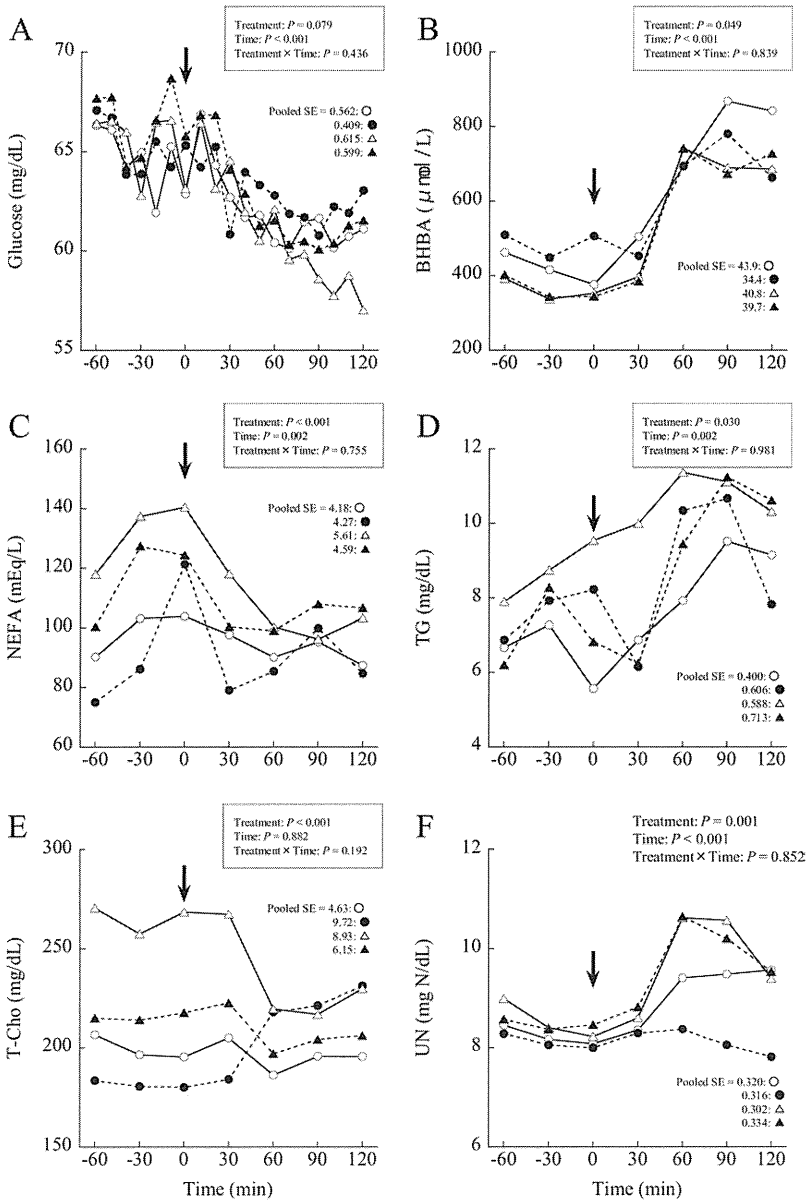

Fig. 2. Plasma concentrations of metabolite [glucose, A; β-hydroxy butyrate (BHBA), B; NEFA, C; triglyceride (TG), D; total cholesterol (T-Cho), E; and urea nitrogen (UN), F] in cows fed each treatment diet: no–calcium salts of long-chain fatty acids (CLFAs) +non–rumen-protected methionine (RPM; ○ with a solid line), non-CLFAs + RPM (● with a dotted line), CLFAs + non-RPM (Δ with a solid line), and CLFAs + RPM (▲ with a dotted line). Values are expressed as least squares means (n = 4). Probability values are for effects of treatment, time, and the interaction between treatment and time. The horizontal bar and arrow show the milking period and feeding time, respectively.

showed that dietary Met and glycine reduced serum T-Cho and increased fecal sterol excretion in rats. Furthermore, Met, as a sulfur amino acid, suppressed serum concentrations of NEFA and TG through the control of hormone-sensitive lipase activity and the restoration of lipoprotein lipase activity in peripheral tissues of hepatoma-bearing rats [4]. In this study, therefore, suppression of plasma T-Cho, NEFA, and TG with CLFAs plus Met might be induced by enhanced uptake and use of lipids by various tissues.

The increase in plasma ghrelin concentration of cows fed CLFAs is consistent with that of rats that ingested some fatty acids [35]. In addition, RPM decreased plasma ghrelin concentration in cows not fed CLFAs, whereas CLFAs plus RPM compared with CLFAs alone tended to increase plasma ghrelin con-

Table 3
Plasma hormone and metabolite concentrations during feeding period in lactating cows.

	Treatment							
	Non-CLFAs		CLFAs			Contrast P^a		
Item	Non-RPM	RPM	Non-RPM	RPM	SEM	CLFAs	RPM	CLFAs × RPM
Hormones								
Ghrelin (ng/mL)	0.106	0.087	0.116	0.126	0.027	< 0.001	0.301	0.002
GLP-1 (ng/mL)	0.519	0.464	0.452	0.448	0.128	0.061	0.185	0.258
Glucagon (pg/mL)	99.1	93.6	60.3	85.5	46.2	0.002	0.187	0.041
Insulin (ng/mL)	2.51	2.86	2.36	2.41	0.128	0.012	0.092	0.202
Metabolites								
Glucose (mg/dL)	62.9	63.4	62.1	63.6	1.91	0.872	0.001	0.344
BHBA (μmol/L)	595	579	514	517	43.4	0.006	0.796	0.729
NEFA (μEq/L)	95.4	90.2	116	110	7.13	< 0.001	0.150	0.842
TG (mg/dL)	7.57	8.29	9.86	8.41	0.82	0.029	0.501	0.049
T-Cho (mg/dL)	197	200	247	211	14.9	< 0.001	0.009	0.003
UN (mgN/dL)	8.79	8.13	9.26	9.23	0.70	< 0.001	0.121	0.152

Abbreviations: BHBA, β-hydroxy butyrate; CLFAs, calcium salts of long-chain fatty acids; FCM, fat-corrected milk; MEI, metabolizable energy intake; RPM, rumen-protected methionine; TG, triglyceride; T-Cho, total cholesterol; UN, urea nitrogen.
Note: Data are shown by least-squares means and SEMs.
[a] *P* value for factorial contrasts: CLFAs, RPM, and the interaction between CLFAs and RPM.

centration. Thus, only a simultaneous inclusion of CLFAs and RPM may enhance ghrelin secretion via the changes in lipid metabolism. Ghrelin enhances food intake in both nonruminants [36] and ruminants [37,38]. As a result, we could not find the relation between plasma ghrelin concentration and feed intake in this study. Because reports on ghrelin responses to lipid metabolism in ruminants are limited, further research is desirable.

Many researchers have reported that high-fat supplementation increased GLP-1 concentration [20,39,40]. Litherland et al [40] demonstrated that abomasal infusion of fat increased plasma GLP-1 concentration, dependent on dosage. Compared with others, the decrease of plasma GLP-1 concentration with CLFAs in this study could be attributed to a lesser fat ingestion level of 280 g/d. Glucagon-like peptide-1 plays a role in food intake [41], and Relling and Reynolds [20] have reported that dietary fats increased GLP-1 in dairy cows. In addition, Relling et al [42] observed that intrajugular infusion of GLP-1 tended to decrease DMI in growing wethers. In this study however, plasma GLP-1 concentration was decreased by CLFAs, suggesting that GLP-1 did not mediate DMI.

Plasma insulin concentration decreased in cows fed CLFAs with decreasing DMI, consistent with previous reports [23,43]. We consider that insulin depression might be due to lesser production of volatile fatty acids production caused by decreased DMI. Although we did not determine plasma concentrations volatile fatty acids, CLFAs decreased plasma BHBA concentration, derived from butyrate by rumen fermentation. In addition, we also observed that plasma GLP-1 concentration tended to decrease at the same time, suggesting decreased GLP-1, which stimulates insulin secretion, may be related to insulin depression in cows fed CLFAs.

Calcium salts of long-chain fatty acids also decreased plasma glucagon concentration. Our result is consistent with that of Cummins and Sartin [43], but inconsistent with that of Khorasani and Kennelly [44]. In several species, elevated NEFA decreases plasma glucagon concentration [45,46]. However, the present results showed that the decrease of plasma glucagon with CLFAs was attenuated by RPM. In our previous study, glucagon secretion was more strongly enhanced when amino acids and ghrelin were simultaneously administered compared with the administration of ghrelin alone in lactating cows [11]. In addition, RPM tended to depress plasma NEFA concentration of cows fed CLFAs. Therefore, we consider that increased Met absorption, higher plasma ghrelin concentration, and a decreased plasma NEFA concentration might modulate plasma glucagon depression in CLFA-fed cows.

The present study did not show any favorable effects of RPM on feed intake and milk production in cows fed with CLFAs, although plasma ghrelin concentration increased. Further studies are desirable to elucidate the effects of lactating stage and Met supplemental level with CLFAs on DMI and milk production.

In conclusion, responses to CLFAs are associated with increases in plasma concentration of ghrelin and decreases of insulin, glucagon, and GLP-1. Rumen-

protected Met plus CLFAs modulated plasma lipid concentrations and concomitantly elevated plasma ghrelin concentration. These metabolic and endocrine changes were induced by absorbed long-chain fatty acid and Met and not by energy intake because metabolizable energy intake was not different among treatments.

