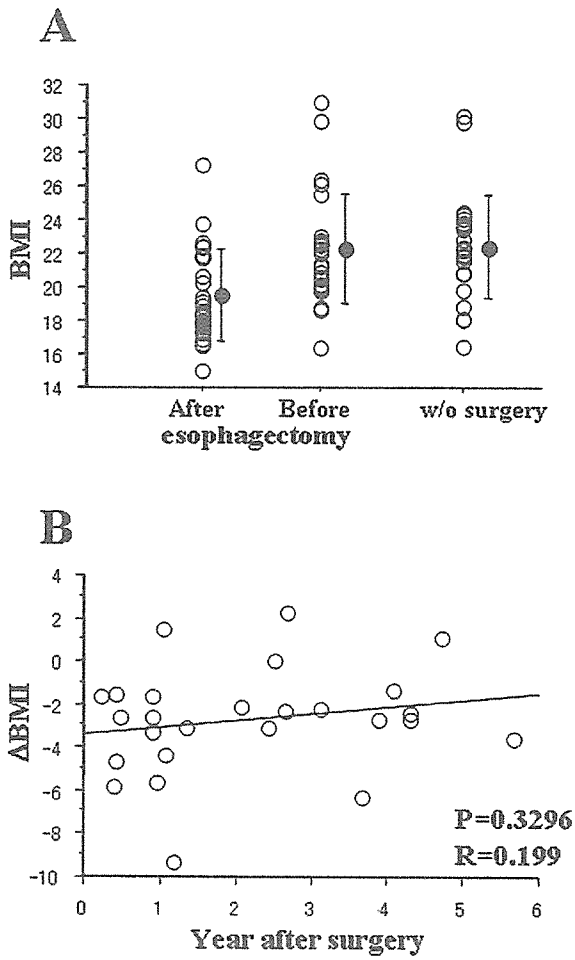

Fig 3. Body weight loss after esophagectomy with gastric tube reconstruction. A, Postoperative body weight standardized as body mass index (BMI) of 26 outpatients, who had previously undergone esophagectomy with gastric tube reconstruction, were compared with those before esophagectomy and of the control group (w/o surgery). BMI of each subject (*open circles*) and mean (*closed circles*) with standard deviation (*bar*) are indicated. Significant differences were observed by the Mann-Whitney *U* test between the patients after surgery and the controls ($P = .0018$) or after and before surgery ($P < .0001$), but not between the control and the patients before surgery ($P = .7334$). B, Postoperative body weight loss (ΔBMI) was correlated with time after surgery by a linear regression model. The regression line is indicated, but the positive correlation was weak although statistically significant ($P = .3296$)

body weight by stimulating appetite and GH secretion. In general, if the stomach is not impaired, ghrelin functions in a negative feedback loop in relation to body weight: In comparison with normal weight individuals, obese individuals show lower ghrelin levels, while lean individuals show higher levels.[32,33] However, after surgery that affects gastric function, reduction of ghrelin seems to be related to postoperative body weight loss. For example, gastric bypass surgery for the morbidly obese successfully reduces body weight and ghrelin concentration, and probably appetite as well, while starvation reduces body weight but increases the ghrelin concentration with hunger pangs.[19,21] Gastric cancer patients lose 80% of their serum ghrelin and 20% of their body weight after total gastrectomy.[34] In the present study, the correlation between body weight loss and ghrelin concentration among esophageal cancer patients within 3 years after surgery suggests that the decline of ghrelin after esophageal reconstruction using the stomach is not a result but a cause of postoperative body weight loss. Consistently, it seems reasonable that ghrelin concentration is correlated not with absolute body weight but with alteration of body weight after surgery in these patients.

The mechanism by which serum ghrelin decreased after esophageal reconstruction using the stomach is not known. Likewise, the decline of serum ghrelin after gastric bypass surgery is not understood, but it is speculated that, although short-term starvation increases serum ghrelin, the fundic gland may decrease ghrelin secretion when it loses contact with food for a long period.[19,21] Vagotomy, which was done for all patients in this study, has been reported to not affect base line ghrelin secretion but to reduce its increase after starvation in animal experiments.[35] Clinically, vagal preservation can prevent postoperative body weight loss after esophagectomy,[36] or vagotomy has an additional effect on body weight reduction at bariatric surgery.[37] It also is unknown what differences in ghrelin production are observed after the same surgical procedure. We could not find a difference between use of the whole stomach and the great curvature gastric tube, despite differences in gastric volume, motility, and symptoms being reported by others.[28] To obtain as long a great curvature gastric tube as possible, we added an incision along the lesser curvature, removing a minimum (approximately 20%) of the gastric area and providing a wide diameter (5-cm) gastric tube. One possibility is the implication of *Helicobacter pylori* infection, which causes atrophic gastritis and impairs ghrelin secretion.[38] *H pylori* infection is widespread among the Japanese and recently has been found to be a high-risk factor for squamous cell carcinoma of the esophagus.[39]

Another interesting observation was a positive correlation between serum ghrelin and the postop-

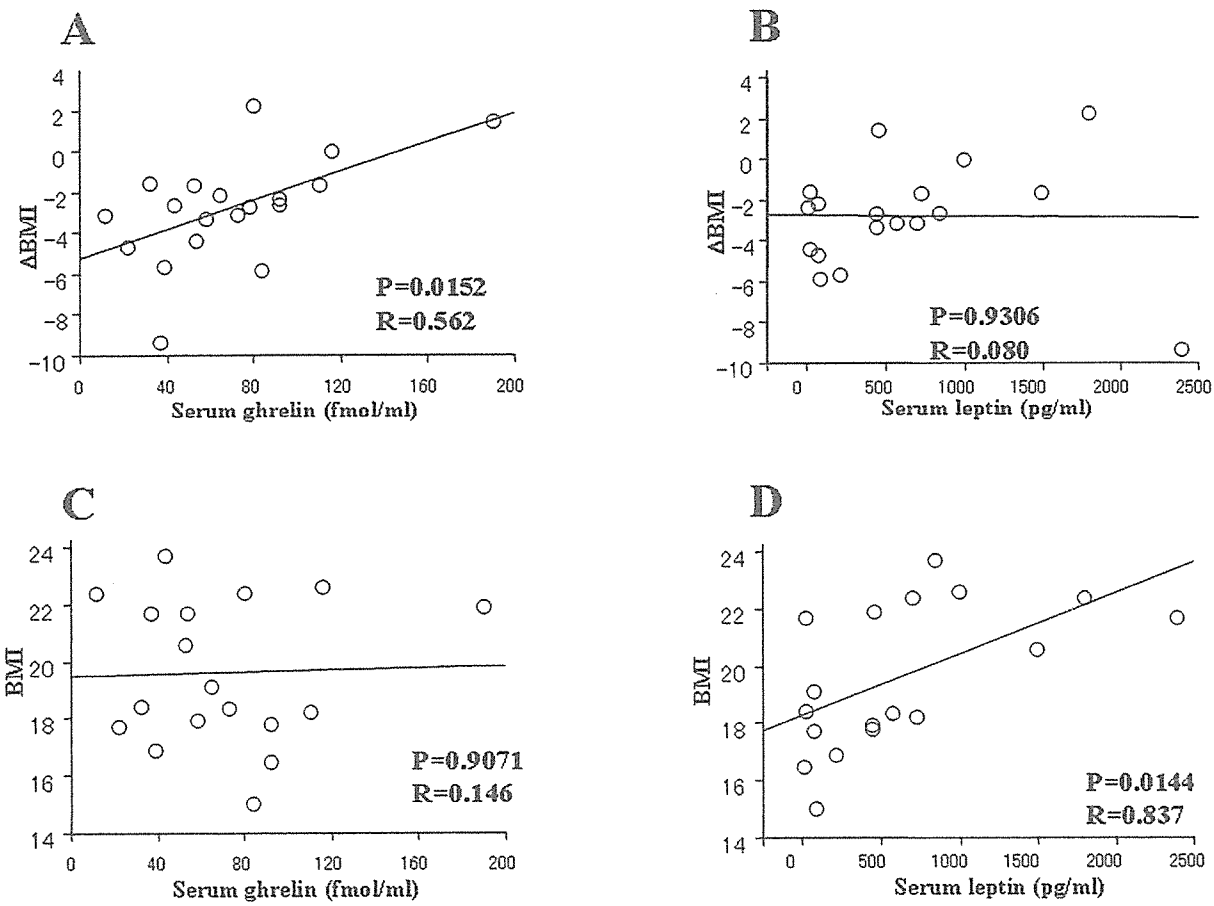

Fig 4. Correlation between weight control hormones and body weight loss after esophagectomy with gastric tube reconstruction. Serum ghrelin (A, C) and leptin (B, D) were correlated with postoperative body weight loss (ΔBMI) (A, B) and body weight (C, D) and evaluated by linear regression, among 18 esophageal cancer patients within 3 years after surgery. The regression line and *P* value are indicated.

erative period, although this finding needs to be confirmed by following each patient for a long period. Transient reduction of ghrelin was observed after distal gastrectomy[34] and reported after partial gastrectomy for the obese.[40] However, it was characteristic of this operation that serum ghrelin exhibited not only recovery from postoperative decline but also overproduction at more than 3 years after surgery. Various factors, including swallowing dysfunction, early satiety, and dumping are involved in body weight loss after this operation. The ghrelin-stimulating signal, raised by persisting hypoglycemia and malnutrition, may cause hypertrophy or hyperplasia of ghrelin-secreting cells and result in its overproduction. Gastric function such as acid secretion[41] or peristalsis[42] was perturbed after this surgery but recovered after 1 to 2 years, when ghrelin secretion also recovered. Ghrelin controls these gastric functions, but this action occurs through the vagal nerve,[16] which was removed by surgery in our patients. We are very interested in this consistency, whether it occurred by chance or whether ghrelin works through pathways other than the vagal nerve to the denerved stomach used to substitute for the esophagus. To investigate this hypothesis, a long-term and continuous survey for each patient in a larger cohort would be necessary as ghrelin showed relatively high deviation among individuals.

Various surgical approaches failed, but parenteral or enteral nutrition[43] is effective for preventing body weight loss, although these treatments may be unpleasant, and the effect disappears when interrupted. Ghrelin physiologically increases appetite and GH, leading to a more comfortable body weight gain. The safety and usefulness of recombinant ghrelin have been confirmed by clinical trials for patients with heart failure.[44] Recently definitive

chemoradiation therapy has emerged as an alternative therapeutic modality of this disease.[45,46] Some esophageal cancer patients may choose chemoradiation therapy, although the survival rate is not always superior to that of surgical therapy, because the QOL after such therapy apparently is better than that after surgery. The surgeon must explain not only the survival but also the QOL after surgery to the patients and should endeavor to improve it. The use of recombinant ghrelin should be a powerful tool for surgeons dealing with upper digestive tract cancers.

