent in both treatment groups during the individualised, open-label study phase than in the patient group who received fixed dose GH treatment during the randomised double-blind study phase. In addition, the incidence of oedema decreased further in both treatment groups in the second half of the open-label study

There were no deaths during the study period and only five serious adverse events were reported in four patients (influenza-like symptoms and convulsions in one patient in the GH–GH treatment group, a recurrent craniopharyngioma in a patient in the GH–GH treat-

Table 5
Numbers of patients (%) in the intent-to-treat population reporting adverse events that occurred in ⩾7% of all patients during the study period

	GH–GH (*n* = 35)	Placebo–GH (*n* = 36)	Total (both groups)
Upper respiratory tract infection	17 (48.6)	19 (52.8)	36 (50.7)
Emotional lability	7 (20.0)	7 (19.4)	14 (19.7)
Depression psychotic	6 (17.1)	6 (16.7)	12 (16.9)
Thinking abnormal	6 (17.1)	6 (16.7)	12 (16.9)
Anxiety	5 (14.3)	6 (16.7)	11 (15.5)
Headache	6 (17.1)	5 (13.9)	11 (15.5)
Alkaline phosphatase increased	5 (14.3)	3 (8.3)	8 (11.3)
Hypertonia	4 (11.4)	4 (11.1)	8 (11.3)
Muscle weakness	2 (5.7)	6 (16.7)	8 (11.3)
Oedema	6 (17.1)	2 (5.6)	8 (11.3)
SGPT increased	4 (11.4)	4 (11.1)	8 (11.3)
Diarrhoea	2 (5.7)	5 (13.9)	7 (9.9)
Gastroenteritis	3 (8.6)	4 (11.1)	7 (9.9)
Pain	6 (17.1)	1 (2.8)	7 (9.9)
SGOT increased	4 (11.4)	3 (8.3)	7 (9.9)
Arthralgia	3 (8.6)	3 (8.3)	6 (8.5)
Back pain	2 (5.7)	4 (11.1)	6 (8.5)
Haematuria	4 (11.4)	2 (5.6)	6 (8.5)
Insomnia	2 (5.7)	4 (11.1)	6 (8.5)
Myalgia	2 (5.7)	4 (11.1)	6 (8.5)
Pharyngitis	4 (11.4)	2 (5.6)	6 (8.5)
Depersonalization	3 (8.6)	2 (5.6)	5 (7.0)
Inflicted injury	4 (11.4)	1 (2.8)	5 (7.0)

SGOT, serum glutamate oxaloacetate transaminase; SGPT, serum glutamate pyruvate transaminase.

ment group, a case of neoplasm (recurrent cervical cord tumour) in the Placebo–GH treatment group and a case of colonic diverticulitis in the GH–GH treatment group). Of these events, the cases of recurrent craniopharyngioma and neoplasm were considered to be related to study treatment. Three patients (4.2%) – those with craniopharyngioma and secondary carcinoma and a patient in the GH–GH treatment group with arthralgia – discontinued treatment due to adverse events.

Adverse event recording included laboratory data analyses that showed mild to moderate changes in haematological or biochemical parameters in small numbers of patients in both treatment groups. This included positive tests for occult blood (seven reports in the GH–GH treatment group and three reports in the Placebo–GH group, increased white blood cell counts in six patients in the GH–GH group, and decreased free thyroxine (two reports in the Placebo–GH group). In addition, there were no clinically significant changes in patients' vital signs or in mean haemoglobin A_{1c} concentrations, which remained at 4.8% in both treatment groups throughout the study.

4. Discussion

The syndrome of adult GHD and the benefits of GH replacement therapy are well recognised in the Caucasian population [9]. This study adds to the growing body of evidence to support the clinical utility of GH therapy in adult Japanese patients [8,10–13].

