

Production of Ghrelin by the Stomach of Patients with Gastric Cancer

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Summary: Poor nutrition and weight loss are important factors contributing to poor quality of life (QOL) after gastrectomy in patients with gastric cancer. Ghrelin is a hormone produced by the stomach that, plays a role in appetite increase and fat storage. The present study aims to clarify the location of ghrelin mRNA in the stomach, changes in blood ghrelin concentrations after gastrectomy and whether or not they are associated with the reconstruction method in patients with gastric cancer. We collected seven normal mucosa samples from different parts of six totally resected stomachs with gastric cancer. We extracted RNA from the normal mucosa, synthesized cDNA from total RNA (1 µg), and then quantified ghrelin mRNA using quantitative real-time polymerase chain reaction (Q-PCR). Ghrelin blood concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kits in 74 patients with gastric cancer (total gastrectomy (TG), n=23; distal gastrectomy (DG), n=30; proximal gastrectomy (PG), n=11; pylorus preserving gastrectomy (PPG), n=10). In order, the ghrelin gene was expressed most frequently in the gastric body, followed by the fornix, cardia, antrum and pylorus ring. Blood ghrelin concentrations after surgery similarly changed in all groups. The average blood ghrelin concentrations were significantly higher in the DG and PPG groups than in the TG group on postoperative days (POD) 1, 7, 30, 90 and 180. However, blood ghrelin concentrations did not significantly differ between the DG and TG groups on POD 270 and 360. Cells that produce ghrelin are supposed to be located mostly in the fundic gland of the stomach. We speculate that the production of ghrelin from other organs increases from around nine months after total gastrectomy. Therefore, evaluating the nutritional status and the weight of patients at nine months after total gastrectomy is important to help these patients improve their QOL.

Key words gastric cancer, ghrelin, quantitative real-time polymerase chain reaction, gastrectomy, enzyme-linked immunosorbent assay

INTRODUCTION

Ghrelin is a 28-amino-acid peptide that was originally isolated from the rat stomach. It is the endogenous ligand for the growth hormone secretagogue receptor (GHSR). It is mainly produced in cells located within the fundus of the stomach [1,2] and it stimulates growth hormone (GH) release in rats when peripherally or centrally administered [3,4]. Ghrelin is also

synthesized in the hypothalamic arcuate nucleus, which is a critical region for the regulation of feeding [5]. Acyl and des-acyl forms of ghrelin have been identified; acyl ghrelin has a posttranslational n-octanoyl modification at serine-3 which is essential for ghrelin-induced biological activities, whereas des-acyl ghrelin is inactive [1]. Ghrelin secretion is upregulated when the energy balance is negative, such as during fasting and insulin-induced hypoglycemia,

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Abbreviations: BMI, body mass index; DG, distal gastrectomy; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GH, growth hormone; GHSR, growth hormone secretagogue receptor; IGF, insulin-like growth factor; IGFBP, GH-IGF-IGF binding protein; PG, proximal gastrectomy; POD, postoperative day; PPG, pylorus preserving gastrectomy; QOL, quality of life; Q-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean; TG, total gastrectomy.

whereas it is downregulated with feeding, hyperglycemia and obesity [6,7]. Ghrelin also regulates interdigestive stomach contractions in rodents [8] and in humans [9].

Poor nutrition and weight loss are important factors contributing to poor quality of life (QOL) for patients with gastric cancer after gastrectomy. Ghrelin is produced in the stomach and has the hormonal function of increasing appetite and fat storage [2,4,6,10-12]. Reduced food intake, appetite loss and altered ghrelin secretion might be related to the loss of body weight that commonly occurs after gastrectomy. Endocrine ghrelin cells represent about 20% of the total population of endocrine cells in the human gastric mucosa, and they are located in the lower parts of the oxyntic glands in the fundus and body of the stomach [13]. Little is known about the relationship between ghrelin production and gastrectomy in patients with gastric cancer.

The present study aims to clarify the location of ghrelin in the resected stomach, changes in the plasma ghrelin concentration after gastrectomy and whether or not those changes depend on surgical method in patients with gastric cancer.