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Transgenic overexpression of intraislet ghrelin does not affect insulin secretion or glucose metabolism in vivo

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Transgenic overexpression of intraislet ghrelin does not affect insulin secretion or glucose metabolism in vivo

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Bando M, Iwakura H, Ariyasu H, Hosoda H, Yamada G, Hosoda K, Adachi S, Nakao K, Kangawa K, Akamizu T. Transgenic overexpression of intraislet ghrelin does not affect insulin secretion or glucose metabolism in vivo. *Am J Physiol Endocrinol Metab* 302: E403–E408, 2012. First published November 22, 2011; doi:10.1152/ajpendo.00341.2011.—Whereas ghrelin is produced primarily in the stomach, a small amount of it is produced in pancreatic islets. Although exogenous administration of ghrelin suppresses insulin secretion in vitro or in vivo, the role of intraislet ghrelin in the regulation of insulin secretion in vivo remains unclear. To understand the physiological role of intraislet ghrelin in insulin secretion and glucose metabolism, we developed a transgenic (Tg) mouse model, rat insulin II promoter ghrelin-internal ribosomal entry site-ghrelin *O*-acyl transferase (RIP-GG) Tg mice, in which mouse ghrelin cDNA and ghrelin *O*-acyltransferase are overexpressed under the control of the rat insulin II promoter. Although pancreatic desacyl ghrelin levels were elevated in RIP-GG Tg mice, pancreatic ghrelin levels were not altered in animals on a standard diet. However, when Tg mice were fed a medium-chain triglyceride-rich diet (MCTD), pancreatic ghrelin levels were elevated to ~16 times that seen in control animals. It seems likely that the gastric ghrelin cells possess specific machinery to provide the octanoyl acid necessary for ghrelin acylation but that this machinery is absent from pancreatic β-cells. Despite the overexpression of ghrelin, plasma ghrelin levels in the portal veins of RIP-GG Tg mice were unchanged from control levels. Glucose tolerance, insulin secretion, and islet architecture in RIP-GG Tg mice were not significantly different even when the mice were fed a MCTD. These results indicate that intraislet ghrelin does not play a major role in the regulation of insulin secretion in vivo.

pancreas; ghrelin D-acyltransferase

GHRELIN IS A 28-AMINO ACID PEPTIDE HORMONE, with a unique modification of acylation at the third serine residue, first described by Kojima et al. (17) in 1999. The acyl modification of ghrelin is mediated by the recently discovered enzyme ghrelin *O*-acyl transferase (29), and the modification is essential for ghrelin binding to its cognate receptor (12). Ghrelin is produced primarily in the stomach, but small amounts of ghrelin are also produced in pancreatic islets (1, 5, 8, 10, 12, 26, 27). Controversy remains about which type of islet cell produces ghrelin (5, 20, 26, 27). Date et al. (5) reported that ghrelin is present in α-cells in humans and rats, whereas Volante et al. (26) reported that ghrelin is produced by β-cells in humans. In contrast, Wierup and colleagues (27, 28) and Prado et al. (20) reported that ghrelin-expressing cells comprise a new islet cell type distinct from α-, β-, and δ-cells and PP cells in human, rat, and mouse islets.

Exogenous ghrelin suppresses insulin secretion from pancreatic β-cells in vitro (4, 9, 22) or in vivo (3, 22, 25). Although several studies have demonstrated contradictory results (1, 5, 11, 18, 24), data from genetically engineered mice are consistent with this concept. Chronic elevation of plasma ghrelin levels suppresses insulin secretion, inducing glucose intolerance in transgenic mice (2, 13, 21), whereas ablation of ghrelin improves glucose tolerance by enhancing insulin secretion in diet-induced obesity (7) or *ob/ob* mouse models (23). Although in vitro studies demonstrate that intraislet ghrelin can suppress insulin secretion from isolated islets (6), the physiological role of intraislet ghrelin on the regulation of insulin secretion in vivo is unclear. Because only minimal amounts of ghrelin are produced by the pancreas compared with that made by the stomach (15), the effect of stomach-derived ghrelin may overpower the effects of intraislet ghrelin in vivo.

In this study, we developed a transgenic mouse model in which the ghrelin and ghrelin *O*-acyltransferase (GOAT) genes are overexpressed by pancreatic β-cells under the control of the rat insulin II promoter (RIP) to ascertain the physiological role of intraislet ghrelin on insulin secretion and glucose metabolism in vivo.

MATERIALS AND METHODS

Generation of RIP-ghrelin-GOAT transgenic mice. We designed a fusion gene comprised of RIP, mouse ghrelin cDNA, internal ribosomal entry site (IRES), and mouse GOAT cDNA coding sequences. The purified fragment (10 μg/ml) was microinjected into the pronuclei of fertilized C57/B6J mouse eggs (SLC, Shizuoka, Japan). Viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (SLC) by using standard techniques. Transgenic founder mice were identified by Southern blot analyses of tail DNA, using a mouse ghrelin cDNA fragment as a probe. For experimentation, we utilized heterozygous transgenic mice. Animals were maintained on a 12:12-h light-dark cycle and fed a standard diet (SD; CE-2, 352 kcal/100 g; Japan CLEA, Tokyo, Japan) or a MCTD containing 45% Dermol M5 (C8:60%, C10:40%; Research Diets, New Brunswick, NJ) as indicated. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

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Measurement of plasma and tissue ghrelin concentrations. Blood was drawn from the proximal end of the portal vein under ether anesthesia, transferred immediately to chilled siliconized glass tubes containing Na_2-EDTA (1 mg/ml) and aprotinin (1,000 KIU/ml), 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 AIA-600 II (Tosoh, Tokyo, Japan).

To measure tissue ghrelin concentrations, pancreata or stomachs were isolated from mice and then boiled for 5 min in the 10-fold vol/wt of water. Acetic acid was added to each solution to adjust the final concentration to 1 M before tissue homogenization. We determine the tissue ghrelin concentration in supernatants obtained after centrifugation by radioimmunoassay (RIA) using anti-ghrelin [13–28] (C-RIA) and anti-ghrelin [1–11] (N-RIA) antisera, as described previously (12, 15).

Real-time quantitative RT-PCR. Total RNA was extracted from pancreata using an RNeasy Protect mini kit (Qiagen, Hilden, Germany). Reverse transcription (RT) was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems), using the following primers and TaqMan probes: mouse ghrelin (sense, 5′-GCATGCTCGGATGGACATG-3′; antisense, 5′-TGGTGGCTTCTTGGATTCCT-3′; TaqMan probe, 5′-AGCCCAGAGCACCAGAAAGCCCA-3′); mouse insulin (sense, 5′-CAGCTATAATCAGAGACCATCAGCAA-3′; antisense, 5′-GGGTAGGAAGTGCACCAACAG-3′; TaqMan probe, 5′-CAGGTCATTGTTTCAAC-3′); GOAT (sense, 5′-AGGGACTCTAGGAAGGACAG-3′; antisense, 5′-CCCATCTGAAAGAAGAAGGT-3′, with Power SybrGreen). Data were normalized to the content of 18S rRNA in each sample.

Glucose tolerance tests. For glucose tolerance testing, the ad libitum-fed mice were injected intraperitoneally with 1.5 g/kg glucose. Blood was sampled from the tail veins before and 30, 60, 90, and 120 min after the injection. Blood glucose levels were determined by the glucose oxidase method using a Glutest sensor (Sanwa Kagaku, Kyoto, Japan).

Insulin release. Ad libitum-fed mice were injected with 3.0 g/kg glucose intravenously. Plasma was sampled from a retroorbital vein before and 2 or 30 min after injections into heparin-coated tubes. Insulin concentrations were measured by a high-range speedy mouse insulin kit (Morinaga, Yokohama, Japan).

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 (14). Serial sections of 5-μm thickness were incubated with anti-COOH-terminal ghrelin (1:1,000) (17) and anti-NH_2-terminal ghrelin (1:2,000) (17), anti-glucagon (1:500), anti-insulin (1:500), anti-somatostatin (1:500), and anti-pancreatic polypeptide (1:500; DAKO, Glostrup, Denmark) antisera.

Statistical analysis. All values are expressed as means ± SE. The statistical significance of the differences in mean values was assessed by ANOVA with a post hoc test (Tukey's test) or Student's *t*-test as appropriate. Differences with $P < 0.05$ were considered significant. Statistical analyses were performed using Statcel2 (OMS, Saitama, Japan).

RESULTS

Generation of RIP-ghrelin-IRES-GOAT transgenic mice. After the RIP-ghrelin-IRES-GOAT transgene was injected into 286 eggs, we obtained three lines (3–4, 9–3, and 11–5) confirmed to be rat insulin II promoter-ghrelin-IRES-GOAT transgenic (RIP-GG Tg) mice. For further analyses, we selected the 9–3 line, which had the highest expression of ghrelin and GOAT mRNA in the pancreas (data not shown). The expression levels of pancreatic ghrelin mRNA in the 9–3 line of RIP-GG Tg mice were ~20-fold higher than those seen in controls (Fig. 1*B*), whereas GOAT mRNA levels were ~80-fold higher than those in controls (Fig. 1*C*). There was also an increment in ghrelin and GOAT mRNA levels in the hypothalamus of RIP-GG Tg mice (non-Tg vs. Tg: ghrelin, 1.0 ± 0.28 vs. 25.6 ± 5.6; GOAT, 1.0 ± 0.26 vs. 5,735.5 ± 1,189.1, arbitrary unit; $n = 8$, $P < 0.01$).