The individualised GH dosing regimen based on patients' serum IGF-I levels used in this study acknowledges the known wide inter-individual variation in sensitivity to GH therapy [4] and the finding that the use of such regimens minimises variation in response for most endpoints, particularly between genders [14–17]. This dosing methodology was shown to allow lower GH dose increases compared with those used in the fixed-dose titration regimen of the double-blind comparative phase that preceded this extended study, as well as in a separate open-label extension study of GH replacement therapy [11] and in similar studies in Caucasian patients [18–19].

The results of this study demonstrate the good tolerability of long-term GH replacement therapy with maintenance of the improvements in body composition and serum lipid profiles seen in patients previously treated with active GH replacement therapy in preceding double-blind placebo controlled studies [8,10], and marked improvements in these parameters with active treatment in patients who had previously received placebo.

In the present study, there was little overall change in body composition in the GH–GH treatment group, with the improvements in patients' mean LBM and BFM observed at the end of the double-blind study being maintained. In contrast, there were marked improvements in these parameters among patients in the Placebo–GH group who had previously received placebo. The changes in body composition, particularly in BFM, in the Placebo–GH group at 24 weeks (Table 2) show some differences from those seen with GH treatment in the previous double-blind study. For example, the mean (SD) increase in LBM in patients with AO GHD was 2.5% (±5.3%) at week 24, compared with a corresponding increase of 5.1% (± 4.1%) in this patient group with GH treatment in the double-blind study [8]. In addition, in the preceding study, the changes in BFM and LBM in patients with AO GHD were larger than the changes in these measures in patients with CO GHD, whereas in the present study, the change in mean LBM was greater in the CO GHD subgroup. These differences in response to GH treatment are likely to be due to the lower doses of GH used during this study. Although the different responses of CO GHD and AO GHD observed in previous studies were not seen in this study, this probably reflects the fact that patients with CO GHD had lower mean IGF levels than those with AO GHD at baseline and thus received higher average doses of GH treatment, with greater effects on body composition.

The benefits of GH treatment on patients' serum lipid profiles observed at the end of the preceding double-blind study in the GH–GH group were largely maintained. The mean total cholesterol level at 48 weeks, although increased from baseline and from week 24, was in the normal range. In the Placebo–GH group, total cholesterol, LDL-cholesterol and triglycerides were all markedly reduced and HDL-cholesterol increased from baseline at week 48 although, as in the GH–GH group, the mean total cholesterol level at week 48 was higher than that at week 24. This phenomenon was also observed in both the GH–GH and Placebo–GH groups in another open-label extension study [11] and has been noted in other clinical trials [12]. In GHD, the beneficial effects of GH on lipid metabolism both in adults and in children [20] are complex and it may be that, at least in the Placebo–GH treatment group, this is reflected in the variation in cholesterol levels as a new metabolic equilibrium was being established with GH treatment. Most but not all studies of adults with GHD have shown increases in HDL-cholesterol and decreases in total and LDL-cholesterol after institution of GH treatment [21], with an overall positive effect on these cardiovascular risk factors [1].

Mean IGF-I values at weeks 24 and 48 were broadly similar in the two patient groups, resulting from a decrease in the mean IGF-I level in the GH–GH group and a marked increase in the Placebo–GH group. This reflects the GH dosing protocol, which resulted in 82% and 84% of patients in the GH–GH and Placebo–GH groups, respectively, having values within the reference

range (−1.96 to 1.96 SD score) at week 48. Notably, the incidences of oedema and high IGF-I levels were less with individualised GH dosing during the open-label phase compared with the forced GH dose titration used during the double-blind study phase.

GH replacement therapy was associated with either maintenance of improvements or further improvements in symptom and QoL-AGHDA scores in the GH–GH treatment group. In the Placebo–GH group there were improvements or no change in symptom scores and improvements in all eight SF-36 domains were recorded at week 48. Thus, the improvements in the patients' body composition with GH therapy were associated with improvements in their quality of life.