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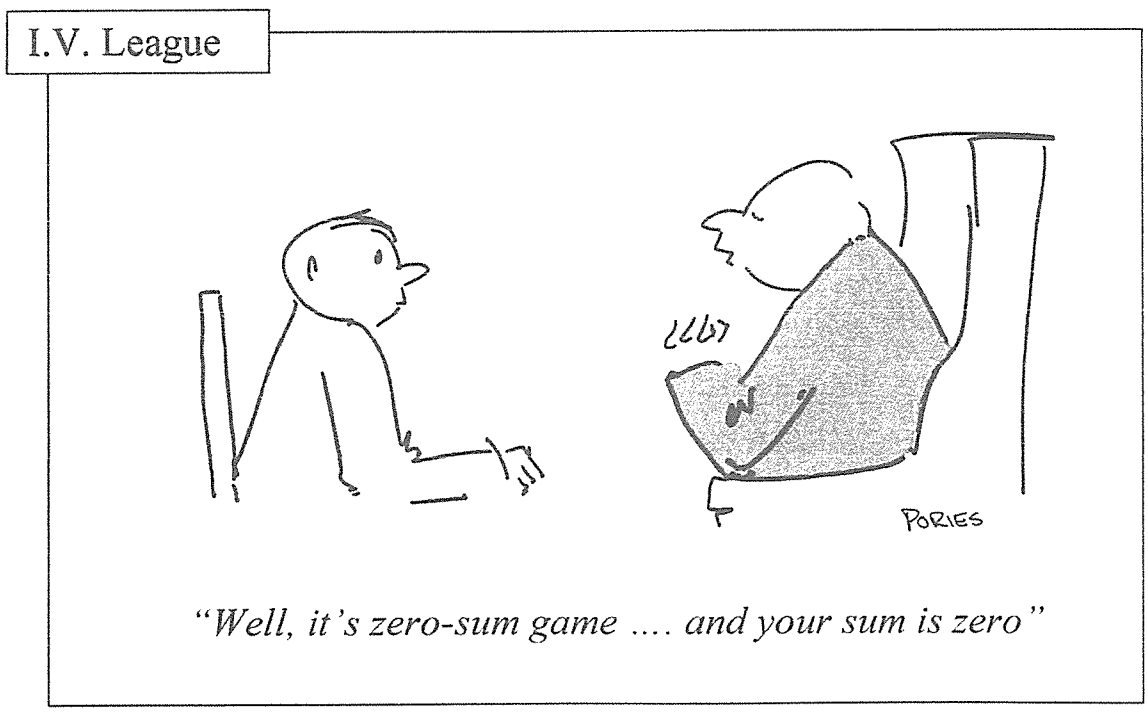

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Analysis of Rat Insulin II Promoter-Ghrelin Transgenic Mice and Rat Glucagon Promoter-Ghrelin Transgenic Mice*

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We developed and analyzed two types of transgenic mice: rat insulin II promoter-ghrelin transgenic (RIP-G Tg) and rat glucagon promoter-ghrelin transgenic mice (RGP-G Tg). The pancreatic tissue ghrelin concentration measured by C-terminal radioimmunoassay (RIA) and plasma desacyl ghrelin concentration of RIP-G Tg were about 1000 and 3.4 times higher than those of nontransgenic littermates, respectively. The pancreatic tissue *n*-octanoylated ghrelin concentration measured by N-terminal RIA and plasma *n*-octanoylated ghrelin concentration of RIP-G Tg were not distinguishable from those of nontransgenic littermates. RIP-G Tg showed suppression of glucose-stimulated insulin secretion. Arginine-stimulated insulin secretion, pancreatic insulin mRNA and peptide levels, β cell mass, islet architecture, and GLUT2 and PDX-1 immunoreactivity in RIP-G Tg pancreas were not significantly different from those of nontransgenic littermates. Islet batch incubation study did not show suppression of insulin secretion of RIP-G Tg *in vitro*. The insulin tolerance test showed lower tendency of blood glucose levels in RIP-G Tg. Taking lower tendency of triglyceride level of RIP-G Tg into consideration, these results may indicate that the suppression of insulin secretion is likely due to the effect of desacyl ghrelin on insulin sensitivity. RGP-G Tg, in which the pancreatic tissue ghrelin concentration measured by C-RIA was about 50 times higher than that of nontransgenic littermates, showed no significant changes in insulin secretion, glucose metabolism, islet mass, and islet architecture. The present study raises the possibility that desacyl ghrelin may have influence on glucose metabolism.

Ghrelin is a 28-amino acid peptide with unique modification of acylation, which is essential for its biological action (1). Ghrelin was originally identified in rat stomach as an endogenous ligand for an orphan receptor, which has been so far called growth hormone secretagogue receptor (GHS-R)[1] (1). Ghrelin expression is detected in the stomach, intestine, hypothalamus, pituitary gland, kidney, placenta, and testis (2–6). Ghrelin is involved in a wide variety of the functions, including the regulation of growth hormone release, food intake, gastric acid secretion, gastric motility, blood pressure, and cardiac output (7–19).

Recently Date *et al.* (20) reported that ghrelin is present in α cells of normal human and rat pancreatic islets. Volante *et al.* (21) described ghrelin-expression in β cells of human islet. Wierup *et al.* and Prado *et al.* reported that ghrelin-expressing cells are a new islet cell type distinct from α, β, δ, and PP cells in human, rat, and mouse islets (22–24). Although there was no apparent change of plasma insulin levels in ghrelin null mouse (25, 26), which may indicate that ghrelin is not a direct regulator of insulin secretion in the physiological condition, there have been several reports of the effect of pharmacological dose of ghrelin on insulin secretion. Broglio *et al.*, Egido *et al.*, and Reimer *et al.* have reported that ghrelin has an inhibitory effect on insulin secretion (27–30). Adeghate *et al.*, Date *et al.*, and Lee *et al.* have reported that ghrelin stimulates insulin secretion (20, 31, 32). Salehi *et al.* have reported ghrelin has both inhibitory and stimulatory effects depending on its concentration (33). Therefore, there is still a lot of controversy about the localization of ghrelin in the pancreas and the effects of ghrelin on the insulin secretion. As for the effects of desacyl ghrelin on insulin secretion, Broglio *et al.* (34) have reported that acute desacyl ghrelin administration has no effect on insulin secretion in human but that it counteracts the inhibitory effect of *n*-octanoylated ghrelin on insulin secretion when co-administrated with *n*-octanoylated ghrelin (35).

Here we developed and analyzed two types of transgenic mice: rat insulin II promoter-ghrelin transgenic mice (RIP-G Tg) and rat glucagon promoter-ghrelin transgenic mice (RGP-G Tg). The purpose of this study was to clarify the effect of transgenic overexpression of ghrelin cDNA in pancreatic islets.

EXPERIMENTAL PROCEDURES

Generating RIP- and RGP-ghrelin Transgenic Mice—Mouse stomach cDNA library was constructed from 1 μg of mouse stomach poly(A)$^+$

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[1] The abbreviations used are: GHS-R, growth hormone secretagogue receptor; RIP-G Tg, rat insulin II promoter-ghrelin transgenic; RGP-G Tg, rat glucagon promoter-ghrelin transgenic mice; RIA, radioimmunoassay; C-RIA, anti-ghrelin [13–28] antiserum; N-RIA, anti-ghrelin [1–11] antiserum; RT, reverse transcription; HDL, high density lipoprotein; PP, pancreatic polypeptide.

RNA with a cDNA synthesis kit (Amersham Biosciences). Mouse ghrelin cDNA was isolated from this library, using rat ghrelin cDNA as a probe. A fusion gene comprising RIP and mouse ghrelin cDNA coding sequences was designed. The purified fragment (10 μg/ml) was microinjected into the pronucleus of fertilized C57/B6J mice (SLC, Shizuoka, Japan) eggs. The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (SLC) using standard techniques. Transgenic founder mice were identified by Southern blot analysis of tail DNAs using the mouse ghrelin cDNA fragment as a probe. RGP-G Tg was generated similarly. Transgenic mice were used as heterozygotes. Animals were maintained on standard rat food (CE-2, 352 kcal/100 g, Japan CLEA, Tokyo, Japan) on a 12-h light/12-h dark cycle. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

Immunohistochemistry—Formalin-fixed, paraffin-embedded tissue sections were immunostained using the avidin-biotin peroxidase complex method (Vectastain "ABC" Elite kit, Vector Laboratories, Burlingame, CA) as described previously (36). Serial sections were used, and the thickness of each section was 5 μm. Sections were incubated with anti-C-terminal ghrelin [13–28] (1:1000 at final dilution), anti-N-terminal ghrelin [1–11] (1:2000) (1), which recognizes the *n*-octanoylated portion of ghrelin, anti-glucagon (1:500), anti-insulin (1:500), anti-somatostatin (1:500), anti-pancreatic polypeptide (PP, 1:500, DAKO, Glostrup, Denmark), anti-PDX-1 (1:2000, kindly provided by Christopher V. E. Wright) (37), and anti-GLUT2 (1:200, kindly provided by Bernard Thorens) (38) antisera. Quantification of β cell area was performed in insulin-stained sections by using Axio Vision (Carl Zeiss, Hallbergmoos, Germany) and Scion Image (Scion Corp., Frederick, MD). Ten sections (200-μm interval) for each mouse ($n = 5$) were analyzed. The percentage of β cell area in the pancreas was determined by dividing the area of all insulin-positive cells in one section by the total area of the section.

Measurements of Plasma and Tissue Ghrelin Concentrations—Plasma was sampled from 10-week-old RIP-G Tg and their nontransgenic littermates under ad libitum feeding states considering the promoter activity. From RGP-G Tg and their littermates, it was sampled after overnight fast. Blood was withdrawn from the retroorbital vein or the proximal end of the portal vein under ether anesthesia, immediately transferred to chilled siliconized glass tubes containing Na_2EDTA (1 mg/ml) and aprotinin (1000 KIU/ml, Ohkura Pharmaceutical, Kyoto, Japan), and centrifuged at 4 °C. Hydrogen chloride was added to the samples at a final concentration of 0.1 N immediately after separation of plasma. Plasma was immediately frozen and stored at −80 °C until assay. Plasma ghrelin concentration was determined by desacyl ghrelin enzyme-linked immunosorbent assay kit and active-ghrelin enzyme-linked immunosorbent assay kit that recognizes *n*-octanoylated ghrelin (Mitsubishi Kagaku Iatron, Tokyo, Japan).

As for measurement of tissue ghrelin concentration, pancreata or stomachs were taken from the 8-week-old male mice. The rumen was removed from the stomach. Samples were diced and boiled for 5 min in the 10-fold v/w of water. Acetic acid was added to each solution so that the final concentration was adjusted to 1 M, and the tissues were homogenized. The supernatants were obtained after centrifugation. Tissue ghrelin concentration was determined by radioimmunoassay (RIA) using anti-ghrelin [13–28] antiserum (C-RIA) and anti-ghrelin [1–11] antiserum (N-RIA) as described previously (39).

Measurements of Body Weight and Food Consumption—Mice were housed individually and were allowed free access to standard rat chow. Body weights of mice were measured weekly. Daily food intake was measured by weighing the pellets between 9:00 and 10:00 a.m.

Measurements of % Body Fat and Visceral/Subcutaneous Fat Mass Ratio—Forty-week-old mice were anesthetized with pentobarbital. Percent body fat and visceral/subcutaneous fat mass ratio of mice were measured by Latheta LTC-100 (ALOKA, Tokyo, Japan).

Glucose and Insulin Tolerance Tests—For the glucose tolerance test, after overnight fast, the mice were injected with 1.5 g/kg glucose intraperitoneally. For the insulin tolerance test, after a 4-h fast, mice were injected with 2.0 milliunits/g human regular insulin (Novolin R; Novo Nordisk, Bagsvaerd, Denmark) intraperitoneally. Blood was sampled from the tail vein before and 15, 30, 60, 90, and 120 min after the injection. Blood glucose levels were determined by glucose oxidase method using Glutest sensor (Sanwa Kagaku, Kyoto, Japan).

Insulin Release—After overnight fast, the mice were injected with 3.0 g/kg glucose or 0.25 g/kg L-arginine intraperitoneally. Plasma was sampled from the tail vein before and 2, 5, 15, 30, and 60 min after the injection using heparin coated tubes. The measurement of insulin concentration was carried out by enzyme-linked immunosorbent assay using ultra-sensitive rat insulin kit (Morinaga, Yokohama, Japan).

Pancreatic Insulin Concentration—As for measurement of pancreatic insulin concentration, pancreata were obtained from the mice under the ether anesthesia and homogenized in acid-ethanol. The supernatants were used for assay after centrifugation.

Batch Incubation of Islet—Under the pentobarbital anesthesia, Type IV collagenase (Worthington, Lakewood, NJ) dissolved in Hanks' balanced salt solution (1.5 mg/ml) was injected into mouse pancreatic duct. Pancreas was removed and incubated at 37 °C for 14 min. After washing out collagenase by Hanks' balanced salt solution, islets were collected by Ficoll gradient and manually picked up so that the sizes of the islets were equal. Islets were incubated at 37 °C in RPMI1640 containing 10% fetal calf serum for 2 h and then in Krebs-Ringer bicarbonate buffer containing 3.3 mM glucose and 0.2% bovine serum albumin for 30 min. Five islets were incubated at 37 °C in 500 μl of Krebs-Ringer bicarbonate buffer containing 0.2% bovine serum albumin and 3.3 or 8.7 or 16.7 mM glucose for 1 h. After centrifugation, the supernatants were collected. Insulin concentrations in supernatants were determined by rat insulin kit (Morinaga, Yokohama, Japan).