Pancreatic and plasma ghrelin levels in RIP-GG Tg mice. Total ghrelin levels measured by C-RIA were significantly elevated in the pancreata of RIP-GG Tg mice on a SD or MCTD (Fig. 2*A*). However, the ghrelin levels measured by N-RIA were elevated only when RIP-GG Tg mice were fed a MCTD (Fig. 2*B*). Although ghrelin levels 16-fold higher than those seen in control littermates were observed in the pancreata of RIP-GG Tg mice fed a MCTD, these absolute levels were low compared with those isolated from stomach (Fig. 2, *D* and *E*). Furthermore, the ratio of ghrelin to total ghrelin in the pancreas of RIP-GG Tg mice was significantly low on SD, which was elevated on a MCTD (Fig. 2*C*). Still, the level was significantly lower compared with that of the stomach (Fig. 1*F*).

Immunohistochemistry showed that the ghrelin-like immunoreactivities were increased in the core of the islets of RIP-GG Tg mice on a MCTD (Fig. 3), indicating that increased tissue levels of pancreatic ghrelin were originated from β-cells.

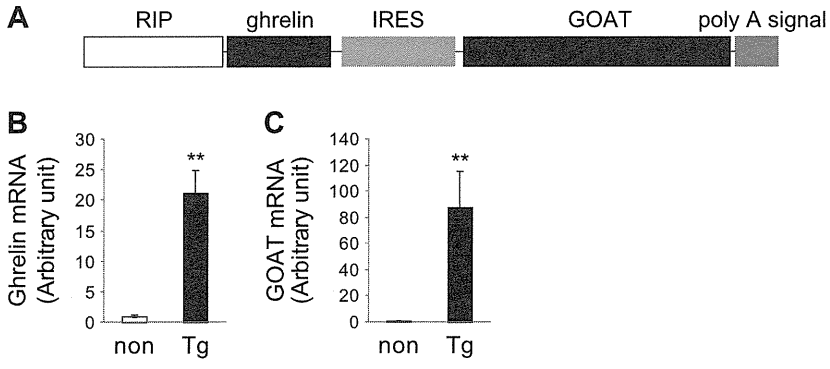

Fig. 1. Constructs of rat insulin II promoter (RIP)-ghrelin-internal ribosomal entry site (IRES)-ghrelin *O*-acyltransferase (GOAT) transgenic (RIP-GG Tg) mice and the expression levels of ghrelin and GOAT mRNA in the pancreas. *A*: we designed a fusion gene containing RIP, mouse ghrelin cDNA, IRES, and mouse GOAT cDNA. *B* and *C*: the resultant expression levels of ghrelin (*B*) and GOAT mRNA (*C*) in the pancreata of RIP-GG Tg mice. Non, nontransgenic littermate; Tg, RIP-GG Tg mice; $n = 7–11$, $^{**}P < 0.01$ compared with non.

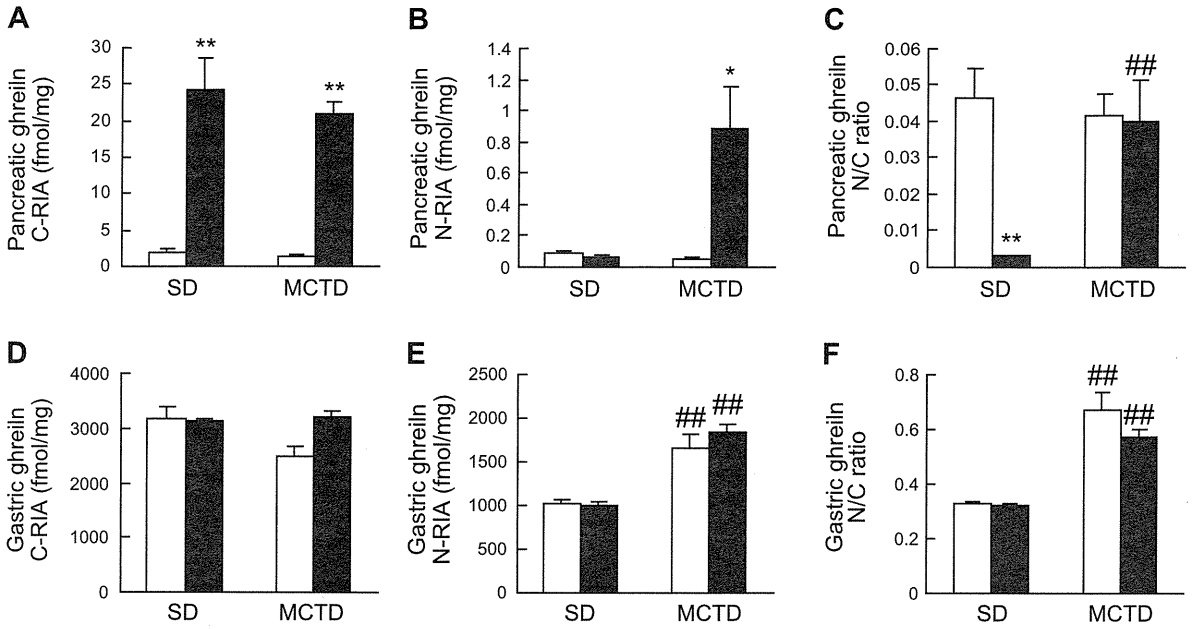

Fig. 2. Pancreatic and gastric ghrelin levels in RIP-GG Tg mice on standard (SD) or medium-chain triglyceride-rich diet (MCTD). *A* and *B*: pancreatic ghrelin levels in RIP-GG Tg mice (black bars) and nontransgenic controls (open bars) measured by using anti-ghrelin [13–28] (C-RIA; *A*) and anti-ghrelin [1–11] (N-RIA; *B*). Although total ghrelin levels measured by C-RIA were elevated in RIP-GG Tg mice on both SD and MCTD, ghrelin levels measured by N-RIA were elevated only when RIP-GG Tg mice were fed MCTD. *D* and *E*: gastric ghrelin levels of RIP-GG Tg mice (black bars) and nontransgenic controls (open bars) measured by C-RIA (*D*) or N-RIA (*E*) were significantly higher than pancreatic levels, regardless of diet. *C* and *F*: The ratio of N-RIA/C-RIA (N/C). $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with controls. $\#\#P < 0.01$ compared with SD; $n = 5$–7.

We measured plasma ghrelin levels in the portal veins of RIP-GG Tg mice fed a MCTD to determine whether this level of ghrelin overexpression in islets could affect plasma ghrelin levels. No significant changes were observed in either ghrelin or desacyl ghrelin levels in the portal veins of RIP-GG Tg mice (Fig. 4, *A* and *B*), indicating that ghrelin overexpression from the transgene in islets produces minimal effect on plasma ghrelin levels.

Glucose metabolism and insulin secretion in RIP-GG Tg mice. No significant changes in blood glucose levels were seen by intraperitoneal glucose tolerance tests between 10-wk-old RIP-GG Tg mice and controls on a MCTD (Fig. 5*A*). Plasma insulin levels before and after a glucose load were not altered significantly in 15-wk-old RIP-GG Tg mice on a MCTD (Fig. 5*B*). There were also no significant changes in blood glucose or plasma insulin levels after glucose load in old mice (≈84-wk old) or in female mice (Fig. 5, *C–F*).

Islet architecture. There were no obvious abnormalities in intraislet cytoarchitecture or in the cell numbers of insulin-, glucagon-, somatostatin-, and polypeptide-producing cells in the islets of RIP-GG Tg mice on MCTD (Fig. 6, *A–D*).

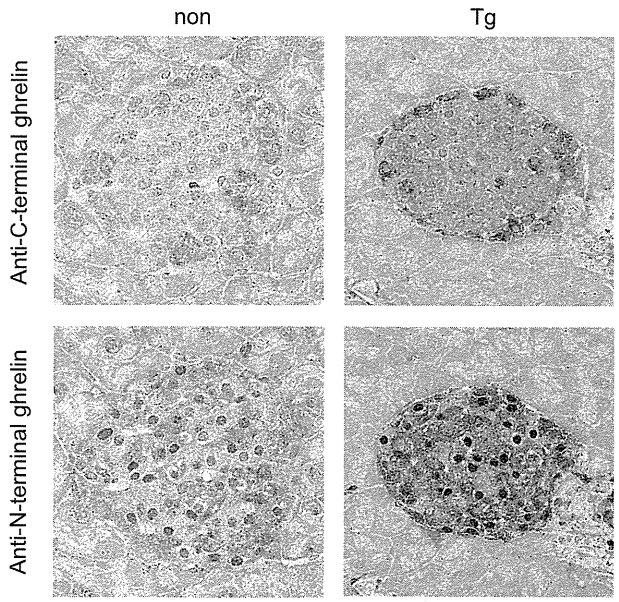

Fig. 3. Immunohistochemical analysis of the expression of ghrelin in the islets of RIP-GG Tg mice. Ghrelin-like immunoreactivities were increased in the core of the islets of RIP-GG Tg mice on MCTD.

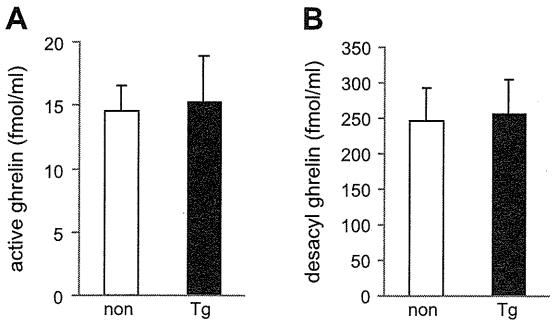

Fig. 4. Portal ghrelin levels of RIP-GG Tg mice. *A* and *B*: portal ghrelin (*A*) and desacyl ghrelin levels (*B*) in male Tg (black bars) and non (open bars) fed MCTD; $n = 7$–8.