Consistent with previous findings in Caucasian patients, higher mean doses of GH were required to achieve IGF-I levels within the age- and sex-adjusted reference range in females than in males [15–17]. Similarly, the higher GH dose requirement of patients with CO GHD, which has been observed in previous studies in Caucasian patients [22], confirms the findings of our previous study of long-term GH treatment in Japanese patients [11]. Studies in European patients extending over several years have shown that differences in the body composition response to treatment between CO and AO GHD reduce with time [23]. Extended follow-up of Japanese patients with GHD will help clarify the long-term effects of individualised GH treatment based on IGF-I levels in this patient population.

Long-term GH replacement therapy was well tolerated, with most adverse events of mild or moderate severity. Only a small percentage of patients in either treatment group discontinued due to adverse events during the 48-week study period. For two serious adverse events (a recurrence of a cervical cord tumour and a craniopharyngioma), the possible relationship to treatment could not be excluded. Hypopituitarism, ranging from isolated GHD to panhypopituitarism, is a common consequence of brain tumours, particularly pituitary tumours, or their treatment. Large epidemiological studies have demonstrated a link between high concentrations of IGF-I and some common cancers, including prostate, lung, colon, and breast cancer [24,25]. Data from KIMS (Pfizer International Metabolic Database) and other studies indicate that GH therapy does not increase recurrence of hypothalamic–pituitary tumours or the risk of *de novo* neoplasms in GH-deficient adults [26–32]. Patients with GHD require life-long GH replacement, however, so although the available data are reassuring, in terms of incidence of neoplasms, continued long-term surveillance is essential with regular measurement of IGF-I concentrations in patients receiving treatment.

Current guidelines for GH replacement therapy in adults recommend individualisation of GH dosing with titration to maximise benefits and minimise adverse effects [21,33]. Serum IGF-I is considered the best biochemical marker of GH action, and age-related normal values should be used for monitoring GH treatment. This approach is not, however, without limitations [33]. Adverse effects of GH replacement are more likely to occur, for example, in those patients with a large increment in serum IGF-I SD score whilst receiving GH replacement, even if IGF-I levels remain within the normal reference range [34]. In addition, a normal serum IGF-1 during GH treatment does not exclude the possibility that the GH dose is too high for an individual patient. The population-based normal range of IGF-1 is too wide, even when adjusted for age, to define normality for an individual patient [35]. Thus, while individualised dose titration based on serum IGF-I response is recommended, it should be integrated with clinical monitoring [33]. Nevertheless, overall, this study demonstrated the good tolerability of the individualised dosing regimen for long-term GH replacement therapy and confirms the excellent safety profile of GH therapy reported in other clinical studies in both Japanese and Caucasian adult patients.

In conclusion, this study has further demonstrated the good tolerability and efficacy of long-term GH therapy in adult Japanese patients with GHD. GH treatment maintained the improvements in body composition and lipid profiles in the patients previously treated in the double-blind study (GH–GH group) and improved these parameters in previously untreated patients (Placebo–GH group). Individualised GH administration based on IGF-I levels was well-tolerated and effective.

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Synergistic action of gastrin and ghrelin on gastric acid secretion in rats

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ABSTRACT

Gastrin and ghrelin are secreted from G cells and X/A-like cells in the stomach, respectively, and respective hormones stimulate gastric acid secretion by acting through histamine and the vagus nerve. In this study, we examined the relationship between gastrin, ghrelin and gastric acid secretion in rats. Intravenous (iv) administration of 3 and 10 nmol of gastrin induced transient increases of ghrelin levels within 10 min in a dose-dependent manner. Double immunostaining for ghrelin and gastrin receptor revealed that a proportion of ghrelin cells possess gastrin receptors. Although (iv) administration of gastrin or ghrelin induced significant gastric acid secretion, simultaneous treatment with both hormones resulted in a synergistic, rather than additive, increase of gastric acid secretion. This synergistic increase was not observed in vagotomized rats.

These results suggest that gastrin may directly stimulate ghrelin release from the stomach, and that both hormones may increase gastric acid secretion synergistically.