Northern Blot Analysis and Real-time Quantitative RT-PCR—Total RNA was extracted from pancreata using RNeasy mini kit (Qiagen K.K., Tokyo, Japan). Filters containing 5 μg of total RNA were prepared. Northern blot analyses were performed as described previously (36) using the mouse insulin II cDNA and human β-actin cDNA (Clontech, Palo Alto, CA) as probes. To confirm that approximately equal amounts of total RNA were assayed in Northern blot hybridization analysis, the density of 18 S rRNA in the gel and signal of β-actin in each lane was used. The hybridization signal intensity was quantitated using an image analyzer BAS-2500 (Fuji Photo Film, Tokyo, Japan). Reverse transcription (RT) was performed with random hexamer and SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed with ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The following primers and TaqMan probes were used: mouse GHS-R (sense, 5′-CACCAACCTCTACCTATCCAGCAT-3′; antisense, 5′-CTGACAAACTGGAAGAGTTTGCA-3′; TaqMan probe, 5′-TCCGATCTGCTCATCTTCCTGTGCATG-3′); mouse ghrelin (sense, 5′-GCATGCTCTGGATGGACATG-3′; antisense, 5′-TGGTGGCTTCTTGGATTCCT-3′; TaqMan probe, 5′-AGCCCAGAGCACCAGAAAGCCCA-3′).

Lipid Measurements—Blood was collected from the retroorbital vein of 35-week-old RIP-G Tg and their nontransgenic littermates. After separation of serum, total cholesterol, triglyceride, free fatty acid, and HDL-cholesterol levels in serum were determined by Cholesterol E-test Wako, Triglyceride E-test Wako, NEFA C-test Wako, and HDL-cholesterol E-test Wako (Wako Pure Chemical Industries, Osaka, Japan).

Statistical Analysis—All values were expressed as means ± S.E. Statistical significance of difference in mean values was assessed by repeated measures analysis of variance or Student's *t* test.

RESULTS

Distribution of Ghrelin in Normal Mouse Pancreas—We first examined which cell type of islet cells expresses ghrelin in mouse by immunohistochemistry using anti-C-terminal ghrelin antiserum. In the most of the islets no ghrelin-like immunoreactivity was detected. C-terminal ghrelin-like immunoreactivity was observed in the periphery of minor proportion of islets of wild type mice (Fig. 1*A*). Most of the ghrelin-positive cells were also glucagon-positive by serial section analysis (Fig. 1*B*), whereas most of the glucagon-positive cells were not ghrelin-positive.

Generation of RIP- and RGP-ghrelin Transgenic Mice—A fusion gene comprising RIP and mouse ghrelin cDNA coding sequences was designed so that ghrelin expression might be targeted to the pancreatic β cells (Fig. 2*A*). The ghrelin mRNA level of RIP-G Tg in pancreas determined by quantitative RT-PCR was about 215 times higher than that of nontransgenic littermates (215.3 ± 40.6 *versus* 1.0 ± 0.025 arbitrary units, $n = 5$, $p < 0.01$). There was also an increase in ghrelin mRNA levels in brain of RIP-G Tg (242.6 ± 17.6 *versus* 89.1 ± 27.3 arbitrary unit, $n = 5$, $p < 0.01$). To confirm the expression of ghrelin transgene in pancreatic β cells, we performed an immunohistochemical analysis using anti-C-terminal ghrelin antiserum. C-terminal ghrelin-like immunoreactivity was observed in the nearly whole area of the islets of the RIP-G Tg (Fig. 2*C*), whereas it was only seen in the periphery of the islets

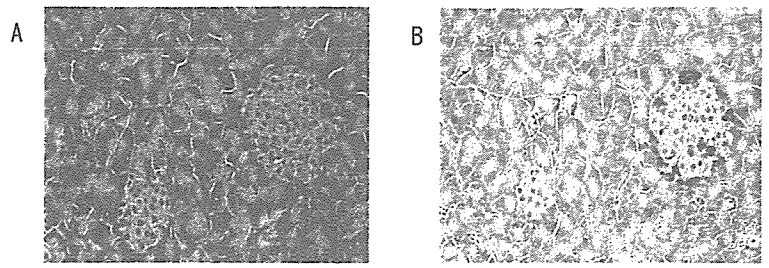

FIG. 1. *A*, C-terminal ghrelin-like immunoreactivity in adult mouse islet. The staining was observed in the peripheral region of the islet. *B*, glucagon-like immunoreactivity in serial section.

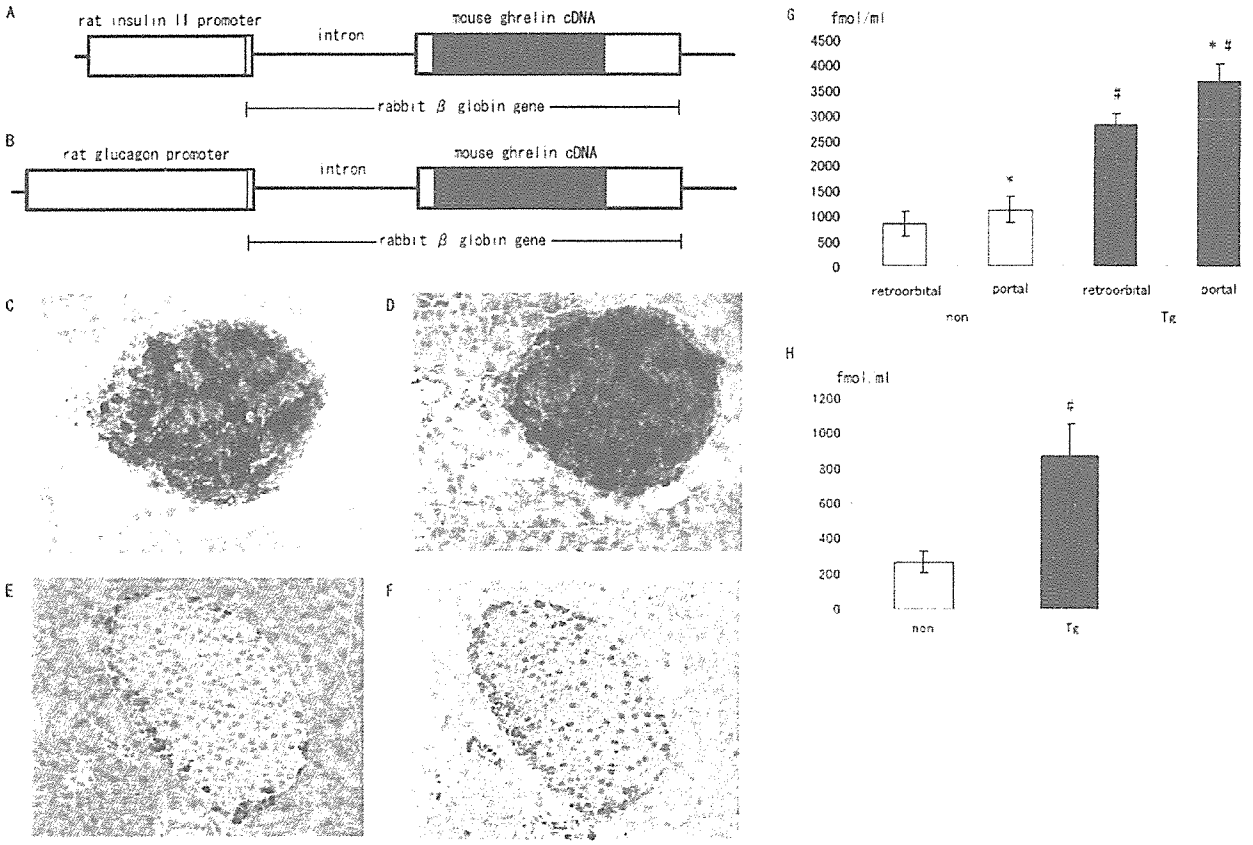

FIG. 2. *A*, structure of RIP-ghrelin transgene. *B*, structure of RGP-ghrelin transgene. *C* and *D*, pancreatic islet of RIP-ghrelin transgenic mouse stained with anti-C-terminal ghrelin (*C*) and anti-N-terminal ghrelin antisera (*D*). *E* and *F*, pancreatic islet of RGP-ghrelin transgenic mouse stained with anti-C-terminal ghrelin (*E*) and anti-N-terminal ghrelin antisera (*F*). *G*, plasma ghrelin levels collected from retroorbital and portal veins in RIP-G Tg. *, $p < 0.05$ compared with retroorbital vein. #, $p < 0.01$ compared with nontransgenic littermates. *H*, the step-up of ghrelin concentration from retroorbital vein to portal vein in RIP-G Tg. #, $p < 0.01$ compared with their nontransgenic littermates.

of their nontransgenic littermates (Fig. 1*A*). Immunohistochemical analysis using anti-N-terminal ghrelin antiserum showed the same staining pattern (Fig. 2*D*), indicating that *n*-octanoylated ghrelin may be produced in β cells of this transgenic mouse. We also stained the brain section of RIP-G Tg. No ghrelin-like immunoreactivity was detected either with anti-C-terminal or anti-N-terminal ghrelin antisera (data not shown). The pancreatic tissue ghrelin concentration of RIP-G Tg measured by C-RIA was about 1000 times higher than that of their nontransgenic littermates (1024 ± 108.9 fmol/mg *versus* 1.2 ± 0.1 fmol/mg, $n = 5, p < 0.01$). This concentration was about one third of the nontransgenic stomach concentration (3558.1 ± 51.0 fmol/mg, $n = 5$). The pancreatic tissue ghrelin concentration of RIP-G Tg measured by N-RIA tended to be also higher than that of their nontransgenic littermates (0.054 ± 0.017 fmol/mg *versus* 0.038 ± 0.006fmol/mg, $n = 5$, NS; not significant), but it did not reach statistical significance. Plasma desacyl ghrelin concentration of RIP-G Tg was about 3.4 times higher than that of nontransgenic littermates under the ad libitum feeding states (2805.5 ± 236.4 *versus* 825.9 ± 244.4 fmol/ml, $n = 5, p < 0.01$, Fig. 2*G*). We also measured desacyl ghrelin levels in portal vein of the mice. In the nontransgenic mice, the portal desacyl ghrelin level was significantly higher than that in retroorbital vein (1108.0 ± 257.3 fmol/ml *versus* 825.9 ± 244.4 fmol/ml, $n = 5, p < 0.05$, Fig. 2*G*). The desacyl ghrelin concentration collected from portal vein of RIP-G Tg at the same time was much higher than that of nontransgenic littermates (3671.8 ± 328.6 *versus* 1108.0 ± 257.3 fmol/ml, $n = 5, p < 0.01$, Fig. 2*G*). The step-up of desacyl ghrelin concentration from retroorbital vein to portal vein of RIP-G Tg was significantly higher than that of nontransgenic littermates (866.3 ± 182.2 fmol/ml *versus* 262.9 ± 59.8 fmol/ml, $p < 0.01$, Fig. 2*H*). Plasma *n*-octanoylated ghrelin levels in retroorbital and portal vein of RIP-G Tg tended to be higher than those of their nontransgenic littermates (retroorbital: 78.5 ± 13.4 *versus* 66.1 ± 7.1 fmol/ml, $n = 5$, NS; portal: 104.6 ± 15.3 *versus*