MATERIALS AND METHODS

Quantitative real-time polymerase chain reaction (PCR)

We collected seven normal mucosa samples from the cardia (A), fundus (B), the lesser (C) and greater (D) curvatures of the middle stomach body, the lesser (E) and greater (F) curvatures of the antrum, and the pylorus ring (G) of the stomachs resected from six patients (age, 42 to 71 y; mean, 61 y; male, n=4) with gastric cancer during total gastrectomy between June and October 2006.

We synthesized cDNA using SuperScript II reverse transcriptase (Invitrogen, Tokyo, Japan) from total RNA (1 µg) extracted from each sample using TRIzol

(Invitrogen). Quantitative real-time PCR (Q-PCR) proceeded using SYBR Green PCR Core Reagent (PE Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was measured as the internal control for normalization. Differences in expression were calculated using a PRISM 7000 Sequence Detection System (PE Applied Biosystems).

The gene-specific and GAPDH primers were as follows: sense, 5'-GGGCAGAGGATGAACTGGAA-3' and anti-sense, 5'-CCTGGCTGTGCTGCTGGTA-3'; sense, 5'-GGGAAGCTTGTTCATCAATGG-3' and antisense, CATCGCCACCTGATTTT-3', respectively.

Plasma ghrelin measurements

We used Active Ghrelin and Desacyl-Ghrelin ELISA kits (Mitsubishi Chemical Corporation, Tokyo, Japan) to measure ghrelin concentrations in plasma samples from 74 patients (mean age, 64.3 y; range, 39 to 79 y; male, n=47; female, n=27) who underwent gastrectomy to treat gastric cancer between June 2006 and August 2007. Blood samples were collected into tubes including EDTA·2Na 1.25 mg/mL and aprotinin 500 U/mL on postoperative days (POD) 1, 7, 14, 30, 90, 180, 270, and 360. The number of patients who underwent distal (DG), total (TG), proximal (PG) and pylorus-preserving (PPG) gastrectomy were 30, 23, 11 and 10, respectively (TABLE 1). Plasma was extracted from blood samples, 1 N HCl was immediately added and the samples were stored below -40°C. Measurement of plasma ghrelin concentrations using ELISA kits proceeded as follows. Assay buffer (150 µL) was mixed with standard or sample (50 µL) and placed in antibody-coated plates at room temperature for two hours. After three washes, HRP-conjugated antibody (200 µL) was added to the plates and left at room temperature for one hour. After six washes, substrate (200 µL) was added and the plates were placed in the dark at room temperature for thirty minutes.

TABLE 1.
Background of patients in whom plasma ghrelin concentrations were measured

Type of gastrectomy	n	Age range (y, mean)	Male:female
Total (TG)	23	42 - 74 (64.6)	18:5
Distal (DG)	30	39 - 78 (62.4)	19:11
Proximal (PG)	11	56 - 79 (69.0)	5:6
Pylorus-preserving (PPG)	10	49 - 71 (64.2)	5:5

This table shows that the background of patients were dependent on the type of gastrectomy. Plasma ghrelin concentrations were measured in these patients after gastrectomy.

Thereafter, 0.5 mol/L sulfuric acid (50 μ L) was added and absorbance was measured at 450 nm.

Body mass index (BMI)

We also measured the body mass index (BMI) of the same patients on POD 0, 4, 14, 30, 90, 180, 270, 360. The BMI on POD 0 was defined as 100%.

Statistical analysis

Data are shown as means \pm standard error of the mean (SEM). Data were statistically analyzed using Student's t-test and Welch's t test. $P < 0.05$ was considered significant.