Ghrelin, recently purified from rat and human stomachs as an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R), is a 28-amino-acid peptide with an *n*-octanoylation modification at the Ser3 residue [1]. This octanoylation is essential for stimulation of GH secretion from the pituitary gland [1]. Although cells showing immunostaining for ghrelin are distributed widely in the stomach, hypothalamus, pituitary gland, liver, kidney, pancreas and placenta, the main source of circulating ghrelin has been considered to be the gastrointestinal tract, especially in X/A-like cells of fundic glands [2–6]. Several studies on the physiological function of ghrelin have demonstrated that, in addition to stimulating GH secretion, ghrelin also stimulates food intake, body weight gain, stomach motility, insulin release, cell proliferation and gastric acid secretion [7–13].

It is well known that ghrelin secretion is stimulated by starvation, and that subsequently this increase of ghrelin induces food intake [7–9,14]. Certainly, central and peripheral treatment with ghrelin causes initiation of food intake within 5 min in rats [9,14]. Therefore, ghrelin has been considered an appetite-promoting hormone. In addition to regulation of ghrelin secretion by the autonomic nervous system and nutrients, some previous studies have demonstrated that ghrelin secretion is influenced by many gastrointestinal hormones, such as glucagon, insulin, cholecystokinin, glucagon-like peptide, gastrin and somatostatin [15–21]. However, the relationship between ghrelin secretion influenced by these gastrointestinal hormones and the physiological role of secreted ghrelin is still largely unknown. Circulating ghrelin levels exhibit a diurnal pattern, with bimodal peaks occurring before the dark and the light periods, respectively [14]. These two peaks are consistent with the minimum (emptiness) and maximum (satiety) volumes of the gastric content, respectively. This indicates that ghrelin secretion increases not only when the stomach is empty but also when it is full. Therefore, it has been assumed that the function of the ghrelin peak observed at stomach emptiness is to stimulate food intake, whereas that of the satiety peak is to stimulate gastric acid secretion [14]. If this hypothesis is correct, it is important to clarify what stimulates ghrelin secretion under satiety conditions. We have suspected that gastrin may be involved in this ghrelin increase at satiety, since gastrin is released at this time and stimulates gastric acid secretion, whereas somatostatin and cholecystokinin inhibit gastric acid secretion [22].

In this study using rats, therefore, we examined whether intravenous injection of gastrin affects ghrelin release from the stomach, or whether ghrelin and gastrin stimulate gastric acid secretion independently or cooperatively.

Materials and methods

Animals. Male Wistar rats weighing 400–500 g were used in all experiments. The rats were maintained under a 12:12-h light/dark

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cycle (lights on at 07.00 h) and a room temperature of 23 ± 1 °C with standard laboratory food and water provided *ad libitum*. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Gastrin administration. Rats were anesthetized with an intraperitoneal (ip) injection of pentobarbital. To exclude the possibility that several treatments, such as implantation of a polyethylene tube into a stomach vein, or injection of saline and gastrin, might affect ghrelin secretion by stimulating the endings of sensory neurons in the stomach or vagus nerve, rats were firstly vagotomized at the subphrenic esophagus level using the method described previously [23] and were then maintained at a body temperature at 37 °C by a small-animal heat controller while monitoring the rectal temperature (Unique Medical Co., Ltd., Tokyo, Japan). Next, to analyze ghrelin secretion into the gastric vein, a heparin-treated PE10 tube (inner diameter 0.28 mm: Becton–Dickinson, NJ, USA) was inserted and fixed to the gastric vein [20]. Blood samples were collected at 10-min intervals. After the third blood sample collection, 0.2 ml of 3 or 10 nmol gastrin (Peptide Institute, Osaka, Japan), or saline as a control, was injected into the femoral vein. Thereafter, blood sampling was continued for 60 min.

Measurement of plasma ghrelin. Ghrelin concentration was measured by the method described previously [12]. Briefly, blood samples were collected into chilled polypropylene tubes containing a protease inhibitor, aprotinin (Sigma–Aldrich, St. Louis, USA), and EDTA-2Na, and immediately centrifuged at 14,000 rpm for 3 min. Plasma samples were acidified with a 10% volume of 1 N HCl and stored at −80 °C until assay. Acyl-ghrelin was measured using an active ghrelin ELISA kit (Mitsubishi Kagaku Iatron, Tokyo, Japan).