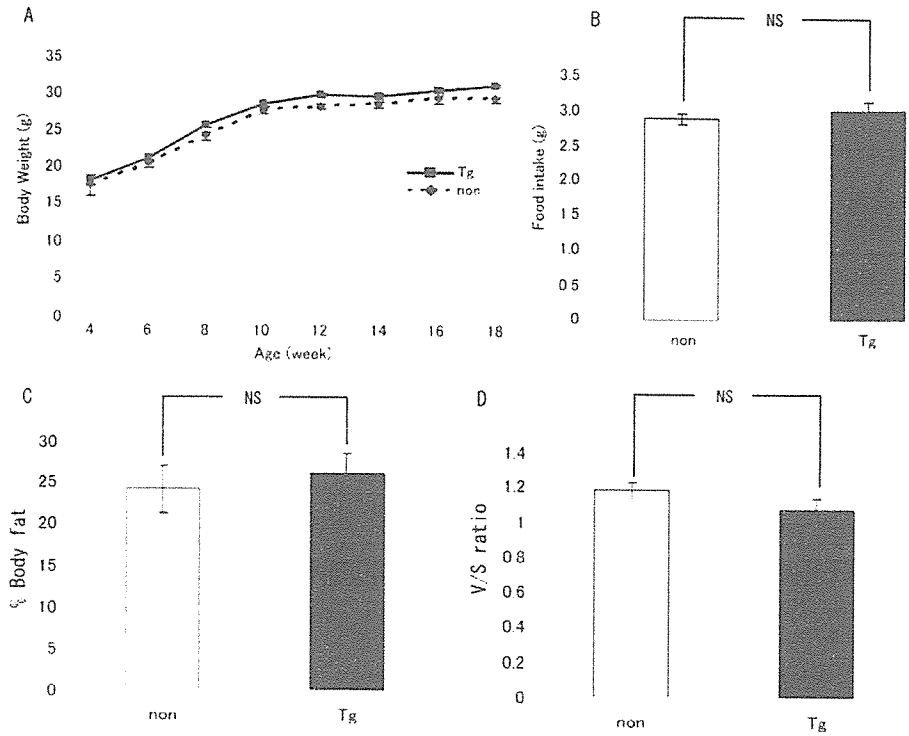

FIG. 3. *A*, body weight of RIP-G Tg (*Tg*) and their nontransgenic littermates (*non*). *B*, food intake of RIP-GTg (*Tg*) and their nontransgenic littermates (*non*). Percent body fat (*C*) and visceral/subcutaneous fat ratio (*D*) of RIP-GT_g and their nontransgenic littermates (*non*).

71.4 ± 9.0 fmol/ml, $n = 5$, NS), but it did not reach statistical significance.

We also generated RGP-G Tg, in which ghrelin expression is targeted to the pancreatic α cells (Fig. 2*B*). The ghrelin mRNA level Tg in pancreas of RGP-G determined by quantitative RT-PCR was about 16 times higher than that of nontransgenic littermates (16.3 ± 1.7 *versus* 1.0 ± 0.24 arbitrary unit, $n = 5$, $p < 0.01$). The ghrelin mRNA level in duodenum of RGP Tg was not statistically different from that of nontransgenic littermates (520.1 ± 111.1 *versus* 379.1 ± 37.6 arbitrary unit, $n = 5$, NS). The ghrelin mRNA level in brain of RGP Tg was not distinguishable from that of nontransgenic littermates (72.0 ± 6.4 *versus* 71.8 ± 7.8 arbitrary unit, $n = 5$, NS). Immunohistochemical analysis showed ghrelin-like immunoreactivity in the periphery of the pancreatic islet of RGP-ghrelin transgenic mouse by both anti-C-terminal ghrelin and anti-N-terminal ghrelin antisera (Fig. 2, *E* and *F*). The pancreatic tissue ghrelin concentrations of RGP-G Tg measured by C-RIA were about 50 times higher than those of their nontransgenic littermates (48.9 ± 2.5 fmol/mg *versus* 1.2 ± 0.1 fmol/mg, $n = 5$, $p < 0.01$). The pancreatic tissue ghrelin concentration of RGP-G Tg measured by N-RIA tended to be higher than that of their nontransgenic littermates (0.076 ± 0.019 fmol/mg *versus* 0.038 ± 0.006 fmol/mg, $n = 5$, NS), but it did not reach statistical significance. The plasma desacyl ghrelin concentrations in retroorbital vein were not elevated in RGP-G Tg after over night fasting compared with nontransgenic littermates (661.6 ± 38.0 *versus* 1024.7 ± 27.1 fmol/ml, $n = 5$). The portal desacyl ghrelin concentrations of RGP-G Tg were also indistinguishable from those of their nontransgenic littermate (1320.6 ± 164.7 *versus* 1442.9 ± 361.5 fmol/ml, $n = 5$, NS). Plasma *n*-octanoylated ghrelin levels in retroorbital and portal vein of RGP-G Tg were indistinguishable from those of their nontransgenic littermates (retroorbital: 98.3 ± 18.7 *versus* 133.5 ± 25.3 fmol/ml, $n = 5$, NS; portal: 154.3 ± 20.7 *versus* 198.9 ± 34.9 fmol/ml, $n = 5$, NS).

Body Weight, Food Consumption, and Percent Body Fat—There was no significant difference in body weight and food intake between RIP-G Tg and their nontransgenic littermates (Fig. 3). Percent body fat and visceral/subcutaneous ratio of RIP-G Tg were not different from those of nontransgenic littermates (Fig. 2, *C* and *D*). No significant changes were observed in RGP-G Tg, either (data not shown).

Glucose Metabolism and Insulin Secretion—Although no significant differences in blood glucose levels were noted between RIP-G Tg and their nontransgenic littermates on the fasting state and intraperitoneal glucose tolerance tests (Fig. 4, *A* and *C*), plasma insulin levels 2 and 30 min after the glucose injection were significantly decreased in RIP-G Tg compared with those in their nontransgenic littermates (Fig. 4*D*). Suppression of insulin secretion was not observed in RIP-G Tg on intraperitoneal injection of arginine (Fig. 4*G*). Blood glucose level of RIP-G Tg in the insulin tolerance test tended to be lower than those of their nontransgenic littermates, but it did not reach statistical significance (Fig. 4*H*).

No significant differences in blood glucose or insulin levels were observed between RGP-G Tg and their nontransgenic littermates on the fasting state, ad libitum feeding, or intraperitoneal glucose or arginine injection (Fig. 4, *B*, *E*, and *F*, and data not shown). Blood glucose levels on insulin tolerance test showed no differences between RGP-ghrelin and their nontransgenic littermates (data not shown).

Islet Architecture and β Cell Mass—We studied the tissue sections of RIP-G Tg to explore the effect of ghrelin on the islet architecture and β cell mass. There were no obvious abnormalities in the intra islet cytoarchitecture and cell number of insulin, glucagon, somatostatin, and PP cells in the islets of the RIP-G Tg (Fig. 5*A–D*). The intensity of staining of these four islet hormones in the islets of the RIP-G Tg was not apparently different from those of nontransgenic littermates. The ratio of the β cell area to whole pancreas was not changed significantly

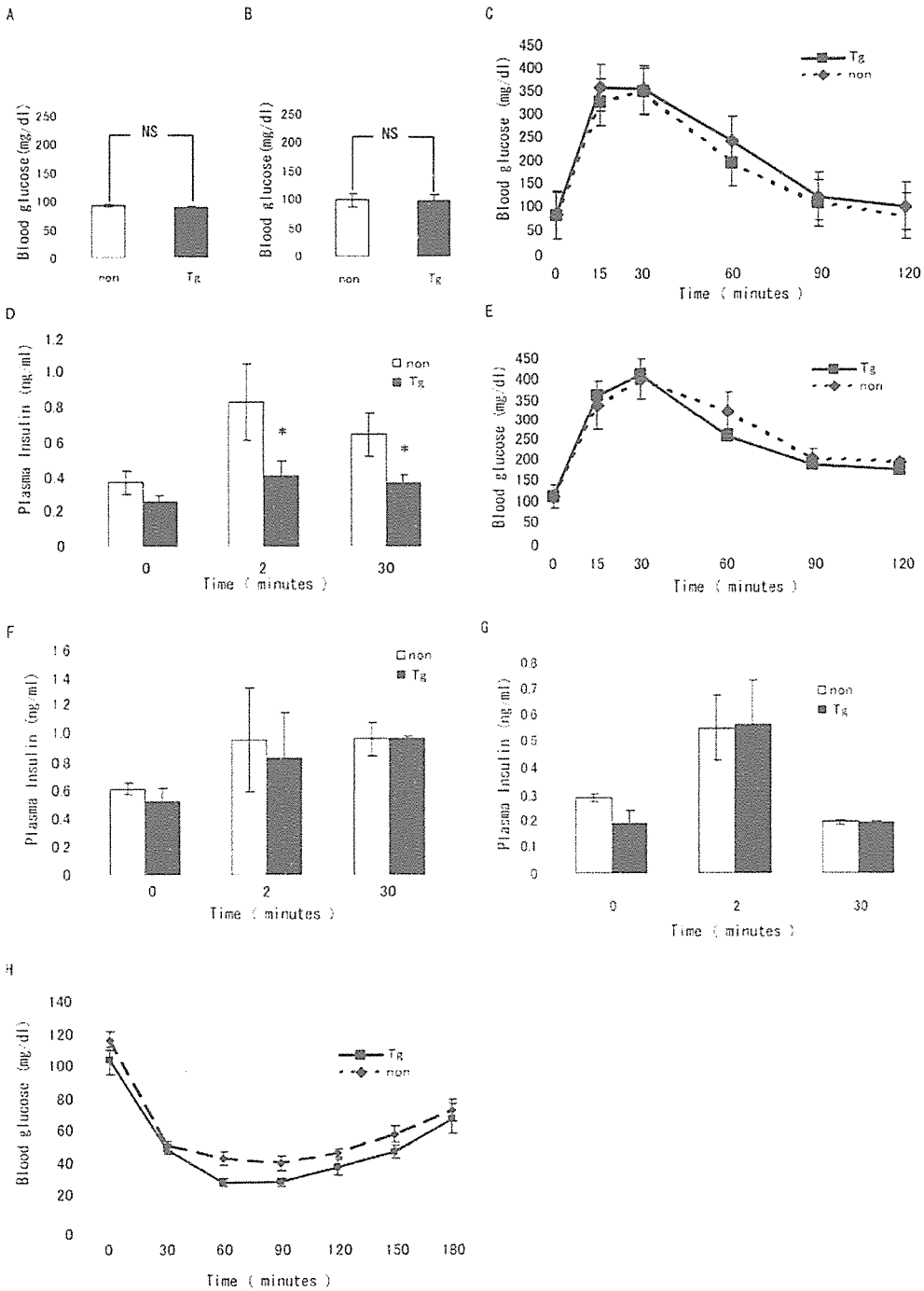

FIG. 4. *A* and *B*, blood glucose levels after overnight fast in RIP-G Tg (*A*) and RGP-G Tg (*B*) (*Tg*) and their nontransgenic littermates (*non*). *C* and *E*, intraperitoneal (*IP*) glucose tolerance test (1.5 g/kg) in RIP-G Tg (*C*) and RGP-G Tg (*E*) (*Tg*) and their nontransgenic littermates (*non*). *D* and *F*, plasma insulin concentration after intraperitoneal glucose (3g/kg) injection in RIP-G Tg (*D*) and RGP-G Tg (*F*) (*Tg*) and their nontransgenic littermates (*non*). *G*, plasma insulin concentration after intraperitoneal arginine (0.25g/kg) injection in RIP-G Tg (*Tg*) and their nontransgenic littermates (*non*). *H*, insulin (2.0 units/kg) tolerance test in RIP-G Tg (*Tg*) and their nontransgenic littermates (*non*). Values are represented as mean ± S.E. *, $p < 0.05$ compared with nontransgenic littermates.

(Fig. 5*I*). We also studied the tissue sections of RGP-G Tg and found no significant differences (Fig. 5, *E–H*, and *J*).

Expression of Insulin mRNA and Insulin Content—Because RIP-G Tg showed suppression of insulin secretion, we examined pancreatic mRNA expression and peptide content of insulin in RIP-G Tg and their nontransgenic littermates by Northern blot analysis and RIA. The insulin mRNA in RIP-G Tg did not differ from those of their nontransgenic littermates (Fig. 6, *A* and *B*). No significant differences of insulin contents were observed between RIP-G Tg and their nontransgenic littermates (Fig. 6*C*).