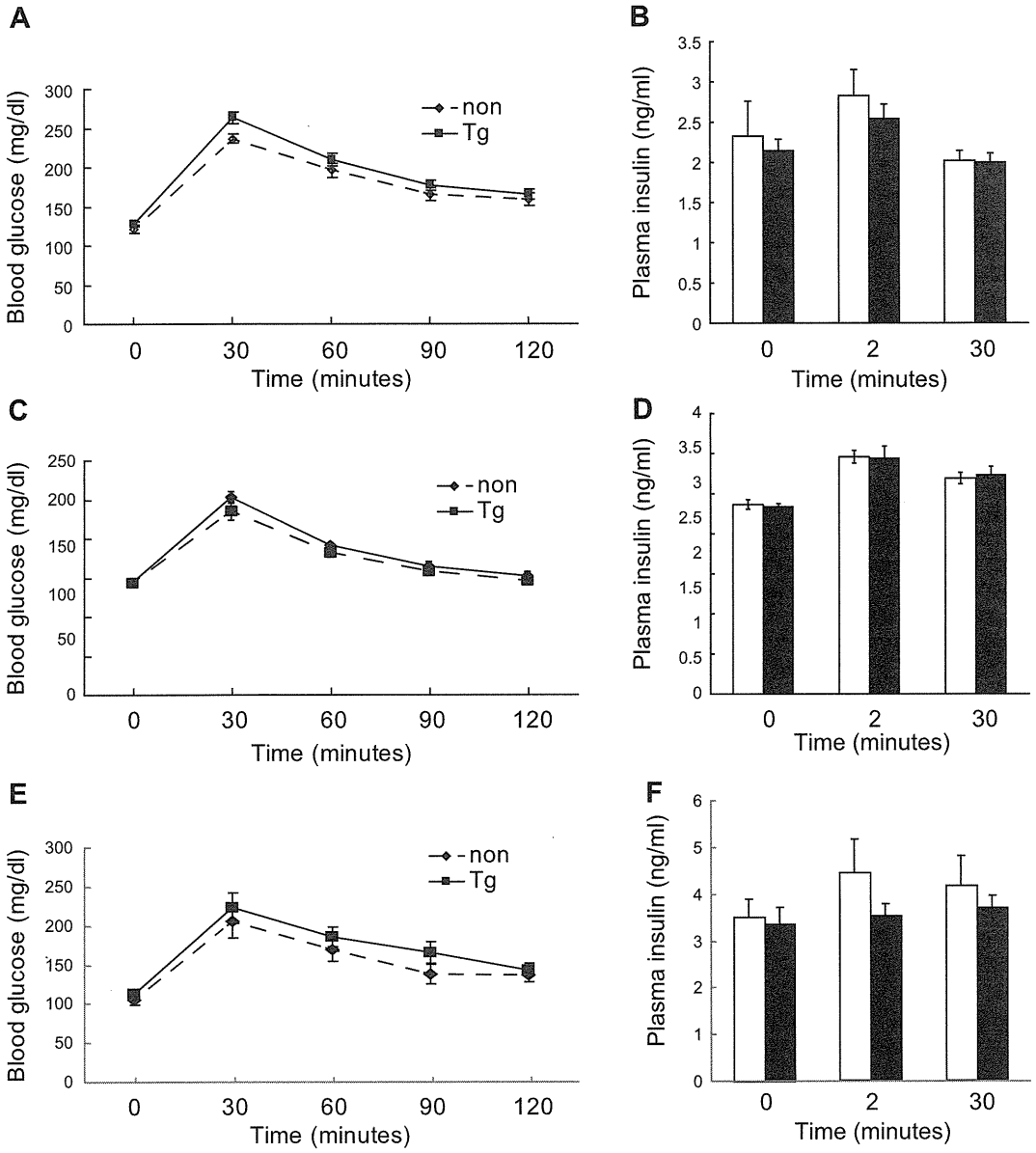

Fig. 5. Glucose metabolism in RIP-GG Tg mice. *A*, *C*, and *E*: glucose tolerance tests in 10-wk-old male (*A*), 11-wk-old female (*C*), or 83-wk-old male (*E*) Tg on MCTD (■) and non (♦); n = 7–10. *B*, *D*, and *F*: serum insulin levels at baseline and at 2 or 30 min after intravenous glucose injection in 15-wk-old male (*B*), 10-wk-old female (*D*), or 84-wk-old male (*F*) Tg fed MCTD (black bars) and in non (open bars); n = 5–10.

Staining intensities for these four islet hormones within islets of RIP-GG Tg mice did not differ from those of nontransgenic littermates.

DISCUSSION

In previous studies, we developed transgenic mice in which mouse ghrelin cDNA is overexpressed in pancreatic β-cells under the control of the rat insulin II promoter to identify the effect of ghrelin on pancreatic islets (15). However, these Tg mice displayed elevated expression of desacyl ghrelin only within the pancreas. At that time, the mechanism by which ghrelin received an *n*-octanoyl modification was unknown. Recently, Yang et al. (29) identified GOAT as the enzyme mediating this modification. In this study, we developed a transgenic mouse in which ghrelin produced in the pancreas might be both overexpressed and modified, with the overexpression of both mouse ghrelin and GOAT cDNA in pancreatic β-cells under the control of the rat insulin II promoter.

To our surprise, whereas pancreatic desacyl ghrelin levels were elevated in RIP-GG Tg mice, pancreatic levels of (active, modified) ghrelin were unchanged on a SD. Ghrelin levels were elevated only when mice were fed a MCTD. Similar results were reported by Kirchner et al. (16), who created a transgenic mouse in which ghrelin and GOAT cDNA were overexpressed in the liver under the control of

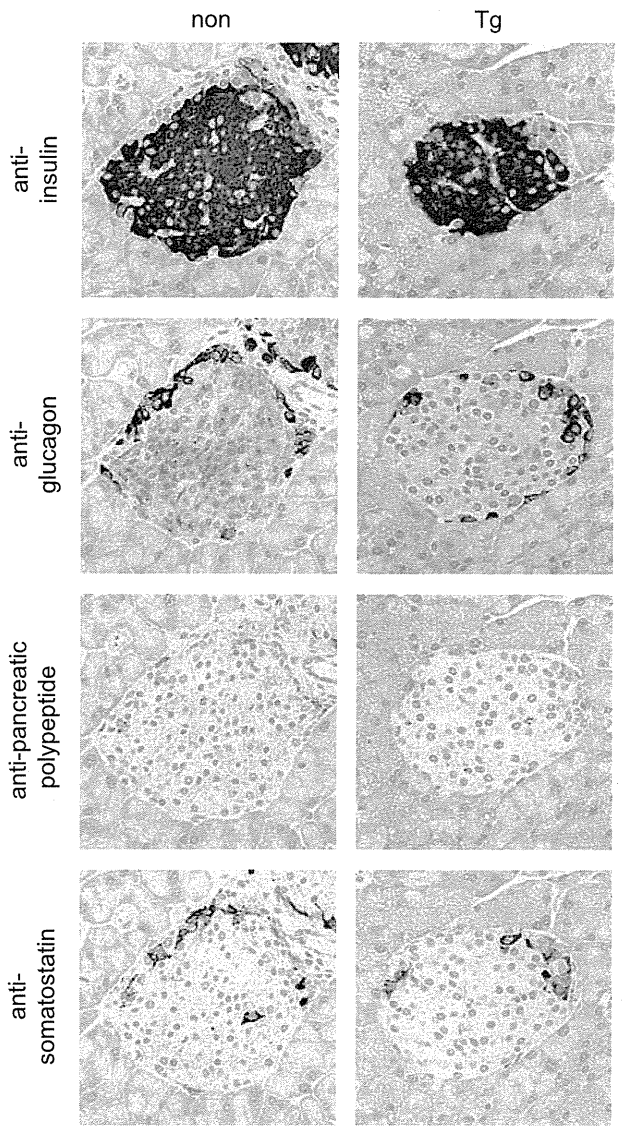

Fig. 6. Islet morphology in RIP-GG Tg mice. The pancreatic sections from RIP-GG Tg mice and non were stained with anti-insulin, anti-glucagon, anti-somatostatin, or anti-pancreatic polypeptide antibodies. Representative images are presented.

the apolipoprotein E promoter. These mice demonstrated elevated plasma ghrelin levels only when mice were fed a medium-chain fatty acid-rich diet. Considering that gastric ghrelin-producing cells can produce ghrelin regardless of diet, even in a fasting state, it is likely that these gastric cells possess a specific machinery to generate the octanoyl acid necessary for acylation, which is lacking from pancreatic β-cells or hepatocytes.

In previous studies, we demonstrated that the chronic elevation of plasma ghrelin levels at ~10-fold higher than the normal range suppresses insulin secretion and induces glucose intolerance in mice (13). In this study, RIP-GG Tg mice, which produce 16-fold higher ghrelin levels from the pancreas as normal mice, exhibited normal glucose tolerance and insulin secretion. The pancreatic ghrelin levels in RIP-GG Tg mice, although elevated, were still considerably lower than the gastric ghrelin level. We tried to compare the ghrelin levels in pancreatic vein with those in artery, as Dezaki et al. (7) did using rats, but it was difficult to determine the ghrelin levels in pancreatic veins of mice due to the small body size. We measured ghrelin levels in the portal vein instead, which were not elevated in RIP-GG Tg mice. We cannot determine the exact concentration of ghrelin in the microenvironment surrounding β-cells, but these levels still seem to be overpowered by the circulating ghrelin produced by the stomach. Although it is possible that additional overproducing of ghrelin in islets could eventually suppress insulin secretion, further enhancement of ghrelin expression by islets would not be in the realm of physiological relevance. In vitro, intraislet ghrelin may suppress insulin secretion in a paracrine (or autocrine) manner where the effect of circulating ghrelin is eliminated (6). However, this study indicates that intraislet ghrelin does not play a major role in controlling insulin secretion in vivo, where high levels of circulating ghrelin are generated by the stomach.