Double immunostaining for ghrelin and gastrin receptor. A trimmed part of the glandular stomach was washed using saline and placed in 0.34% formalin for 4 days at 4 °C, and then transferred to 0.1 M phosphate buffer containing 20% sucrose. Sections were cut at a thickness of 18 μm with a cryostat at a temperature of −20 °C. The sections were fixed with 40% paraformaldehyde for 20 min and blocked for 1 h in 5% normal donkey serum in PBST and then incubated overnight at 4 °C with rabbit antiserum against rat ghrelin together with goat antiserum against CCKB-R C18 (Santa Cruz Biotechnology, Santa Cruz, CA). This anti-rat ghrelin antibody (antiserum #G606) specifically recognizes ghrelin with *n*-octanoylated Ser-3 and does not recognize des-acyl-ghrelin. After washing, the sections were incubated with a second antibody solution of Alexa-488-labeled anti-rabbit IgG antibody and Alexa-555-labeled donkey anti-goat IgG antibody solution for 30 min. The samples were observed using a fluorescence microscope (Axioskop 2plus: Zeiss, MA, USA). Digital images were contrasted and color-adjusted using Adobe Photoshop 7.0 for Windows.

Measurement of gastric acid secretion. Intact and vagotomized rats were used in this study. Vagotomy was performed at the subphrenic esophagus level under pentobarbital anesthesia as mentioned above. Four days after the operation, the rats were used for the following experiment. After anesthesia with urethane, two gastric perfusion cannulae were implanted in the pyloric and cardiac parts of the stomach by insertion from the duodenum and esophagus, respectively. The body temperature was maintained at 37 °C until the end of sampling. Five milliliters of water warmed at 37 °C was perfused from the cardiac side to the pyloric part side with an infusion pump at a flow rate of 2.5 ml/min at 10-min intervals. Acidity of the perfusate was recorded by the method reported previously [24]. After acidity had been stabilized at between pH5 and pH7, 3 nmol of either gastrin or ghrelin, or 3 nmol of both gastrin and ghrelin was injected into the femoral vein. Injection of saline was used as a control. After treatment with the agents, the perfusate was collected at 10-min intervals for 60 min.

Data analysis. Data are expressed as means ± SEM for the groups. Comparisons between groups were performed using unpaired *t*-test or ANOVA followed by the Tukey–Kramer test for multiple comparisons. Changes in plasma ghrelin levels over time were analyzed using repeated measures ANOVA followed by Dunnett's test. Differences at $P < 0.05$ were considered significant.

Result and discussion

The effect of intravenous injection of gastrin on ghrelin secretion from the stomach in vagotomized rats is shown in Fig. 1C. The plasma concentration of ghrelin in the gastric vein at the start of the experimental period, at 0 min in Fig. 1C, was 44.7 ± 5 fmol/ml. Saline administration did not cause significant changes in plasma ghrelin levels after treatment. On the other hand, gastrin induced a significant dose-dependent increase in ghrelin levels. The peak level of ghrelin was observed 10 min after treatment with 3 and 10 nmol of gastrin, thereafter falling rapidly to below the control level (Fig. 1C). This decrease of the ghrelin level to below the baseline may be due to exhaustion of stored ghrelin after secretion in quantity. These results indicate that gastrin may play an important role in ghrelin secretion, and that the effect is probably limited to stimulation of secretion, and not synthesis.