PDX-1 and GLUT2 Immunoreactivity—We examined the immunoreactivity of PDX-1 and GLUT2 in RIP-G Tg. The staining intensities of PDX-1 and GLUT2 in the RIP-G Tg (Fig. 7, *A*

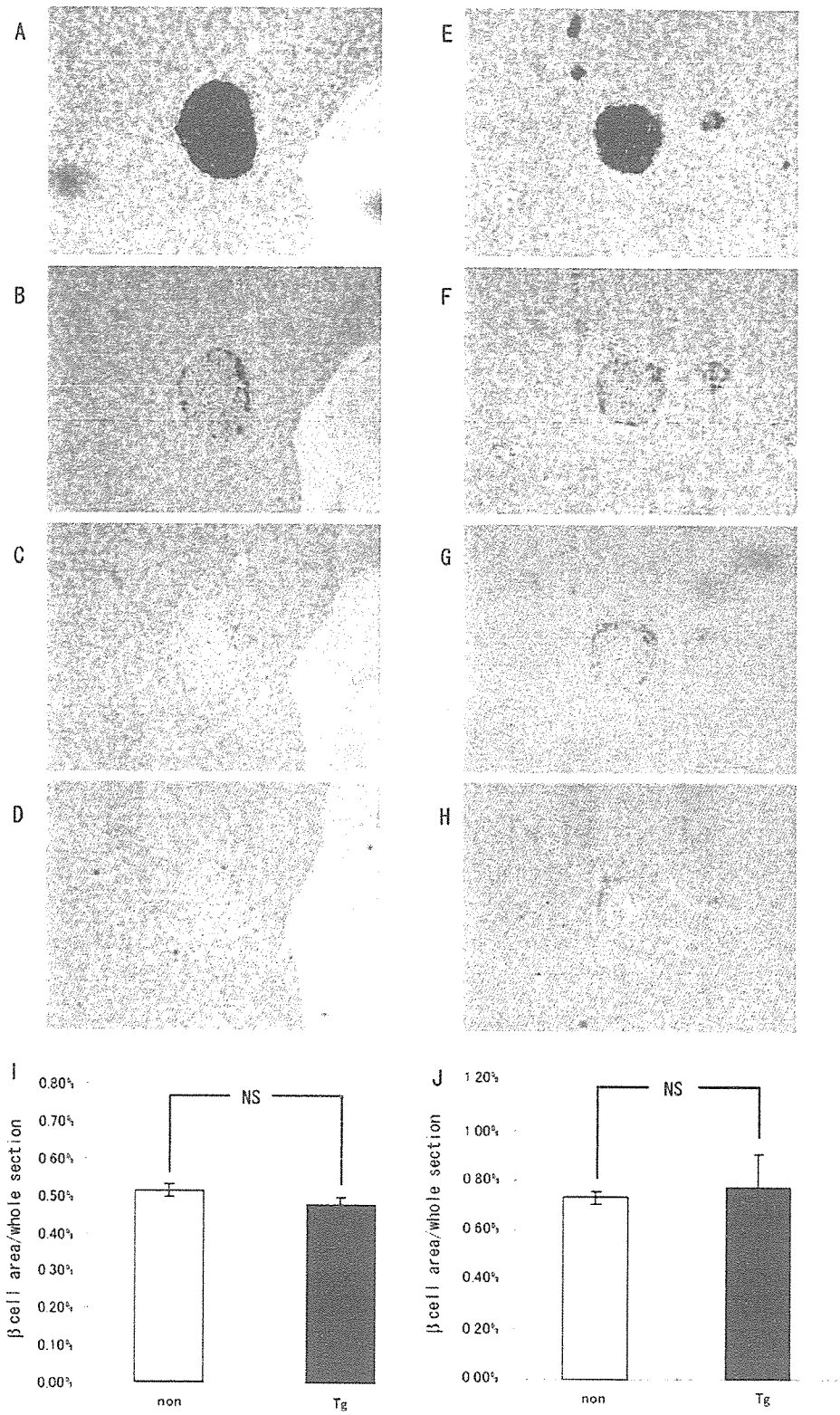

FIG. 5. **Islet morphology and β cell area in RIP-G Tg (*A–D*) and RGP-G Tg (*E–H*).** The sections were stained with anti-insulin (*A* and *E*), anti-glucagon (*B* and *F*), anti-somatostatin (*C* and *G*), and anti-PP antiserum (*D* and *H*). *I* and *J*, the ratio of β cell area to that of whole section in RIP-G Tg (*I*) and RGP-G Tg (*J*). *non*, nontransgenic littermates; *Tg*, RIP-G Tg; *NS*, not significant.

and *C*) were not apparently different from those in the nontransgenic littermates (Fig. 7, *B* and *D*).

Expression of GHS-R mRNA—To rule out possible down-regulation of GHS-R due to chronic exposure to high level ghrelin, we measured the expression level of GHS-R mRNA in pancreas and pituitary by real-time quantitative RT-PCR.

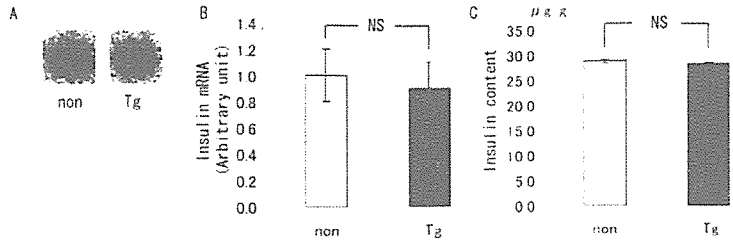

FIG. 6. **mRNA level and peptide content of insulin in RIP-G Tg (*Tg*) and their nontransgenic littermates (*non*) pancreas.** *A*, representative blot of Northern blot analysis of insulin; *B*, insulin mRNA levels; *C*, insulin peptide contents. *NS*, not significant.

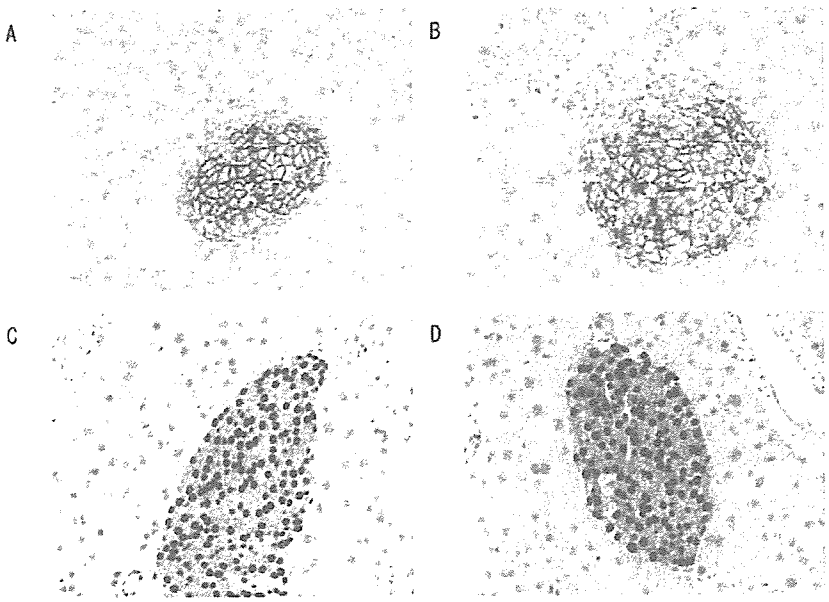

FIG. 7. *A* and *B*, immunoreactivity of Glut-2 in the islet of RIP-G Tg (*A*) and nontransgenic littermates (*B*). *C* and *D*, immunoreactivity of PDX-1 in the islet of RIP-G Tg (*C*) and nontransgenic littermates (*D*).

There were no significant differences in GHS-R mRNA levels between RIP-G Tg and their nontransgenic littermates either in pancreas (Fig. 8*A*) or in pituitary (Fig. 8*B*).

Batch Incubation of Islets—The insulin secretion from isolated islet of RIP-G Tg by batch incubation was indistinguishable from that of nontransgenic littermates, in 3.3 or 8.7 or 16.7 mM glucose conditions (Fig. 9).

Lipid Metabolism—Plasma total cholesterol level of RIP-G Tg tended to be lower than those of nontransgenic littermates, but it did not reach statistical significance (total cholesterol: 85.4 ± 6.9 *versus* 79.4 ± 7.5 mg/dl, $n = 6$, NS). The plasma triglyceride level of RIP-G Tg tended to be lower than that of nontransgenic littermates, but it did not reach statistical significance (154.5 ± 11.0 *versus* 136.9 ± 10.3 mg/dl, $n = 6$, NS). Free fatty acid level and HDL-cholesterol level of RIP-G Tg were not significantly different from those of nontransgenic littermates (free fatty acid; 0.44 ± 0.05 *versus* 0.48 ± 0.07 mEq/liter, $n = 6$, NS, HDL-cholesterol; 46.1 ± 2.3 *versus* 44.9 ± 3.4 mg/dl, $n = 6$, NS).

DISCUSSION

In wild-type mice, no ghrelin-like immunoreactivity was detected in most of the islets. C-terminal ghrelin-like immunoreactivity was observed in the periphery of minor proportion of islets of wild type mice, which is consistent with a previous report (24). By the serial section analysis, most of the ghrelin-producing cells also showed glucagon-like immunoreactivity. These findings indicate that ghrelin was expressed in minor proportion of mouse pancreatic α cells. Expression of ghrelin was not detected in pancreatic β cells of wild type mice.

In the present study we developed RIP-G Tg, in which pancreatic ghrelin concentration measured by C-RIA was ~1000 times higher than that of nontransgenic littermates. By immu-

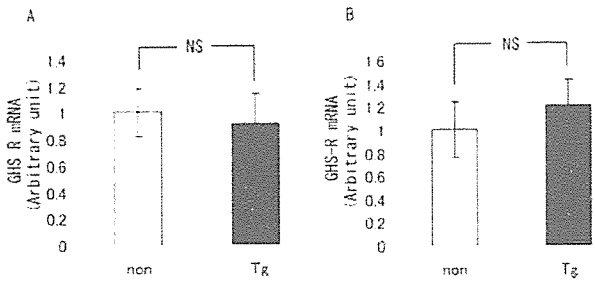

FIG. 8. **mRNA level of GHS-R determined by quantitative RT-PCR in pancreas (*A*) and pituitary (*B*) of RIP-G Tg (*Tg*) and their nontransgenic littermates (*non*).** *NS*, not significant.

nohistochemistry using anti-C-terminal ghrelin [13–28] antiserum we detected C-terminal ghrelin-like immunoreactivity in almost the whole area of islets. Therefore, because ghrelin was not detected in β cells of control mice by immunohistochemistry, ghrelin transgene driven by RIP was considered to be expressed in β cells.

We also found about 3 times higher expression level of ghrelin mRNA in the brain of RIP-G Tg compared with that of nontransgenic littermates, which could not be detected by immunohistochemistry. Although a small amount of ghrelin has been reported to be expressed in brain, which can be detected by immunohistochemistry only after colchicine treatment (1), there have been controversies as to whether this small amount of ghrelin in the brain has a biological role. Because the food intake of RIP-G Tg was not different from that of nontransgenic littermates, the ghrelin produced by transgene in the brain seems not to show bioactive effect of *n*-octanoylated ghrelin.