One drawback of this study is that elevated pancreatic ghrelin levels in RIP-GG Tg mice could not be obtained without feeding mice a MCTD. The MCTD consists of medium-chain fatty acids (C6–C10) that can enter mitochondria without the carnitine shuttle. Medium-chain triglycerides generally have favorable effects on obesity or diabetes (19), suppressing fat accumulation and improving insulin sensitivity. We cannot exclude the possibility that a MCTD may have interfered with the effects of ghrelin within islets. In addition, ghrelin and GOAT mRNA levels were increased not only in the islet but also in the hypothalamus of RIP-GG Tg mice. There is a possibility that the overexpressed ghrelin in the hypothalamus may have influenced the effects of overexpressed ghrelin in the islet.

In summary, we have developed RIP-GG Tg mice, in which intraislet ghrelin levels were elevated to ~16 times the control levels when mice were fed a MCTD. The glucose tolerance and insulin secretion of RIP-GG Tg mice were unchanged, indicating that intraislet ghrelin does not play a major role in regulating insulin secretion in vivo.

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DISCLOSURES

The authors have nothing to declare.

AUTHOR CONTRIBUTIONS

M.B., H.I., and H.H. performed the experiments; M.B. and H.I. analyzed the data; M.B., H.I., H.A., H.H., G.Y., K.H., S.A., K.N., K.K., and T.A. interpreted the results of the experiments; M.B. and H.I. prepared the figures; M.B., H.I., and T.A. drafted the manuscript; M.B., H.I., H.A., K.H., S.A., K.N., K.K., and T.A. edited and revised the manuscript; M.B., H.I., K.N., K.K., and T.A. approved the final version of the manuscript; H.I., K.H., K.N., K.K., and T.A. did the conception and design of the research.

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Review article

The physiological significance and potential clinical applications of ghrelin

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ABSTRACT

Ghrelin, a natural ligand for the growth hormone (GH)-secretagogue receptor (GHS-R), is now known to play a role in a number of different physiological processes. For example, ghrelin increases GH secretion, feeding, and body weight when administered centrally or peripherally. These unique effects of ghrelin should be invaluable for the development of novel treatments and disease diagnostic techniques. Clinical trials have already been performed to assess the utility of ghrelin for the treatment of several disorders including anorexia, cachexia, and GH-related disorders. This review summarizes the recent advances in this area of research.

1. Introduction

Ghrelin is a peptide hormone that was discovered in 1999 as an endogenous ligand for the growth hormone (GH)-secretagogue receptor (GHS-R) [1]. Ghrelin is a 28-amino-acid peptide and possesses a unique fatty acid modification, *n*-octanoylation, at Ser 3. There are two circulating forms of ghrelin, acylated and unacylated (desacyl), and the acylated form is essential for ghrelin's biological activity through GHS-R. Recently, however, desacyl ghrelin was reported to influence both cell proliferation and adipogenesis through another unknown receptor [2–5]. Ghrelin is produced primarily in the stomach and circulates in the blood at a considerable plasma concentration. Expression of ghrelin is also detectable in the hypothalamus, intestine, pituitary, placenta, and other tissues [1,6–8]. Ghrelin is now known to play a role in a number of different physiological processes; for example, ghrelin increases GH secretion and feeding, and decreases insulin secretion [1,9–19].

These unique effects of ghrelin and growth hormone secretagogues (GHS) should be invaluable for the development of novel treatments and disease diagnostic techniques [20–22]. Clinical trials have already been performed to assess the utility of ghrelin for the treatment of various disorders including anorexia [23–26], cachexia [27–29], malnutrition [30], GH-related disorders [31], and postgastrectomy/esophagectomy [32,33]. Because many excellent reviews concerning basic and clinical research on ghrelin have already been published, we will summarize and discuss recent clinical trials of ghrelin in this work.

2. Physiological actions of ghrelin

2.1. Orexigenic action

Ghrelin has a well-established role in stimulating appetite and increasing food intake [34,35]. Peripheral administration of ghrelin stimulates GH secretion and appetite in both animals and humans [10,18]; it is the only hormone known to have this effect. Ghrelin increases c-fos expression in the arcuate nucleus, and also activates hypothalamic neuropeptide Y (NPY)/Y1 receptors and agouti-related peptide (AgRP) pathways [36–38]. In addition, ghrelin induces food intake via the orexin pathway [39]. These functions are mediated at least in part by vagal nerve pathways [40]. Repeated administration of ghrelin resulted in significant weight gain in rats [41] and patients with chronic obstructive pulmonary disease (COPD) [28].

2.2. Stimulation of GH secretion

Ghrelin strongly stimulates GH secretion in humans [12,16,17,42], several-fold more potently than GHRH under similar conditions. Furthermore, ghrelin and growth hormone releasing hormone (GHRH) synergistically increase GH release [17]. Ghrelin might also play a role in GH release in a non-acute setting [43,44]. GH regulates IGF-I levels, promotes anabolism, and increases muscle strength [45,46].

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While GH enhances lipolysis, IGF-1 stimulates protein synthesis, myoblast differentiation, and muscle growth.

2.3. Anti-inflammatory action

Evidence that ghrelin exerts anti-inflammatory actions has been accumulating. Ghrelin suppresses the production of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α both *in vitro* [47,48] and *in vivo* [49–51]. In clinical trials, daily administration of ghrelin for 3 weeks decreased inflammatory cytokine levels and neutrophil density in sputum from patients with chronic respiratory infections [52]. In contrast, ghrelin induces the anti-inflammatory cytokine IL-10 [49,53].

Ghrelin inhibits the activation of NF-κB, a transcription factor known to control the production of multiple pro-inflammatory cytokines during inflammatory insults [48,50,53]. Although the molecular mechanisms and cellular targets mediating ghrelin inhibition of NF-κB activation remain to be determined, the vagus nerve may play an important role in the ghrelin-mediated inhibition of pro-inflammatory cytokine release [50,54]. Cachexia and muscular wasting occur via protein degradation by the ubiquitin–proteasome pathway [55]. Two muscle-specific ubiquitin ligases, muscle RING-finger protein-1 (MuRF1) and atrogin-1/muscle atrophy F-box (MAFbx), are up-regulated under catabolic conditions. NF-κB activation may regulate skeletal muscle proteasome expression and protein degradation. The elevation in MuRF1 and MAFbx expression seen in skeletal muscle after thermal injury, arthritis, and dexamethasone administration was normalized, attenuated, and prevented, respectively, by ghrelin or GHS administration [56–58]. IGF-1 prevents the expression of MuRF1 and MAFbx by inhibiting Forkhead box O (FOXO) transcription factors via stimulation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway. The IGF-1 receptor triggers activation of several intracellular kinases, including phosphatidylinositol-3-kinase (PI3K) [59]. Thus, the effects of ghrelin on NF-κB activation and IGF-1 synthesis are favorable for minimizing inflammatory responses and sarcopenia in patients with cachexia.

2.4. Other actions

The role of ghrelin in stimulating gastric emptying and acid secretion is well-established [60]. This effect may ameliorate gastrointestinal symptoms in patients with anorexia–cachexia syndrome. Ghrelin also increases endogenous nitric oxide (NO) release [61,62], which may influence its orexigenic and anti-inflammatory actions [63,64].

3. Potential clinical applications of ghrelin

3.1. Appetite-related disorders

3.1.1. Anorexia nervosa (AN) and related disorders

Anorexia nervosa (AN) is an eating disorder characterized by chronically decreased caloric intake, resulting in self-induced starvation. Plasma ghrelin levels are elevated in lean patients with anorexia nervosa, consistent with a state of negative energy balance [65–67]. Only a few preliminary studies have been performed to examine the effects of ghrelin in individuals with AN. Miljic et al. infused ghrelin (300-min intravenous infusion of 5 pmol/kg/min ghrelin) into nine AN patients with very low body weights, six AN patients who had partially recovered their body weights but who remained amenorrheic, and ten constitutionally thin female subjects [68]. The fifteen AN patients felt significantly less hungry compared with the constitutionally thin subjects, suggesting that AN patients are less sensitive to the orexigenic effects of ghrelin than healthy controls. In another paper, however, six of nine patients with restrictive AN were reported to have been hungry after ghrelin administration (1.0 μg/kg as an intravenous bolus), a similar ratio to that seen in normal subjects (five of seven) [69]. We examined the effects of ghrelin on appetite, food intake, and nutritional parameters in AN patients [26]. Five female patients who met the Diagnostic and Statistical Manual IV (DSM-IV) criteria for restricting-type AN [70] and desired to recover from the disorder participated in this study. The patients were hospitalized for 26 days (6 days pre-treatment, 14 days ghrelin infusion, and 6 days post-treatment). The patients received an intravenous infusion of 3 μg/kg ghrelin twice a day (before breakfast and dinner). Attitudes toward food were evaluated by visual analogue scale (VAS) questionnaires and daily energy intake was calculated by dieticians. Ghrelin infusion improved epigastric discomfort or constipation in four patients, whose hunger scores on VAS also increased significantly after ghrelin administration. Daily energy intake during ghrelin administration increased by 12–36% compared with the pre-treatment period. The change in body weight of the five patients ranged from +1.5 to 2.4 kg. Nutritional parameters such as total protein and triglyceride levels improved. There were no serious adverse effects, including psychological symptoms. All patients who did not gain weight during hospitalization did so after discharge. These findings suggest that ghrelin may have therapeutic potential in AN patients who cannot gain weight because of gastrointestinal dysfunction. Clearly, further studies, including randomized controlled trials, are needed to determine whether ghrelin is useful for the treatment of AN.