RESULTS

Ghrelin mRNA in gastric mucosa

Figure 1 shows the levels of endogenous ghrelin

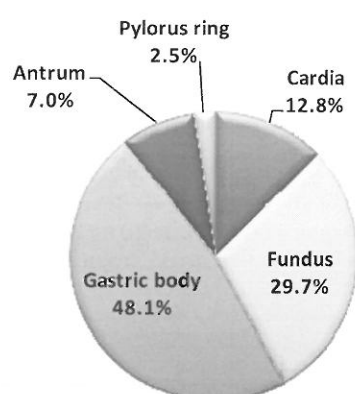


Fig. 1. Location of ghrelin gene mRNA expression. If ghrelin gene mRNA expression in patients with gastric cancer is defined as 100%, then 48.1%, 29.7%, 12.8%, 7.0%, and 2.5% of ghrelin gene mRNA is expressed in gastric body (C+D), fundus (B), cardia (A), antrum (E+F), and pylorus ring (G), respectively.

mRNA expression measured in the stomach using RT-PCR. The ghrelin/GAPDH ratios were 1.00 ± 1.81 , 3.94 ± 8.45 , 1.90 ± 2.51 , 1.16 ± 1.55 , 0.05 ± 0.06 , 0.10 ± 0.10 and 0.03 ± 0.03 at areas A, B, C, D, E, F and G, respectively (n=6 each). When the total amount of ghrelin gene expression in patients with gastric cancer was defined as 100%, then the gastric body (C+D), fundus (B), cardia (A), antrum (E+F) and pylorus ring (G) expressed 48.1%, 29.7%, 12.8%, 7.0%, and 2.5% of the mRNA, respectively. Expression levels of ghrelin mRNA on the gastric body were the highest in the stomach, and decreased in the order of the fundus, cardia, antrum, and pylorus ring (Fig. 1).

Changes in the concentration of plasma ghrelin

We summarized the plasma concentrations of des-acyl and acyl ghrelin in Tables 2 and 3, respectively. The average concentration of plasma des-acyl ghrelin was the lowest in the TG group and was significantly higher in the DG and PPG, than in the TG group at POD 1, 7, 30, 90, and 180 ($P < 0.05$, each). The value was significantly higher in the DG than in the TG group at POD 14, and in the PPG vs. TG group at POD 270 ($P < 0.05$, each). However, at POD 270 and 360 there was no significant difference in the plasma concentrations of des-acyl ghrelin between the DG and TG groups. The des-acyl ghrelin plasma concentrations changed in the same manner regardless of the surgical procedure. Plasma concentrations of des-acyl ghrelin changed remarkably after each surgical procedure before POD 30, but not thereafter (TABLE 2). The average concentration of plasma acyl ghrelin was significantly higher in the PPG than in the TG group at POD 30 ($P < 0.05$). The plasma concentrations of acyl ghrelin and des-acyl ghrelin similarly changed, but those of acyl-ghrelin did not increase after POD 270 in the TG group (TABLE 3).

TABLE 2.
Plasma des-acyl ghrelin concentrations (fmol/mL) in four groups from POD 0-360

POD	0	1	7	14	30	90	180	270	360
PG	60.0 \pm 5.88	42.5 \pm 2.48	45.6 \pm 3.38	49.2 \pm 4.44	48.6 \pm 3.34	50.0 \pm 6.42	42.6 \pm 2.41	44.3 \pm 1.00	44.8 \pm 2.02
DG	60.9 \pm 4.86	47.1 \pm 2.70 ^a	53.9 \pm 5.47 ^a	54.5 \pm 3.55 ^a	50.5 \pm 3.19 ^a	51.2 \pm 3.31 ^a	52.0 \pm 2.75 ^a	49.7 \pm 4.16	53.5 \pm 4.80
TG	59.3 \pm 5.06	37.4 \pm 1.69 ^b	42.9 \pm 4.47 ^b	43.7 \pm 2.14 ^b	41.2 \pm 1.86 ^b	38.9 \pm 1.92 ^b	39.6 \pm 1.74 ^b	40.8 \pm 2.09 ^b	49.1 \pm 4.89
PPG	56.3 \pm 6.29	47.5 \pm 3.04 ^a	48.3 \pm 3.29 ^a	49.4 \pm 4.28	52.7 \pm 6.07 ^a	47.6 \pm 3.64 ^a	50.2 \pm 5.61 ^a	55.9 \pm 9.84 ^a	47.1 \pm 1.04