By immunohistochemistry using anti-ghrelin [1–11] anti-

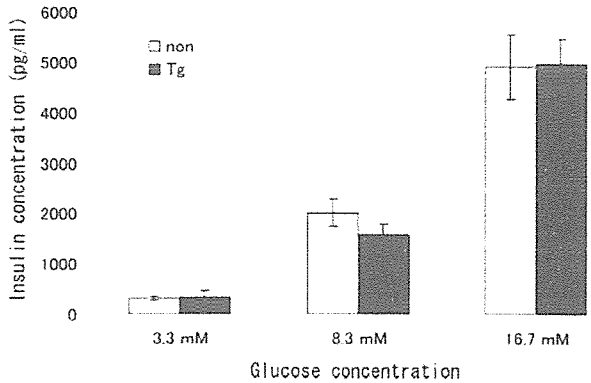

FIG. 9. **Batch incubation study of isolated islets of RIP-G Tg (*Tg*) and their nontransgenic littermates (*non*).**

serum that recognizes the *n*-octanoylated portion of ghrelin, ghrelin-like immunoreactivity was also demonstrated in nearly whole area of islets of RIP-G Tg, indicating the production of *n*-octanoylated ghrelin in β cells. This finding indicates that the mechanism of acylation may exist not only in pancreatic α cells but also in β cells. This is reasonable, because α and β cells are pancreatic endocrine cells derived from common precursor cells (40). Because the N-RIA/C-RIA ratio of the pancreatic tissue ghrelin concentration of RIP-G Tg was much lower than that of the stomach (0.0053% *versus* 11.67%, $p < 0.01$), the ability of acylation in β cell might be lower than that of in ghrelin-producing cell in the stomach (X/A-like cell). It is possible that exocrine pancreatic enzymes might interfere with the results, although these were inactivated by boiling before extraction. The other possibility is that because of the formalin fixation of ghrelin in the tissue section the epitope recognized by immunohistochemistry using anti-ghrelin [1–11] antiserum might not be exactly the same as that recognized by N-RIA or enzyme-linked immunosorbent assay. Because the amount of *n*-octanoylated ghrelin was so little that it could not be detected by RIA if any, we considered that the phenotype of these transgenic mice is due to the effect of desacyl ghrelin. Desacyl ghrelin has been shown not to activate GHS-R (39). There have been several reports saying that desacyl ghrelin has biological activities, such as promoting adipogenesis (41), inhibition of cell proliferation, inhibition of apoptosis (42), and counteracting the effect of *n*-octanoylated ghrelin (35).

We showed here that the ghrelin level in portal vein is significantly higher than that in retroorbital vein in wild type mouse. Ghrelin has been reported to be mainly synthesized in stomach and intestine. The step-up of plasma ghrelin level in gastric vein has been reported previously (43), but there has been no report showing the step-up of plasma ghrelin level in portal vein as compared with that in systemic circulation. The present study is the first report of the step-up of plasma ghrelin levels in portal vein. Moreover, the step-up of desacyl ghrelin in RIP-G Tg was much higher than that in control littermates, indicating overproduction of desacyl ghrelin by transgene in the pancreas.

The body weight, percent body fat, and food consumption of RIP-G Tg were not significantly different from those of nontransgenic littermates. Recently, we and Asakawa *et al.* have reported the studies of β-actin promoter ghrelin transgenic mouse (44, 45), in which plasma desacyl ghrelin levels were 30 and 50 times higher than those of their nontransgenic littermates. These transgenic mice were reported to show small phenotype, although some discrepancy of interpretation regarding on etiology exists. Asakawa *et al.* reported that the triglyceride level of β-actin promoter ghrelin transgenic mouse was lower, but that cholesterol level and free fatty acid level were not changed compared with their nontransgenic littermates. The triglyceride levels of our RIP-G Tg only showed lower tendency compared with that of nontransgenic littermates. The lack of small phenotype and milder phenotype of lipid metabolism in RIP-G Tg may result from the fact that plasma desacyl ghrelin level of RIP-G Tg was only 3.4 times higher than those of nontransgenic littermates.

The tissue sections of the pancreas of these transgenic mice showed no apparent disarrangement in the islet architecture and in β cell mass. There have been several reports on the transgenic mice overexpressing humoral factors in the β cells, such as parathyroid hormone-related peptide, hepatocyte growth factor, and insulin-like growth factor-I (46–49). Some of these transgenic mice showed islet hypertrophy or disarrangement of the endocrine cells in the islet (46–49). Our observation showed that desacyl ghrelin might have no apparent effects on the islet architecture and β cell mass.

In the present study plasma insulin levels after the 3.0 g/kg glucose injection were significantly lower in RIP-G Tg than those in nontransgenic littermates, although there was no significant difference in plasma insulin levels between RIP-G Tg and nontransgenic littermates on the fasting state. To rule out the decreased production of insulin caused by exogenous insulin promoter, we measured insulin mRNA level and content in the pancreata of our transgenic mice. The insulin mRNA level and content from the transgenic mice were not significantly different from those from nontransgenic littermates. Therefore, the insulin production might not be disturbed in these mice either in transcriptional or translational levels. The immunoreactivity of PDX-1, which is the master regulator of the pancreas development and essential for insulin transcription, in RIP-G Tg β cell was not different from that in β cells of nontransgenic littermates. These results suggest that the suppression of glucose-stimulated insulin secretion in RIP-G Tg might not be due to the transcriptional dysregulation of insulin caused by injection of exogenous insulin promoter.

RIP-G Tg did not show decreased-insulin secretion in response to arginine. Arginine is known to stimulate insulin secretion by the mechanisms that are different from those used by glucose, although the detail remains controversial (50, 51). However, it seems certain that arginine somehow evoked Ca^{2+} influx into the β cell, and that leads to the exocytosis of insulin-containing vesicles (52, 53). So at least, the decreased insulin secretion in RIP-G Tg might not be due to disorders in exocytosis process. Egido (28) reported that ghrelin inhibits insulin secretion from rat pancreas in response to arginine *in vitro*, however, there has been no report on the effect of desacyl ghrelin on arginine-induced insulin secretion.

The immunoreactivity of GLUT2, glucose transporter in the pancreatic β cell, in RIP-G Tg β cells, was indistinguishable from that in the β cells of nontransgenic littermates. Although immunohistochemistry is not so suitable for quantitative analysis, at least no apparent decreased expression or disposition of GLUT2 in RIP-G Tg β cell exists. Chronic exposure to the high level of desacyl ghrelin may not influence on GLUT2 expression.

We performed a batch incubation study of RIP-G Tg islet. The insulin secretion from isolated islets of RIP-G Tg was indistinguishable from that of nontransgenic littermates. This finding indicates that insulin secretion was not affected by overexpression of ghrelin transgene *in vitro* but was affected *in vivo*. The different observations *in vitro* and *in vivo* may be explained by dilution of ghrelin produced by transgene with the incubation buffer. Alternatively, suppression of insulin secretion of RIP-G Tg was not due to the effect of desacyl ghrelin on

insulin secretion from β cell but on insulin sensitivity. Recently Gauna *et al.* (55) reported that co-administration of desacyl ghrelin and active ghrelin improves insulin sensitivity in humans (54) and that desacyl ghrelin suppress glucose output from liver. Although an insulin tolerance test did not show a statistically significant difference in blood glucose levels between RIP-G Tg and their nontransgenic littermates, there was a tendency for lower blood glucose levels of RIP-G Tg. Moreover, plasma triglyceride levels of RIP-G Tg showed lower tendency. Taken together, these results may indicate that desacyl ghrelin may improve insulin sensitivity of RIP-G Tg. The suppression of insulin secretion of RIP-G Tg is likely due to the effect of desacyl ghrelin on insulin sensitivity.

To explore if chronic exposure to high level desacyl ghrelin may influence the expression level of GHS-R, we investigated the mRNA level of GHS-R in the pancreas and pituitary of RIP-G Tg. No significant differences were found in GHS-R mRNA levels in pancreas or in pituitary between RIP-G Tg and their nontransgenic littermates. These findings indicate that chronic exposure to high level desacyl ghrelin might not influence the GHS-R mRNA expression level.

We also developed RGP-G Tg. The pancreatic tissue ghrelin concentrations determined by C-RIA of RGP-G Tg were about 50 times higher than those of their nontransgenic littermates, indicating that ghrelin was overexpressed in RGP-G Tg. However, there was no obvious phenotype regarding insulin secretion and pancreatic morphology. Considering the observation that portal ghrelin levels were not elevated in RGP-G Tg compared with those in their nontransgenic littermates, the amount of secreted ghrelin from α cell may not outstrip the amount from stomach.

In summary we developed RIP-G Tg, in which pancreatic desacyl ghrelin content was ~1,000 times higher than that in control littermates. We detected *n*-octanoylated ghrelin-like immunoreactivity in pancreatic β cells by immunohistochemistry, indicating that the mechanism of acylation may exist not only in pancreatic α cells but also in β cells. The glucose-stimulated insulin secretion of RIP-G Tg was decreased. There were no abnormalities with the arginine-induced insulin secretion, pancreatic histology, pancreatic insulin mRNA levels, and insulin content in the RIP-G Tg. The absence of insulin suppression in the islet batch incubation study, lower tendency of blood glucose levels in insulin tolerance test, and lower tendency of plasma triglyceride level may indicate that the suppression of insulin secretion of RIP-G Tg is likely due to the effect of desacyl ghrelin on insulin sensitivity. Although we also developed RGP-G Tg with a 50-fold increase of pancreatic desacyl ghrelin content, we did not find obvious phenotype regarding insulin secretion and pancreatic morphology. The present study raises the possibility that desacyl ghrelin may have an influence on glucose metabolism.

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CLINICAL STUDY

An individualized GH dose regimen for long-term GH treatment in Japanese patients with adult GH deficiency

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Abstract

Objectives: To investigate the effects of growth hormone (GH) treatment, using a dose-adjustment regimen based on serum insulin-like growth factor (IGF)-I concentrations, in adult Japanese hypopituitary patients with GH deficiency.

Study design: Japanese patients who had initially been administered GH ($n = 31$) or placebo ($n = 28$) in a 24-week double-blind study received individualized GH treatment in an open-label study for 48 weeks. Body composition from dual-energy X-ray absorptiometry (DXA) and serum IGF-I, IGF-binding protein 3 (IGFBP-3) and lipid levels were determined centrally.

Results: Significant increases in lean body mass (4.5%) and decreases in fat mass (−10.5%) were observed in the group that received individualized GH doses in the present open-label study following placebo in the double-blind study. This was comparable with the changes observed in these parameters (4.7 and −9.2%, respectively) with fixed-dose GH treatment in the double-blind study; this latter group maintained these improvements throughout the open-label study. Individualized dose adjustment allowed for more moderate dose increases than the fixed-dose titration method. Individualized dosing also resulted in a lower mean dose for adult-onset compared with childhood-onset GH-deficient patients (0.032±0.019 versus 0.061±0.023 mg/kg per week for patients treated with GH for 48 weeks in the open-label study following placebo in the double-blind study). Dosing patterns in the two groups were paralleled by the changes in IGF-I and IGFBP-3. The incidence of oedema and cases with high IGF-I level were less frequent under the IGF-I controlled regimen compared with those during the fixed-dose titration method.

Conclusion: Individualized GH administration based on IGF-I levels was safe and effective. This regimen demonstrated differences in dose requirements between adult- and childhood-onset patients. An individualized dose regimen is recommended in adult Japanese GH-deficient patients.

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Introduction

Growth hormone (GH) is not only essential for growth in childhood but is also necessary in adults for normal metabolic regulation. GH replacement for adult patients with hypopituitarism was introduced into clinical practice in Europe and the USA almost a decade ago. Treatment modalities, such as GH dose, as well as efficacy and safety results during treatment and follow-up, have become established with experience (1–3). In Japan, the adult GH-deficiency (GHD) indication has not yet been approved but the existence of adult GHD syndrome in Japanese subjects has been demonstrated. At present, long-term data on efficacy and safety of GH replacement in adult Japanese patients is lacking.

In a recent report, we demonstrated the short-term efficacy and safety of GH treatment in 64 Japanese adult GHD patients in a placebo-controlled, double-blind study (4). In that study, adult patients with adult-onset (AO) or childhood-onset (CO) GHD were administered GH for a period of 24 weeks by an escalating, fixed-dose method culminating with 0.084 mg/kg per week (12 μg/kg per day) administered subcutaneously to all GH-treated patients. The GH treatment

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effects on body composition and lipids were significant compared with placebo and were qualitatively and quantitatively comparable with those seen in Caucasians using the same GH dose.

In the present study, which was an extension of the initial study, GH was continuously administered to all patients for 48 weeks. The primary objectives of this report were to evaluate the efficacy and safety of a GH dose-adjustment method based on insulin-like growth factor (IGF)-I concentrations for individual patients. Efficacy was evaluated from changes for patients starting individualized GH treatment and against changes observed previously with a fixed GH dose (4).