Functional dyspepsia (FD) is a disorder characterized by the presence of chronic or recurrent symptoms of upper abdominal pain or discomfort [71]. Although no known specific organic abnormalities are present in FD, abnormalities in gastrointestinal motility and sensitivity are thought to play a role in a substantial subgroup of patients. In addition, some patients suffer from anorexia and body-weight loss. We found that levels of plasma acylated, but not desacyl, ghrelin correlated with a subjective symptom score in FD patients, suggesting that acylated ghrelin may play a role in the pathophysiology of FD [72]. We attempted to evaluate the clinical response to repeated ghrelin administration in patients with anorexia caused by functional disorders, such as FD and 'other eating disorders' or 'unspecified eating disorders' [24]. The inclusion criteria in this study were subjects who 1) were diagnosed with functional anorexia, including FD or other eating disorders with the exception of anorexia nervosa, 2) were lean (BMI<22 kg/m^2), and 3) exhibit decreased food intake. Subjects received an intravenous infusion of ghrelin for 30 min twice a day (before breakfast and dinner) for 2 weeks, and we investigated the effects on food intake, appetite, hormones, and metabolic parameters. Six patients with FD were enrolled in this study. Ghrelin administration tended to increase daily food intake in comparison to levels before and after completion of treatment, but this difference, which was the primary endpoint of the study, did not reach statistical significance. Hunger sensation was significantly elevated at the end of drip infusion. No severe adverse effects were observed. These results suggest that ghrelin administration is safe and that this treatment has stimulatory effects on appetite in patients with FD. Further studies remain necessary to confirm the efficacy of ghrelin treatment for anorexia-related disorders.

3.1.2. Cachexia and related disorders

A number of trials seeking to utilize ghrelin for the treatment of cachexia have recently been performed [73]. These studies have sought to evaluate ghrelin as a treatment for patients with cachexia associated with congestive heart failure (CHF), COPD, cancer, and End-stage renal disease (ESRD). Cachexia manifests as excessive weight loss in the setting of an underlying chronic disease [74], and is typically associated with anorexia as a major cause of weight loss. Weight loss and decreased appetite are the major causes of morbidity and mortality in patients with anorexia–cachexia syndrome. There is an immediate need for effective, well-tolerated treatments to

stimulate appetite [75], prompting several trials to explore the application of ghrelin as a treatment for patients with cachexia.

3.1.2.1. CHF-associated cachexia. Ghrelin induces a positive energy balance state through both GH-dependent and -independent mechanisms and has protective cardiovascular effects [76]. GH treatment may be especially useful in a subgroup of patients with cardiac cachexia [77]. Ghrelin stimulates food intake, induces adiposity, regulates the central nervous system to decrease sympathetic nerve outflow, and inhibits apoptosis of cardiomyocytes and endothelial cells in a GH-independent manner. Nagaya et al. investigated the effects of ghrelin on cardiac cachexia in 10 patients with CHF [27] (Table 1). Daily administration of ghrelin for 3 weeks increased both food intake and body weight. This study also demonstrated improvements in patient exercise capacity, muscle wasting, and left ventricular function. Ghrelin treatment also resulted in significantly decreased plasma norepinephrine levels. Although this study was neither randomized nor placebo-controlled, the eight CHF patients who did not receive ghrelin (control group) were followed to rule out any time-course effects during hospitalization. None of the aforementioned parameters changed in patients with CHF who did not receive ghrelin therapy. Further studies will be necessary to identify the pathways involved in this use of ghrelin and to determine the best therapeutic strategies for ghrelin use to combat the wasting process found in cardiac cachexia patients [77]. Clinical trials are currently attempting to reproduce these data in a double-blind, placebo-controlled fashion.

3.1.2.2. COPD-associated cachexia. Patients with COPD often exhibit some degree of cachexia [78], which is an independent risk factor for mortality in COPD; GH treatment increases muscle mass in such patients. COPD and CHF are both associated with multiple pathophysiological disturbances, including anemia and neurohormonal activation [79]. In COPD patients, ghrelin exhibits anti-inflammatory effects. Chronic respiratory infections, characterized by neutrophil-dominant airway inflammation, lead to end-stage cachexia [80]. The cytotoxicity of accumulated neutrophils against bronchial and alveolar epithelial cells induces a deterioration of pulmonary function in COPD, resulting in excess energy expenditure and weight loss in patients. Intravenous ghrelin treatment for 3 weeks reduced neutrophil counts in sputum samples as well as the volume of sputum, suggesting that ghrelin suppressed excess neutrophil influx [52].

An open-label pilot study examined the ability of ghrelin to improve cachexia and functional capacity in patients with COPD; ghrelin was administered intravenously for 3 weeks to seven cachectic patients with COPD [28]. Repeated ghrelin administration significantly increased food intake, body weight, lean body mass, and peripheral and respiratory muscle strength. Ghrelin treatment ameliorated exaggerated sympathetic nerve activity, as indicated by marked decreases in plasma norepinephrine levels. Subsequently, another placebo-controlled trial demonstrated that ghrelin increased both appetite and body weight with an apparent dose-dependent trend towards improved physical performance (chair stand score) [81]. A larger clinical trial is currently being conducted to confirm these data in a double-blind, placebo-controlled fashion. Comparisons of this treatment to current standard medications will be required [79].

3.1.2.3. Cancer cachexia. Anorexia is frequently encountered in cancer patients, and is one of the major causes of malnutrition and cachexia in this patient population. Ghrelin administration resulted in significant increases in weight and food intake in rodent models of cancer-associated cachexia [82–84]. DeBoer et al. determined that weight gain resulted from a reversal in the loss of lean body mass, a critical component of cachexia [82].

Several randomized, double-blind placebo-controlled trials have demonstrated the efficacy and safety of ghrelin or GHS in patients with cancer-associated cachexia [23,25,85]. Nearry et al. performed a randomized, placebo-controlled, cross-over clinical trial to determine whether ghrelin could stimulate appetite in seven cancer patients with severe anorexia [23]. Ghrelin infusion resulted in a marked increase in energy intake in comparison to saline-treated controls; all patients in the study demonstrated increased food consumption. The meal appreciation score was also higher in ghrelin-treated individuals. Strasser et al. detailed a randomized, double-crossover, phase 1/2 study in 21 patients with advanced cancer [25]. They infused a low or high dose of ghrelin or placebo before lunch daily for 4 days in each course. Nutritional intake and eating-related symptoms did not differ between the ghrelin- and placebo-treated groups. More patients, however, preferred ghrelin to placebo at the middle and end of the study, although this finding was not dose-dependent. In contrast to the results of Neary et al., this study did not demonstrate any increases in food intake. As the patient characteristics and study designs were very different in the two studies, further investigation is required.

An important concern regarding the use of ghrelin in cancer-associated cachexia is that ghrelin may increase the levels of growth factors, such as GH and IGF-1, that stimulate tumor growth. Additionally, ghrelin itself may have mitogenic potential. As far as we know, no *in vivo* data has examined the differences in tumor growth following ghrelin or GHS treatment. Long-term, large-scale clinical trials are required to determine whether ghrelin treatment promotes tumor growth.

3.1.2.4. End-stage renal disease (ESRD). ESRD is a chronic condition frequently associated with nutritional dysfunction [86]. This type of malnutrition is highly resistant to intervention and is a major predictor of morbidity and mortality for patients on either peritoneal dialysis (PD) or hemodialysis. Wynne et al. sought to determine whether a single injection of ghrelin could enhance food intake in patients with evidence of malnutrition receiving maintenance peritoneal dialysis [30]. Nine PD patients exhibiting mild to moderate malnutrition were subcutaneously administered either ghrelin or a saline placebo in a randomized, double-blind, cross-over protocol. Ghrelin

Table 1
Clinical studies of ghrelin.

Diseases	Reference	Published year	Study design	Number of patients	Ghrelin administration
CHF	[27]	2004	Open-label pilot study	10	2 μg/kg b.i.d. for 3 wks, i.v.
COPD	[28]	2005	Open-label pilot study	7	2 μg/kg b.i.d. for 3 wks, i.v.
Cancer cachexia	[23]	2004	Acute, randomized, placebo-controlled, cross-over study	7	5 pmol/kg/min i.v. for > 180 min
Cancer cachexia	[25]	2008	Randomized, placebo-controlled, cross-over study	21	2 or 8 μg/kg, i.v. for 4 days, once a day
ESRD	[65]	2005	Acute, randomized, placebo-controlled, cross-over study	9	3.6 nmol/kg, s.c.
ESRD	[8]	2009	Randomized, placebo-controlled, cross-over study	12	12 μg/kg, s.c. for 1 wk, once a day
AN	[26]	2009	Open-label pilot study	5	3 μg/kg b.i.d. for 2 wks, i.v.
FD	[24]	2008	Open-label pilot study	6	3 μg/kg b.i.d. for 2 wks, i.v.
THR for OA	[31]	2008	Randomized, placebo-controlled, double-blind study	32	2 μg/kg b.i.d. for 3 wks, i.v.
Total gastrectomy	[32]	2010	Randomized, placebo-controlled, double-blind study	21	3 μg/kg b.i.d. for 10 days, i.v.
Esophagectomy	[33]	2010	Randomized, placebo-controlled, double-blind study	20	3 μg/kg b.i.d. for 10 days, i.v.

CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; FD, functional dyspepsia; ESRD, End-stage renal disease; THR, total hip replacement; OA, osteoarthritis.

administration significantly increased mean absolute energy intake during the study meals and non-significant increases in energy intake were observed over the first 24 h without a subsequent rebound. This research group subsequently sought to analyze the efficacy of repeated ghrelin administration in malnourished dialysis patients [87] by performing a double-blind randomized cross-over study of a week of daily subcutaneous ghrelin injections in a group of 12 malnourished dialysis patients. Ghrelin administration significantly increased appetite, with increases in energy intake noted at the first study meal. Persistence of this effect throughout the week was confirmed by food diaries and final study meals, indicating that daily ghrelin treatment resulted in a sustained positive change in energy balance in malnourished dialysis patients. In support of this data, experiments using a nephrectomized rat model of renal cachexia demonstrated that daily treatment for 2 weeks with ghrelin or two GHS agents (BIM-28125 and BIM-28131) resulted in increased food intake, improved lean body mass accrual, and decreased circulating inflammatory cytokines [88]. Long-term studies are needed to demonstrate efficacy in improving appetite, weight gain, lean body mass, and quality of life.

3.2. GH deficiency-related disorders

Strong stimulation of GH secretion by ghrelin has been well documented in humans [12–16,21,42]. As with GHS, ghrelin may be useful for the diagnosis and treatment of short stature and GH deficiency. Elderly individuals may be particularly suitable candidates for ghrelin treatment, as aging is associated with progressive decreases in GH secretion, appetite, and energy intake [89–92]. This reduced GH secretion is called "somatopause" and may be a cause of age-related metabolic and physiologic changes including reduced lean body mass and expansion of adipose mass. Sarcopenia is associated with functional decline and death. Altered blood lipid profiles also favor the development of vascular diseases that may increase overall mortality. The age-related reduction in energy intake has been termed "the anorexia of aging" and predisposes to the development of under-nutrition, which has been implicated in the development and progression of chronic diseases commonly affecting the elderly, as well as in increasing mortality. Growth hormone therapy increases IGF-I levels, promotes anabolism, and increases muscle strength in healthy elderly individuals, as well as in selected patient groups [93–95]. Therefore, ghrelin and GHS may also have therapeutic potential to assist in the recovery of frail patients who require nutritional support and conventional rehabilitation [96]. We evaluated the effects of ghrelin administration on physical performance and body composition in patients undergoing elective total hip replacement (THR) as treatment for osteoarthritis (OA) in a randomized, double-blind, placebo-controlled, phase II study [31]. Thirty-two patients were assigned to two groups of 16 subjects each; the ghrelin group received intravenous injections of 2 μg/kg ghrelin twice daily for 3 weeks beginning 1 week before surgery, while the placebo group received vehicle alone. While ghrelin significantly increased lean body mass after the three-week injection period, it did not affect muscle strength or walking ability. Significant decreases in fat mass and GH responses to ghrelin injection were also observed. No severe adverse effects occurred in response to ghrelin treatment. Despite increased lean tissue reserves, ghrelin administration using this study protocol did not provide any favorable effect on physical performance in patients with OA undergoing THR. Further studies are necessary to examine the efficacy of ghrelin treatment in such patients.

We found that plasma levels of acylated ghrelin in healthy elderly female subjects tended to be low and were correlated positively with IGF-1 levels, suggesting that negative feedback mechanism does not function properly in elderly subjects [97]. Further, acylated ghrelin concentrations in elderly females correlated with both systolic blood pressure and the frequency of bowel movements. These findings suggest that, in elderly females, acylated ghrelin may play a role in the regulation of the GH/IGF-1 axis, blood pressure, and bowel movements.

3.3. Post-gastrectomy and -esophagectomy

Body weight loss is common and is a serious outcome in patients who have undergone total gastrectomy and esophagectomy. Such weight loss correlates with decline in postoperative quality of life and is the most reliable indicator of malnutrition, which impairs immune function, susceptibility to infection, and survival [32,33]. Plasma ghrelin levels decreased after total gastrectomy and esophagectomy [65,98,99]. Moreover, a significant correlation between ghrelin concentration and postoperative weight loss suggested a role for loss of ghrelin. To examine this, Adachi et al. evaluated the efficacy of ghrelin in 21 patients undergoing total gastrectomy [32]. Food intake and appetite were significantly higher in the ghrelin group (3 μg/kg, twice daily for 10 days after starting oral food intake following surgery) compared with the placebo group, and BW loss was significantly lower in the ghrelin group than in the placebo group. Fat mass, lean body mass, and basal metabolic rate decreased significantly in the placebo group; however, the reductions in lean body mass and basal metabolic rate were not significant in the ghrelin group, although that of fat mass was significant. Thus, short-term administration of synthetic ghrelin successfully lessened postoperative body weight loss and improved appetite and food intake after total gastrectomy. Subsequently, the same research group performed a similar study in 20 patients who underwent esophagectomy [33]. Again, they found that administration of ghrelin after esophagectomy increased oral food intake and attenuated weight loss together with maintenance of lean body weight. Thus, ghrelin administration may be useful in minimizing the side effects of these operations.

3.4. Other disorders

Reflecting the wide expression patterns of both ghrelin and its receptor, this peptide is now known to play a role in a number of different physiological processes including cellular proliferation and differentiation, pancreatic exocrine and endocrine function, glucose metabolism, sleep and behavior, immune regulation, and cardiovascular function. For example, as discussed above, repeated administration of ghrelin in patients with CHF significantly improved left ventricular function as well as food intake. A large number of studies have been performed by investigators worldwide to elucidate the various activities of ghrelin. We believe that some of these may lend support to the development of clinical applications of ghrelin to disorders other than those described above in the future.

4. Conclusions

More than ten years have passed since the discovery of ghrelin, and abundant evidence now indicates that it plays a role in a variety of physiological functions. In parallel, clinical trials have proceeded to exploit these activities in the treatment and diagnosis of human disease. There are several characteristic features of the clinical applications of ghrelin: 1) the multiplicity and uniqueness of its function, 2) its unique structure and fatty acid modification, and 3) the paucity of severe adverse effects [100]. These characteristics should allow us to develop novel and unique therapies for a variety of disorders, including many currently intractable and serious diseases. Indeed, research on clinical applications of ghrelin is a challenging and potentially rewarding avenue for the future.

5. Learning points

- Ghrelin plays a critical role in a variety of physiological processes, including the stimulation of food intake and growth hormone secretion.
- The effects of ghrelin should be invaluable for the development of novel treatments and disease diagnostic techniques.
- Clinical trials have already been performed to assess the utility of ghrelin for the treatment of several disorders including anorexia, cachexia, and GH-related disorders.
- This review summarizes the recent advances in this area of research.

Conflict of interest statement

The authors state that they have no conflicts of interest.

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ORIGINAL ARTICLE

Mapping Analysis of Ghrelin Producing Cells in the Human Stomach Associated with Chronic Gastritis and Early Cancers

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Abstract
Objective The majority of ghrelin producing cells (GPC) are present in the fundic gland of the stomach and recognized as X/A like cells. The detailed distribution of GPC in the stomach is still unknown in healthy and pathological subjects.
Methods We investigated the detailed distribution of GPC in the stomach, especially in relation with chronic gastritis, using surgical specimens from 12 patients with early gastric cancer. Either the anterior or posterior half of the whole stomach, which was a counterpart of the tumor bearing side, was subjected for immunohistochemistry of ghrelin, and the number of total GPC were semi quantitatively evaluated as GPC score. GPC score was compared with the degree of chronic gastritis, serum ghrelin concentration and body weight.
Results GPC was not observed in the pyloric gland, but heterogeneously distributed in the fundic gland mainly in upper body and the greater curvature. The GPC score showed about nine-fold difference, which correlated well with the degree of chronic gastritis by Sydney score ($r = -0.84$, $P < 0.001$). The serum ghrelin concentration was basically determined by the GPC score ($r = 0.75$, $P = 0.0047$); however, the obese patients showed low serum ghrelin concentration in spite of the presence of abundant GPCs. In the low GPC score patients, serum ghrelin was constantly low regardless of their body weight.
Conclusions GPC was inversely correlated with progression of chronic gastritis. Its quantification using immunohistochemistry of the whole stomach was useful to comprehensively evaluate ghrelin profile.

Keywords Ghrelin · Chronic gastritis · Gastric cancer · Immunohistochemistry

Introduction

The 28-amino-acid peptide, ghrelin, is isolated from rat and human stomach [1]. This peptide is an endogenous ligand for the growth hormone secretagogue receptor 1a (GHS-R1a), and stimulates growth hormone release from the pituitary gland. Ghrelin has been reported to function not only in the control of growth hormone secretion but also in the regulation of food intake and energy metabolism [2]. The function of ghrelin is to stimulate the appetite signal in the hypothalamus and gastrointestinal activity, such as peristalsis, gastric acid secretion, and pancreatic excretion, through the vagal nerve [3].