Double-staining immunohistochemistry for ghrelin and gastrin receptor in the lamina propria mucosae of the lower gastric corpus was performed to examine the possible presence of gastrin receptors on ghrelin cells. Cells immunoreactive for ghrelin and gastrin

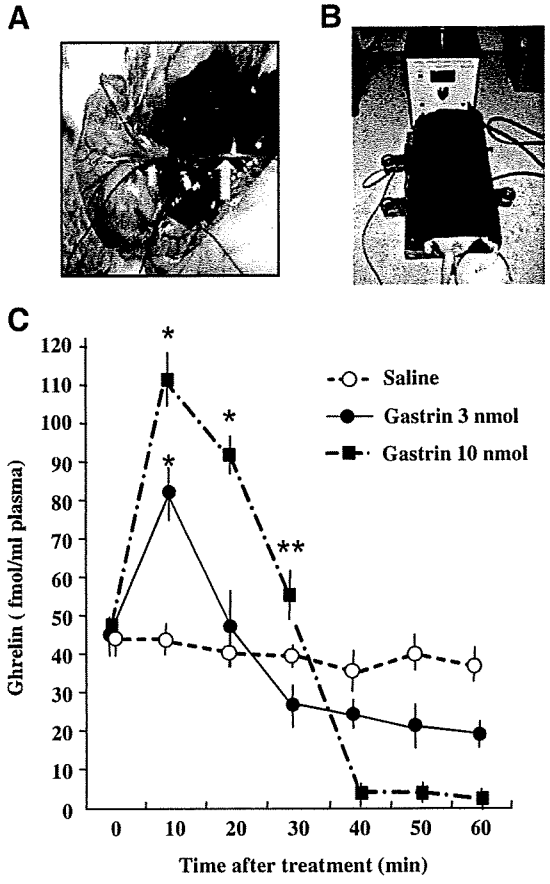

Fig. 1. Effect of peripheral injection of gastrin on plasma ghrelin levels. (A) Photograph showing cannulation of stomach vein (small arrow) by a PE10 tube (large arrow). (B) Body temperature was maintained constantly using a thermostat. (C) Ghrelin levels after injection of saline, and with 3 nmol and 10 nmol gastrin into the femoral vein. Each symbol and vertical bar represent means ± SEM ($n = 6$). Asterisks indicate significant differences ($^{*}P < 0.01$, $^{**}P < 0.05$ vs saline).

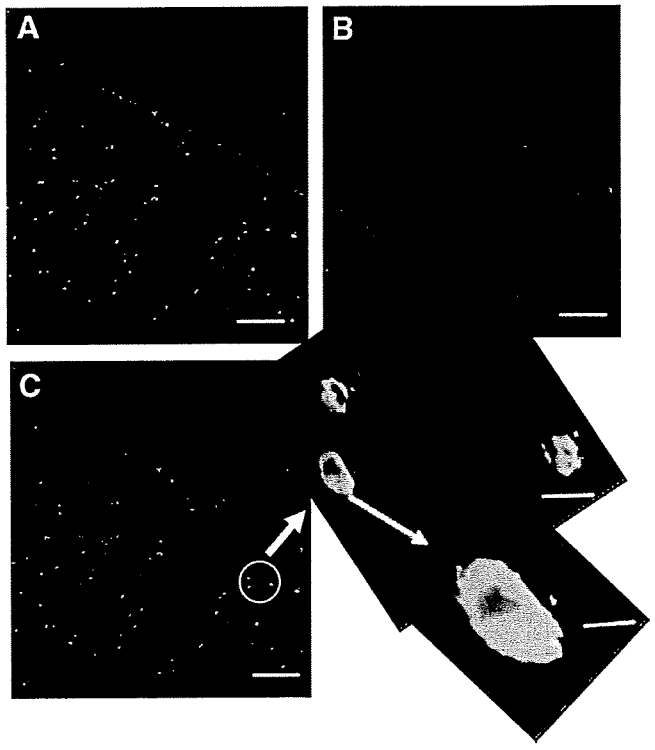

Fig. 2. Double immunostaining for ghrelin and gastrin receptors in rat stomach. Distribution of ghrelin (A: stained green) and gastrin (B: stained red) receptors from the neck to the base of a rat oxyntic gland. The scale bars represent 100 μm. (C) Co-localization of ghrelin and gastrin receptors is observed in a few cells. d,e: Higher magnification of cells stained for both ghrelin and gastrin receptors. The scale bars represent 5 μm.