Patients and methods

This was a 48-week open-label study of GH treatment in Japanese adult GHD patients who had previously taken part in a 24-week placebo-controlled, double-blind study in 25 Japanese study centres (4). All patients gave informed consent and the study was performed with appropriate ethical approval according to the Declaration of Helsinki. In the double-blind study, 64 Japanese patients, with AO (mean age±S.D., 50.8±9.7 years) or CO (age, 28.8±7.3 years) GHD, were enrolled. Details of entry criteria for patients enrolled in the study have been described previously (4). All patients had a peak GH value of less than 3 μg/l during a standard stimulation test (insulin, arginine or glucagon) and any patients with malignancy, diabetes or hypertension were excluded. Of these patients, 33 were treated with GH in an escalating fixed-dose regimen and 31 received placebo during the double-blind study. At the end of this 24-week study, 31 patients who completed the active treatment (GH/GH group) and 28 patients who completed the placebo treatment in the double blind-phase (PL/GH group), entered an open-label 48-week GH dose-adjustment phase (Fig. 1). A total of five subjects had discontinued prior to 24 weeks of treatment in the double-blind study.

Patients were administered recombinant human GH (Humatrope; Eli Lilly and Company, Indianapolis, IN, USA) using cartridge pens. At the beginning of the open-label study, all patients, including those who had been given a fixed GH dose during the double-blind study, received a GH dose of 0.021 mg/kg per week for 8 weeks. Thereafter, the dose level was adjusted for each individual to between 0.021 and 0.084 mg/kg per week according to the serum IGF-I level measured during the previous visit, taking into account any side effects of GH treatment. This dose adjustment, tailored to the features of individual patients, was based on the Growth Hormone Research Society Consensus Guidelines (1) where the serum IGF-I level was maintained at between −1.96 S.D. and +1.96 S.D. (the normal range of IGF-I level by gender and age (5)).

During the open-label study, patient visits were scheduled every 4 weeks for the first 24 weeks followed by two 12-week intervals at 36 and 48 weeks. Measurements of IGF-I for dose adjustment, IGF-binding protein 3 (IGFBP-3) and other laboratory determinations were performed at the scheduled visits. At weeks 0, 24 and 48 of the open-label study, lean body mass (LBM) and fat mass were measured by

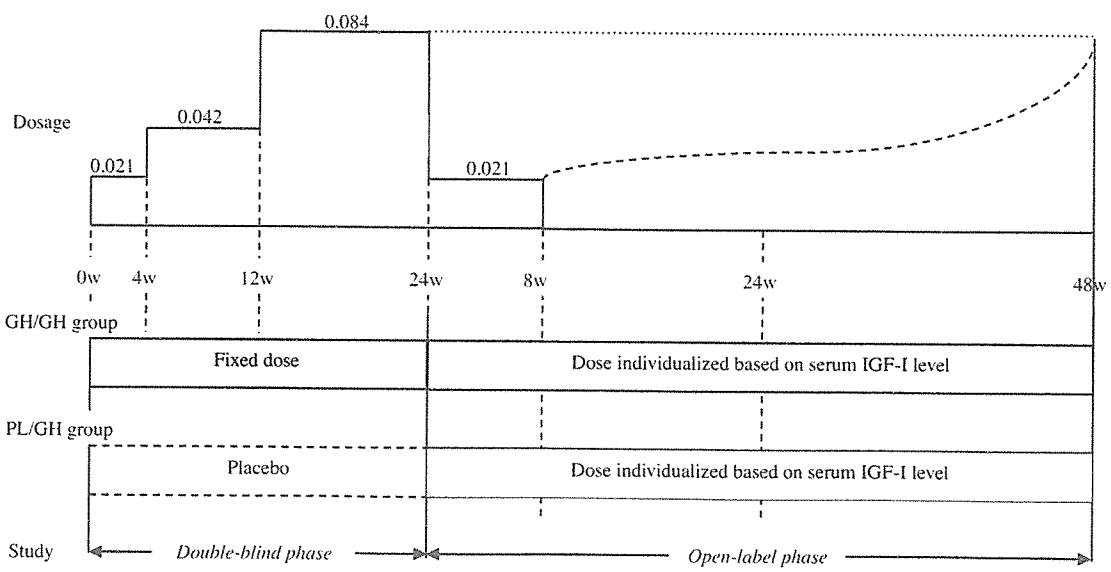

Figure 1 Dosing (mg/kg per week) and nomenclature for double-blind and open-label studies of GH administration to Japanese adult GHD patients.

Table 1 Baseline characteristics of Japanese adult GHD patients at the start of the open-label, individualized dose-adjustment study.

	PL/GH ($n = 28$)	GH/GH ($n = 31$)
Age (mean±S.D.; years)	39.6±13.7	36.2±13.3
Gender (male/female)	14/14	15/16
Onset (AO/CO)	11/17	12/19
Body weight (mean±S.D.; kg)	60.6±13.8	66.4±16.0
Body mass index (mean±S.D.; kg/m^2)	22.9±3.6	24.8±4.8
LBM (mean±S.D.; kg)	38.6±9.7	42.2±9.6
Fat mass (mean±S.D.; kg)	19.0±5.1	20.8±8.6
IGF-I (mean±S.D.; ng/ml)	61±39	243±114
IGF-I SDS (mean±S.D.)	−2.33±1.42	0.82±2.28
IGFBP-3 (mean±S.D.; μg/ml)	1.9±1.0	3.3±1.0
IGFBP-3 SDS (mean±S.D.)	−3.90±3.61	−0.17±2.42

dual-energy X-ray absorptiometry (DXA) in each investigative centre and evaluated centrally (Department of Radiology, Kawasaki Medical School, Kurashiki City, Okayama, Japan). Serum IGF-I and IGFBP-3, triglycerides, total cholesterol and low-density lipoprotein (LDL)-cholesterol concentrations were measured centrally (BML Inc., Shibuya-ku, Tokyo, Japan) using standard immunoradiometric assays for IGF-I and IGFBP-3 and enzymatic methods for cholesterol concentrations.

Safety was assessed by treatment-emergent adverse events (coded according to MedDRA, version 5.1) and by evaluation of laboratory test values and blood pressure. Laboratory determinations were performed centrally and included measurements of thyroid hormones and glycosylated haemoglobin (HbA1c) concentrations.

SD scores (SDSs) for serum IGF-I and IGFBP-3 concentrations were calculated by comparison to age- and gender-matched subjects from a healthy Japanese reference population (5). Cholesterol concentrations were originally determined in mg/dl with the assay kit; the conversion factor for mM is 0.02586 and in-text values have been converted and are shown in parentheses for reference. All results were analysed on an intent-to-treat basis and assessed at a two-tailed significance level of 5%. For all parameters, the baseline used to assess changes during the open-label study was the value observed at the end of the double-blind study. Changes in the open-label study were evaluated using last observation carried forward for all patients who started the open-label study, except for GH dose, IGF-I and IGFBP-3 values when only observed values were assessed. Differences between baseline and the end of study for body composition and lipid parameters were assessed by paired *t*-tests or Wilcoxon signed rank tests. All statistical analyses used SAS version 8.2 (SAS Institute, Cary, NC, USA).

Results

Baseline data

The baseline characteristics of the patients at the start of the open-label study are given in Table 1. The mean ages and numbers of male and female and AO and CO patients were similar in the GH/GH and PL/GH groups. The baseline characteristics of the PL/GH group at the beginning of the open-label study were similar to those reported for the start of the double-blind study (4). IGF-I and IGFBP-3 concentrations were below normal in the PL/GH group; in contrast, the IGF-I and IGFBP-3 concentrations and SDS values were improved in the GH/GH group at baseline of the open-label study following treatment in the double-blind study.

GH dose adjustment and IGF-I response

The time course of dose adjustments is given in Table 2 by GHD onset group. The starting dose was 0.021 mg/kg per week (3 μg/kg per day) and constant in both treatment groups for the first 8 weeks. In the subsequent dose-adjustment period the mean doses increased until week 24 and then stabilized through to week 48. Similar mean doses were being administered to the PL/GH (0.049±0.026 mg/kg per week) and the GH/GH (0.05±0.024 mg/kg per week) groups overall at the end of the open-label study. The mean doses in both groups were lower at the end of the open-label study than that being administered to the GH/GH group at the end of the escalating fixed-dose, double-blind study (0.078±0.015 mg/kg per week). The final dose adjusted according to IGF-I levels was lower in patients with AO

Table 2 Time course of GH dose in adult Japanese patients with AO or CO GHD in the individualized-dose (open-label) study (mean±S.D. (*n*)).

	Dose (mg/kg per week)			
	PL/GH		GH/GH	
Week in study	AO	CO	AO	CO
Week 8	0.021±0.001 (11)	0.021±0.001 (17)	0.021±0.001 (12)	0.021±0.001 (19)
Week 16	0.029±0.019 (11)	0.045±0.018 (17)	0.028±0.012 (12)	0.046±0.018 (18)
Week 24	0.034±0.015 (10)	0.058±0.022 (17)	0.041±0.025 (11)	0.059±0.021 (18)
Week 36	0.034±0.02 (11)	0.059±0.023 (17)	0.036±0.025 (12)	0.058±0.02 (18)
Week 48	0.032±0.019 (11)	0.061±0.023 (16)	0.035±0.025 (12)	0.059±0.019 (18)

compared with CO GHD in both the PL/GH (0.032±0.019 versus 0.061±0.023 mg/kg per week) and GH/GH groups (0.035±0.025 versus 0.059±0.019 mg/kg per week). The maximum and minimum doses for AO and CO patients at the end of the open-label study, in both the PL/GH (min–max, AO, 0.02–0.084 mg/kg per week; CO, 0.021–0.084 mg/kg per week) and GH/GH (AO, 0.01–0.085 mg/kg per week; CO, 0.032–0.085 mg/kg per week) groups in this IGF-I-controlled regimen, illustrate the wide range necessary for individual optimal doses.

The dosing patterns in the two groups were paralleled by the changes in IGF-I SDS and mean values within the normal range were achieved by both the PL/GH (0.38±1.67) and GH/GH (0.51±1.25) groups (Fig. 2, top panel). Overall, the mean IGF-I SDS values in the GH/GH group were similar to those observed at the endpoint of the escalating fixed-dose, double-blind study. In both treatment groups, IGF-I SDS at the end of the open-label study was consistently higher for AO than for CO patients (AO PL/GH, 1.51±1.11; CO PL/GH, −0.39±1.56; AO GH/GH, 0.86±1.28; CO GH/GH, 0.28±1.21). The introduction of the individualized dose-adjustment regimen eliminated excessively high values of IGF-I SDS seen in the GH/GH group in the double-blind study. The GH/GH group had IGF-I SDS above normal in six subjects (maximum 6.81) after the double-blind study and this declined to three subjects at the end of the open-label study (maximum, 2.82). IGF-I SDS was above normal in three subjects in the PL/GH group (maximum, 4.23) after 48 weeks of GH treatment. The IGFBP-3 SDS values during the open-label study (Fig. 2, bottom panel) reflected the changes in IGF-I and the mean values were within the normal range throughout GH treatment.