The majority of ghrelin is produced in the stomach and a smaller amount is secreted from other organs, such as intestine, pancreas, kidney, and hypothalamus [4, 5]. Therefore, circulating ghrelin levels decreased to 10–20% of the preoperative level immediately after total gastrectomy [6, 7]. By the histological examination, ghrelin producing cells (GPC) were immunohistochemically identified in the fundic glands, which had been regarded as X/A-like

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cells [4]. X/A-like cells were named as they morphologically resemble pancreatic A-cells (glucagon producing cell), therefore considered as a kind of neuroendocrine cell. X/A-like cells are abundant next to histamine producing cells as endocrine cells in the stomach [4, 8], but the product had been unknown for a long time. There were few studies concerning distribution of GPC in human stomach including one using three autopsy patients [4] and another investigated only the lesser curvature of the stomach [9]. Other studies investigating the relationship between GPC and clinicopathological features used endoscopic biopsy samples [10, 11]. Thus, the detailed distribution of GPC in the stomach is still unknown in healthy and pathological subjects.

The circulating ghrelin level is greatly varied by diurnal rhythm, food intake and body weight in healthy individuals [12, 13]. Among various pathological status that affect circulating ghrelin level, the chronic gastritis caused by *Helicobacter pylori* (*H. pylori*) infection is the most commonly observed [14, 15]; its prevalence is estimated in more than 70% of Japanese people aged 50 and over [16]. Chronic gastritis by *H. pylori* is well-known to be a causative condition for gastric cancers, which is the most common malignant tumor in the Eastern countries [17]. Moreover, *H. pylori* infection should be a clue to explain the huge difference of nutritional status between Western and Eastern countries. Clinical observation suggests some symptoms of chronic gastritis, such as appetite loss, delayed gastric emptying and malnutrition, should be caused by ghrelin and GPC reduction. A few studies have reported that GPC is decreased in the chronic gastritis patients [10, 11]. However, the detailed relationship is still unclear since those studies have used only few endoscopic biopsy samples for the evaluation. The degree of chronic gastritis, probably that of GPC as well, is heterogeneous, therefore it is necessary for precise evaluation to analyze the whole gastric specimen. Thus, we investigated the detailed distribution of GPC and its association with chronic gastritis using human whole stomach, which were surgically removed due to early gastric cancers. This is the first study of precise mapping analysis of GPC in the human stomach and should help to understand the association of chronic gastritis and ghrelin.

Materials and Methods

Patients and Gastric Specimens

Twelve patients who underwent total gastrectomy due to early gastric cancer at Osaka University Hospital between September 2007 and September 2009 were enrolled in the study. There were nine males and three females with average age of 64.5 years, ranging from 45 to 83 years. Average BMI was 24.1 kg/m^2 ranging from 19.3 to 28.3 kg/m^2. There were three patients medicated by hypertension and two patients by hyperlipidemia; however, those with serious co-morbidity or other cancers were excluded from this study. Gastric cancers of all 12 patients were less invasive (mucosal or submucosal), small (ranging 12–25 mm, median 21 cm in diameter), and localized in the posterior or anterior wall of the stomach, that is, tumors did not reach either the greater or lesser curvature of the stomach. Immediately after surgery, whole stomach was opened by greater curvature incision for observation, then fixed in 10% buffered formalin for three days. After fixation, the stomach was separated into two pieces as posterior and anterior wall by longitudinal incision at the lesser curvature. The tumor bearing side of the stomach was subjected to ordinary pathological examination and the tumor-free half was used for this study. The entire half of the stomach was sliced longitudinally in 8-mm steps, and then cut into 4-cm length pieces. All specimens were embedded in paraffin and used for hematoxylin and eosin (H&E) stain, Giemsa stain and immunohistochemistry. Blood samples collected before surgery and clinicopathological information from patients' charts were also subject for this study.

Atrophic changes in the gastric body on endoscopy were diagnosed on the basis of the atrophic area displaying discoloration with or without blood vessel transparency. The grade of atrophic gastritis was assessed endoscopically using the atrophic pattern system [18, 19]. This classification divides the extent of atrophy into closed type (C-type) and open type (O-type). The C-type indicates that the atrophic border remains on the lesser curvature of the stomach, while the O-type means that the atrophic border no longer exists on the lesser curvature but extends along the anterior and posterior walls of the stomach.

The study was approved by the Osaka University Ethics Committee, and all patients gave written informed consent before study entry in accordance with the Helsinki Declaration. The study was registered at UMIN (http://www.umin.ac.jp with clinical trial number UMIN000002902).

Immunohistochemistry of Ghrelin Producing Cells

Immunohistochemical staining of GPCs was performed with the streptavidin–biotin-peroxidase-complex method (Histofine® SAB-PO(R) Kit, Nichirei Biosciences Inc., Tokyo, Japan). The following steps were performed at room temperature unless otherwise specified. Paraffin-embedded specimens were sectioned at 4-μm thickness, deparaffinized and dehydrated. To enhance immunoreactivity of ghrelin, antigens were retrieved at 95°C for 40 min in citric acid buffer. After blocking of endogenous

peroxidase activity for 20 min with methanol containing 1% H_2O_2, the sections were reacted for 15 min with normal goat serum to prevent nonspecific binding. They were then incubated overnight with the Anti-Rat Ghrelin Polyclonal Antibody (Trans Genic Inc. Kumamoto, Japan) at 4°C. This anti-rat ghrelin antibody specifically recognizes the N-terminal fragment of ghrelin and is able to recognize both rat and human ghrelin [20, 21]. On the next day, the sections were washed in 0.01 M phosphate buffered saline (PBS) and incubated for 20 min with 10 μg/ml biotinylated goat anti-rabbit IgG antibody. After washing PBS, the sections were reincubated for 20 min with 100 μg/ml peroxidase-conjugated streptavidin and stained with 3, 3′-diaminobenzidine tetrahydrochloride in 0.05 M tris–HCl buffer containing H_2O_2. The sections were finally washed in PBS and counterstained with hematoxylin. Negative controls were treated identically without the primary antibody.

Semi-quantitative evaluation of GPCs was performed by counting GPC in the microscope. At 100× magnification, the field of microscope was adjusted to the gastric mucosa area and the number of GPC, which were clearly recognized as cells with small cytoplasm and dark brown staining, were counted. In each 4-cm length section, ten fields of mucosa area were randomly chosen and the average number of GPCs was recorded. According to the average number of GPC, each gastric section was classified as an 'extra rich area' (>40), 'rich area' (20–40), 'middle area' (1–20), and 'poor area' (<1). Due to the differences in the size of the stomach, the number of sections for each patient ranged from 37 to 70 (average 51.2) in each half stomach. To adjust for the size of the stomach, the number of sections of each classification was translated to the proportion area (%) of the entire half stomach. Finally, we established GPC score using an original calculating formula to evaluate the number of GPCs in each patient as follows;

GPC score = 70 × (extra rich area %) + 30 × (rich area %) + 10 × (middle area %) + 0.5 × (poor area %). Twelve patients were classified to high and low groups divided by the median GPC score.

Blood Sampling and Hormone Assay

Preoperative blood samples were collected from patients before breakfast after an overnight fast, transferred into chilled tubes, stored on ice during collection, centrifuged, separated as serum, and stored at −50°C until assay. Serum total ghrelin level was measured using an enzyme-linked immunosorbent assay-kit (Human Desacyl-Ghrelin ELISA- kit® and Human Active (acyl) -Ghrelin ELISA-kit®, Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). Total serum ghrelin concentration was calculated as acyl-ghrelin concentration plus desacyl-ghrelin concentration. Serum gastrin levels were measured using RIA (Gastrin RIA kit® II, TFB Inc., Tokyo Japan), and serum pepsinogen (PG) I and II were measured using Chemiluminescent enzyme immunoassay (CLEIA) (LUMIPULSE Presto®, Fujirebio Inc., Tokyo, Japan). Subjects were screened on the basis of the following PG-test positive criteria: PG I level of less than 70 ng/mL and PG I/II ratio of less than 3.0. The cut-off point for atrophic gastritis and gastric cancer screening has been widely accepted in Japan.

Histopathological Examination of Gastritis

All sections of the entire half stomach were stained with H&E stain. One co-author of the pathologist (M.E.) who was unaware of the clinical information and laboratory data examined histopathological features of chronic gastritis according to the updated Sydney system [22]. In brief, five histological parameters including chronic inflammation (mononuclear cells), polymorphonuclear activity (neutrophils activity), glandular atrophy, intestinal metaplasia, and *H.pylori* density were scored semi-quantitatively from 0 to 3 (none, mild, moderate, or marked) then the sum of the score of the five parameters were used as the final score. The presence of *H. pylori* was confirmed by Giemsa staining of the representative six sections near the greater curvature of the corpus and antrum of the stomach by the same pathologist.

Quantitative mRNA Assay

Each three biopsy samples were collected from the fornix, the upper, the middle, and the lower stomach without obvious pathological change by the fiberscope in the representative patient (Case 2 in Fig. 2). They were immediately lysed in 175 ml of RNA lysis buffer (4 M GTC, 0.01 M Tris (pH7.5), 0.97% b-mercaptoethanol; Promega). RNA was extracted using the Promega SV Total RNA Isolation kit (Promega, Southampton, UK) following the protocol recommended by the manufacturers, and contaminating genomic DNA were removed by DNAse. RNA yield and purity were determined using a spectrophotometer at 260 and 280 nm. First strand cDNA synthesis was performed using RNase Reverse Transcriptase (GIBCO BRL, Paisley, UK).

Quantitative PCR was performed on a Roche Light Cycler system (Roche Molecular Biochemicals, Manheim, Germany). PCR reactions were carried out in a reaction mixture consisting of 5.0 ml reaction buffer and 2.0 mM MgCl2 (Biogene Ltd, Cambridge, UK), 1.0 ml of each primer (1 ng/ml), 2.5 ml of cDNA and 0.5 ml of Light Cycler DNA Master SYBR Green I (Roche Molecular Biochemicals). Protocol conditions consisted of