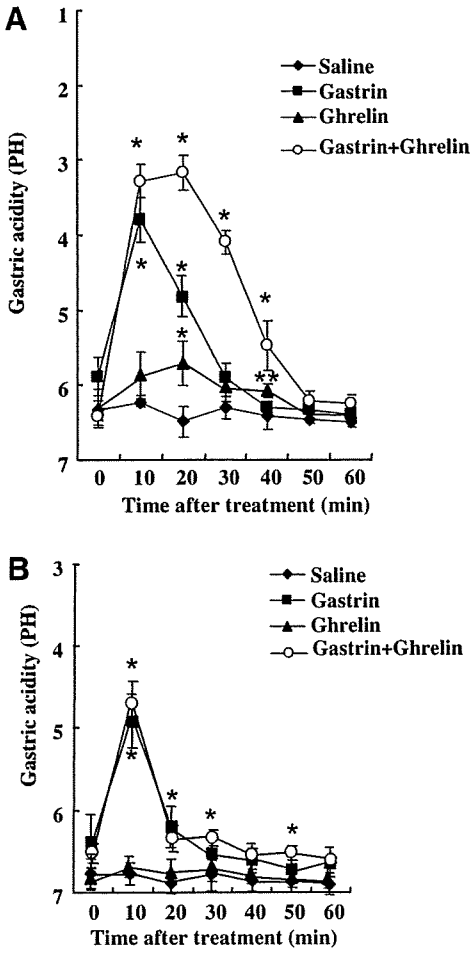

Fig. 3. Effect of peripheral injection of gastrin and ghrelin on gastric acid secretion in intact and vagotomized rats. Ghrelin and/or gastrin were injected into the femoral vein of intact (A) or vagotomized (B) rats under urethane anesthesia. Gastric acidity was determined by measurement the pH of the gastric perfusate. Vagotomy was performed 4 days before the experiment. A synergistic effect on gastric acid secretion was observed upon concomitant treatment with gastrin and ghrelin. Each symbol and vertical bar represent means ± SEM ($n = 6$). Asterisks indicate significant differences ($^{*}P < 0.01$, $^{**}P < 0.05$ vs saline).

receptor were abundant from the neck to the base of the oxyntic glands (Fig. 2A and B). A few cells were reactive for both antibodies (Fig. 2C). At high magnification, gastrin receptors were detected as a spot on the border of ghrelin cells (Fig. 2D and E). This was probably because the antibody for gastrin receptor recognizes the extracellular domain of the receptor molecule, which has a scattered distribution on the cell membrane. These results suggested that a proportion of ghrelin cells express gastrin receptors on their plasma membrane, and that gastrin may stimulate ghrelin secretion by acting directly on ghrelin cells.

In intact rats, injection of 3 nmol of ghrelin or gastrin induced significant gastric acid secretion, gastrin showing the more potent effect (Fig. 3A). Simultaneous administration of ghrelin and gastrin caused an increase of gastric acid secretion, the effect being more potent and longer lasting than that of each agent administered alone. The increase of gastric acid secretion induced by simultaneous injection of ghrelin and gastrin suggested a synergistic rather than an additive effect. These results suggest that gastrin and ghrelin stimulate gastric acid secretion cooperatively. In vagotomized rats, injection of gastrin also increased gastric acid secretion, as seen in intact rats, but acidity at 20 min after treatment was significantly decreased in comparison with that of intact rats (pH 6.23 ± 0.22 in vagotomized rats vs pH 4.83 ± 0.25 in intact rats) (Fig. 3B). In addition, no significant increase was observed after treatment with ghrelin. This lack of ghrelin-induced gastric acid secretion in vagotomized rats is in agreement with a previous report by Masuda et al. [10]. Vagotomy also eliminated the synergistic increase of gastric acid stimulated by simultaneous treatment with gastrin and ghrelin (Fig. 3B). Therefore, the vagus nerve may be involved in the synergistic effect of gastrin and ghrelin on gastric acid secretion.