Efficacy

The overall increases in LBM and decreases in fat mass during the fixed-dose regimen and individualized-dose regimen are summarized in Table 3. There was no significant change in mean LBM or fat mass when the PL/GH group was treated with placebo in the double-blind study. In this group, the mean percentage increases in LBM and mean percentage decreases in fat

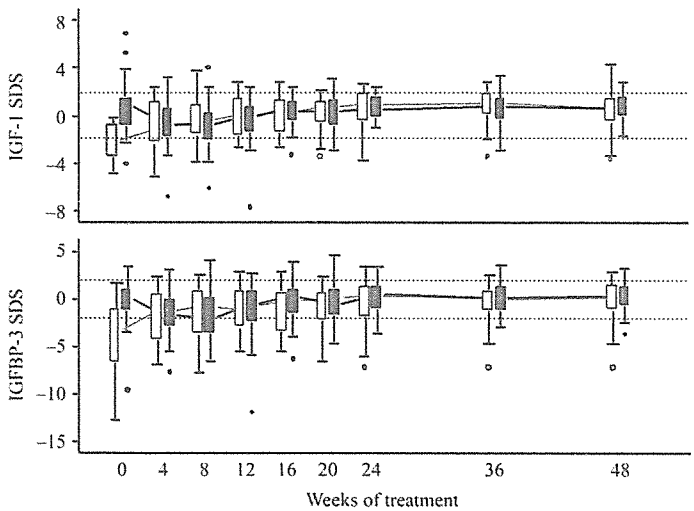

Figure 2 Time course of mean IGF-I SDS (top panel) and IGFBP-3 SDS (bottom panel) during the open-label study with 48 weeks of GH treatment of adult GHD Japanese patients; patients had previously been treated with placebo (open boxes) or GH (shaded boxes) and were then treated with individualized GH dose from week 0. Data show the 25th and 75th percentiles as the top and bottom edges of each box and the vertical lines extend from the box as far as the data extend, to a distance of at most 1.5 interquartile ranges, with values more extreme than this marked as circles; dotted horizontal lines show the normal range of 1.96 to −1.96.

Table 3 Percentage changes from baseline in LBM and fat mass during fixed-dose and individualized regimens of GH treatment in adult Japanese patients with GHD (mean±S.D. (*n*)).

			Percentage change from baseline			
Parameter	**Dose regimen (study)**	**Weeks**	PL/GH	*P* value	GH/GH	*P* value
LBM	Fixed (double-blind)	Up to week 24	−0.5±4.1 (29)	0.519*	4.7±3.9 (32)	<0.001*
	Individualized (open-label)	Up to week 24	3.4±4.8 (28)	<0.001†	0.1±4.7 (31)	–
		Up to week 48	4.5±5.3 (28)	<0.001†	1.2±4.9 (31)	–
Fat mass	Fixed (double-blind)	Up to week 24	1.1±6.9 (29)	0.388*	−9.2±11.8(32)	<0.001*
	Individualized (open-label)	Up to week 24	−8.8±9.2 (28)	<0.001†	2.4±8.8 (31)	–
		Up to week 48	−10.5±11.6 (28)	<0.001†	0.3±9.7(31)	–

* Student's *t*-test; within-group changes from baseline of double-blind study.
† Student's *t*-test; within-group changes from baseline of open-label study.

mass were statistically significantly different after 48 weeks of GH treatment in the open-label study compared with the corresponding changes in the preceding double-blind study ($P = 0.001$ for LBM and $P < 0.001$ for fat mass). Further, the changes from baseline in LBM and fat mass for the PL/GH group were significant in both AO and CO patients (Table 4) at the end of the open-label study. The changes from baseline during the individualized-dose, open-label study in the PL/GH group were similar to those achieved in the GH/GH group at the end of the 24-week, fixed-dose, double-blind study. Mean body weight and body mass index were not changed in the course of GH treatment in these studies.

The reductions in serum total cholesterol levels were significant after 24 weeks in the open-label study but not after 48 weeks in the PL/GH group (Table 5). However, the decreases observed in mean total cholesterol concentrations largely reflected those patients with abnormally high cholesterol levels at baseline. In these patients ($n = 12$) with total cholesterol over 220 mg/dl (equivalent to 5.69 mM), the change after 24 weeks of treatment was significant (baseline, 251±24 mg/dl (6.49±0.62 mM), 24 weeks, 219±31 mg/dl (5.66±0.8 mM); $P = 0.025$) but the change after 48 weeks of treatment was less evident, without statistical significance (226±29 mg/dl (5.84±0.75 mM); $P = 0.062$), in the PL/GH group. Decreases in serum LDL-cholesterol levels, after 48 weeks of treatment, were significant for the PL/GH group as a whole (Table 5). Again, where patients had abnormally high baseline levels (>140 mg/dl (3.62 mM)) for LDL-cholesterol, there were similar reductions at the end of the open-label study (results not shown).

Safety

The treatment-emergent adverse events observed in more than 10% of patients in either group in the double-blind and open-label studies are given in Table 6. Oedema was less frequent in both treatment groups in the individualized-dose, open study than in the GH/GH group during the fixed-dose, double-blind study treatment period. The incidence of oedema declined further in both groups between weeks 24 and 48 of the open-label study. There was an overall tendency that AO patients who received GH ($n = 25$) experienced more adverse events than CO patients who received GH ($n = 36$), such as oedema (16.0% for AO versus 5.6% for CO) and arthralgia (36.0% for AO versus 25.0% for CO). The number of serious adverse events was small (two cases in the GH/GH group and one case in the PL/GH group) in the open-label study and none occurred between weeks 24 and 48. The three serious adverse events were moderate depression (PL/GH group), severe vertigo and severe craniopharyngioma recurrence (GH/GH group). Of these events, depression and vertigo were assessed as having no causal relationship with GH. The serious adverse event of craniopharyngioma resulted in the patient being withdrawn from treat-

Table 4 Percentage changes during individualized-dose regimen of GH treatment in LBM and fat mass by onset in adult Japanese patients with GHD, in the open-label study (mean±S.D.).

			Percentage change			
			PL/GH			
Parameter	**Dose regimen (study)**	**Weeks**	AO ($n = 11$)	*P* value*	CO ($n = 17$)	*P* value
LBM	Individualized (open-label)	Up to week 24	4.7±2.7	<0.001	2.6±5.7	0.074
		Up to week 48	6.1±3.6	<0.001	3.4±5.9	0.030
Fat mass	Individualized (open-label)	Up to week 24	−11.2±11.7	0.010	−7.3±7.2	<0.001
		Up to week 48	−12.1±12.7	0.010	−9.6±11.1	0.003

* Student's *t*-test; within-group changes.

Table 5 Total and LDL-cholesterol values during the open-label, individualized-dose study of GH treatment in Japanese patients with adult GHD (mean±S.D. (*n*)).

Parameter	**Dose regimen** (study)	**Weeks**	**PL/GH** ($n = 28$)	***P* value***
Total cholesterol (mg/dl)	Individualized (open-label)	0	210±42	–
		24	193±38	0.011
		48	199±38	0.103
LDL-cholesterol (mg/dl)	Individualized (open-label)	0	127±34	–
		24	114±36	0.013
		48	116±38	0.032

* Compared with week 0; Wilcoxon signed-rank test.

Table 6 Incidence of adverse events with more than 10% frequency by MedDRA system organ class in adult Japanese GHD patients treated with placebo for 24 weeks followed by GH for 48 weeks (PL/GH) or GH throughout (GH/GH) in the fixed-dose, double-blind and individualized dose, open-label studies.

	Incidence of adverse events, *n* (% of *N*)					
	Fixed-dose study		Individualized dose study			
	24 weeks		0–24 weeks		24–48 weeks	
System organ class/adverse event	PL/GH (*n* = 31)	GH/GH (*n* = 33)	PL/GH (*n* = 28)	GH/GH (*n* = 31)	PL/GH (*n* = 28)	GH/GH (*n* = 30)
Respiratory, thoracic and mediastinal disorders	22 (71.0)	17 (51.5)	15 (53.6)	19 (61.3)	15 (53.6)	17 (56.7)
Nasopharyngitis	17 (54.8)	11 (33.3)	7 (25.0)	13 (41.9)	7 (25.0)	14 (46.7)
Cough	6 (19.4)	7 (21.2)	9 (32.1)	4 (12.9)	3 (10.7)	2 (6.7)
Rhinorrhoea	6 (19.4)	7 (21.2)	5 (17.9)	8 (25.8)	2 (7.1)	4 (13.3)
Pharyngolaryngeal pain	5 (16.1)	6 (18.2)	3 (10.7)	5 (16.1)	2 (7.1)	2 (6.7)
Upper respiratory tract inflammation	4 (12.9)	5 (15.2)	1 (3.6)	2 (6.5)	2 (7.1)	2 (6.7)
General disorders and administration site conditions	16 (51.6)	15 (45.5)	11 (39.3)	13 (41.9)	12 (42.9)	10 (33.3)
Pyrexia	12 (38.7)	7 (21.2)	4 (14.3)	5 (16.1)	7 (25.0)	7 (23.3)
Oedema	2 (6.5)	4 (12.1)	2 (7.1)	0	1 (3.6)	0
Fatigue	3 (9.7)	1 (3.0)	3 (10.7)	1 (3.2)	0	0
Musculoskeletal and connective tissue disorders	4 (12.9)	13 (39.4)	10 (35.7)	7 (22.6)	4 (14.3)	10 (33.3)
Arthralgia	4 (12.9)	6 (18.2)	5 (17.9)	2 (6.5)	1 (3.6)	6 (20.0)
Back pain	0	2 (6.1)	4 (14.3)	0	2 (7.1)	4 (13.3)
Nervous system disorders	6 (19.4)	9 (27.3)	6 (21.4)	9 (29.0)	4 (14.3)	10 (33.3)
Headache	4 (12.9)	6 (18.2)	4 (14.3)	6 (19.4)	3 (10.7)	4 (13.3)
Dizziness	0	1 (3.0)	0	2 (6.5)	1 (3.6)	4 (13.3)
Skin and subcutaneous tissue disorders	10 (32.3)	9 (27.3)	3 (10.7)	5 (16.1)	6 (21.4)	5 (16.7)
Pruritus	4 (12.9)	1 (3.0)	2 (7.1)	2 (6.5)	1 (3.6)	0
Gastrointestinal disorders	12 (38.7)	8 (24.2)	6 (21.4)	7 (22.6)	6 (21.4)	6 (20.0)
Nausea	5 (16.1)	3 (9.1)	1 (3.6)	0	0	2 (6.7)
Diarrhoea (NOS)	5 (16.1)	2 (6.1)	2 (7.1)	1 (3.2)	2 (7.1)	1 (3.3)
Metabolism and nutrition disorders	5 (16.1)	4 (12.1)	0	3 (9.7)	0	1 (3.3)
Anorexia	5 (16.1)	1 (3.0)	0	2 (6.5)	0	1 (3.3)
Investigations	3 (9.7)	5 (15.2)	2 (7.1)	1 (3.2)	3 (10.7)	7 (23.3)
Sputum increased	1 (3.2)	1 (3.0)	2 (7.1)	0	2 (7.1)	3 (10.0)

NOS, not otherwise specified.

ment: the causal relationship with GH was considered to be unknown. The prescribed dose level was reduced in the GH/GH group in five patients and in the PL/GH group in three patients due to adverse events in the open-label study. Of these adverse events, three arthralgia cases (two patients in the GH/GH group), two oedema cases (two patients in the PL/GH group), one hypertension case (GH/GH group) and one case of musculoskeletal stiffness (GH/GH group) were possibly related to GH treatment.

Mean values for all standard clinical safety laboratory parameters were within the normal reference range at the end of the open-label study. No changes from baseline in laboratory parameters or systolic and diastolic blood pressures indicated any safety problems. Following GH treatment in the PL/GH group, there was an increase from 4.5±0.6 to 4.7±0.6% in mean HbA1c, but mean values were within the normal reference range at all visits. One patient in this group had an abnormally high HbA1c level (5.9%) at the end of treatment but the value at baseline (5.6%) was already close to the upper limit of normal. There were almost no changes from baseline to the end of the open-label study in mean thyroid-stimulating hormone (0.0±0.4 mU/l), free 3,3',5triiodothyronine (T_3; 0.3±0.9 ng/l) or free thyroxine (T_4; −0.0±0.7 ng/dl) levels in PL/GH group.

Discussion

The benefits of GH replacement for adult GHD patients are well established for Caucasian patients. Current monitoring indicates that potential safety issues have been identified and addressed in these populations (6). The genetic and environmental backgrounds of Japanese patients differ from their Caucasian counterparts although the incidence of obesity and its related complications are increasing (7, 8). A previous double-blind study indicated the benefits of GH treatment for adult Japanese GHD patients (4). In this open-label study a different approach, using an individualized dosing regimen based on IGF-I levels, was implemented to compare the effects on efficacy and safety. The open-label design of the present study may introduce more bias than a double-blind design and the results must be considered with caution. However, given the demonstrated efficacy in Caucasian patients, it was not