It is well known that gastrin induces gastric acid secretion from parietal cells of the stomach by stimulation of histamine release [25]. The present study suggests that gastrin also induces gastric acid secretion by acting through ghrelin release. Gastrin induced ghrelin release in vagotomized rats. In addition, double immunohistochemistry revealed that the plasma membrane of some ghrelin cells expressed gastrin receptors. Therefore, gastrin-induced ghrelin secretion may be due to the direct action of gastrin on X/A-like ghrelin cells [3]. Lippl et al. [19] have demonstrated that gastrin stimulates ghrelin release in cultured rat stomach cells [19]. However, in that study, the possible involvement of hormones other than gastrin in ghrelin secretion could not be excluded because of the paracrine action of gastrin on other stomach cells, such as enterochromaffin cells.

In this study, we confirmed that ghrelin stimulates gastric acid secretion by acting through the vagus nerve, since vagotomy eliminated this effect of ghrelin. Our findings also indicated that gastric acid secretion induced by gastrin alone may partially involve ghrelin secretion, i.e., the increase of gastric acid secretion after single treatment with gastrin might be induced by both histamine and ghrelin. The diminution of acidity resulting from vagotomy may be attributable to gastrin-induced ghrelin.

When gastrin and ghrelin were administered simultaneously, gastric acid secretion was increased synergistically. Although the mechanism of this synergy was not clarified, total ghrelin including that administered exogenously and that induced endogenously by gastrin might reach a level that is optimal for stimulating the vagus nerve, thereafter leading to secretion of a large amount of gastric acid. Alternatively, gastrin might increase the expression of ghrelin receptors (GHSR-1a) in the nodose ganglion, since we have already reported the possible involvement of gastrointestinal hormone in regulation of GHSR-1a synthesis in the nodose ganglion and for axonal transport of GHSR-1a from the nodose ganglion to nerve endings [26]. The fact that vagotomy eliminated the synergistic effect of concomitant treatment with gastrin and ghrelin, may support these possibilities.

We have previously reported that the level of ghrelin exhibits a diurnal pattern, with bimodal peaks occurring before dark (06:00 h) and before light (15:00 h) [14]. These two peaks were consistent with the maximum and minimum volumes of gastric content, respectively, suggesting that ghrelin secretion is induced by both gastric emptying and filling. The former peak is coincident with the rise before a meal reported in many mammals, and acts to stimulate food intake [27–29]. The present study indicates that this later peak might occur through the action of gastrin.

In summary, the present study has shown that some ghrelin cells in gastric glands of the rat stomach possess gastrin receptors, whereby gastrin acts directly on ghrelin cells to stimulate ghrelin secretion. Our findings suggest that secreted ghrelin stimulates gastric acid secretion, and furthermore that acid secretion stimulated by gastrin may be partly mediated by ghrelin. The increase of ghrelin secretion stimulated by gastrin results in the ghrelin peak observed in the dark period, and is closely related to the diurnal cycle of ghrelin.

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Review

Ghrelin deficiency does not influence feeding performance

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Abstract

Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor that is synthesized predominantly in the stomach. Previous studies demonstrated that ghrelin stimulates growth hormone release and food intake. These data suggested that antagonism of ghrelin could serve as a useful treatment for eating disorders and obesity. To study the role of endogenous ghrelin in feeding performance further, we generated ghrelin-deficient ($ghrl^{-/-}$) mice. Unexpectedly, $ghrl^{-/-}$ mice exhibited normal growth, cumulative food intake, reproduction, histological characters, and serum parameters. There were no differences in feeding patterns between $ghrl^{+/+}$ and $ghrl^{-/-}$ mice. $Ghrl^{-/-}$ mice displayed normal responses to scheduled feedings as seen for $ghrl^{+/+}$ mice. Memory-related feeding performances of $ghrl^{-/-}$ mice were indistinguishable from $ghrl^{+/+}$ littermates. These data indicate that ghrelin is not critical for feeding performance.

Keywords: Ghrelin knockout mouse; Feeding pattern; Memory

Contents

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