POD: postoperative day; PG: proximal gastrectomy; DG: DG, distal gastrectomy; TG: total gastrectomy; PPG: pylorus preserving gastrectomy; $P < 0.05$, a vs. b. Data are expressed as mean \pm SE, n=11, 30, 23 and 10 for PG, DG, TG and PPG, respectively. Average plasma des-acyl ghrelin concentrations were significantly higher in the DG and PPG groups than the TG group on POD 1, 7, 30, 90 and 180 ($P < 0.05$, each), but did not differ between DG and TG groups on POD 270 and 360. The value was significantly higher in the DG than in the TG group at POD 14, and in the PPG than in the TG group at POD 270 ($P < 0.05$, each).

TABLE 3.
Plasma acyl ghrelin concentrations (fmol/mL) in four groups in four groups from POD 0-360

POD	0	1	7	14	30	90	180	270	360
PG	9.82±1.32	7.20±0.82	7.31±1.02	6.77±0.52	7.55±1.14	7.81±1.60	6.80±1.14	7.63±1.19	7.76±1.10
DG	10.8±1.32	8.02±0.74	7.90±0.75	9.60±1.09	8.68±0.86	8.06±0.86	8.62±0.94	8.04±0.79	7.02±0.76
TG	8.72±0.55	6.07±0.65	7.92±1.01	6.99±0.91	5.72±1.01 ^b	7.06±1.19	5.84±0.77	5.89±0.48	4.84±0.90
PPG	10.5±1.56	8.00±0.86	7.01±0.96	7.44±1.05	11.3±1.03 ^a	8.49±1.26	8.90±2.61	8.29±1.70	7.44±0.49

POD: postoperative day; PG: proximal gastrectomy; DG: DG, distal gastrectomy; TG: total gastrectomy; PPG: pylorus preserving gastrectomy; $P < 0.05$, a vs. b. Data are expressed as mean \pm SE, n=11, 30, 23 and 10 for PG, DG, TG and PPG, respectively. Average plasma acyl ghrelin concentration was significantly higher in PPG, than TG group at POD 30 ($P < 0.05$). Plasma acyl and des-acyl ghrelin concentrations similarly changed, but plasma acyl-ghrelin concentrations did not increase in TG group after POD 270.

TABLE 4.
Body mass index (%) in four groups in four groups from POD 0-360

POD	0	4	14	30	90	180	270	360
PG	100±0.0	97.7±2.1	94.4±1.8	92.0±3.0	87.8±3.7	89.3±4.8	90.0±6.3	90.3±7.5
DG	100±0.0	97.2±3.1	93.6±3.1	90.7±4.4	90.0±6.2	90.6±6.7	90.8±5.9 ^a	89.6±6.7
TG	100±0.0	96.6±4.2	93.2±4.0	89.8±5.5	86.8±3.1	88.4±8.7	85.3±6.4 ^b	86.1±4.9
PPG	100±0.0	95.9±3.0	92.5±3.6	91.4±3.6	89.3±6.1	88.7±2.7	91.2±3.0 ^a	89.8±4.0

POD: postoperative day; PG: proximal gastrectomy; DG: DG, distal gastrectomy; TG: total gastrectomy; PPG: pylorus preserving gastrectomy; $P < 0.05$, a vs. b. Data are expressed as mean \pm SE, n=11, 30, 23 and 10 for PG, DG, TG and PPG, respectively. Body mass index (BMI) on POD 0 is defined as 100%. Average BMI was lowest in TG group and significantly higher in DG and PPG, than in TG group at POD 270 ($P < 0.05$). BMI decreased until POD 90 and then stabilized regardless of the surgical procedure, except for that of the TG group, which decreased between POD 180 and 270 and slightly increased thereafter (TABLE 4).

Changes in BMI

TABLE 4 summarizes the BMI values. The average BMI was the lowest in the TG group, and was significantly higher in the DG and PPG groups than in the TG group at POD 270 ($P < 0.05$). The BMI decreased until POD 90 and then stabilized regardless of the surgical procedure, except for that of the TG group, which decreased between POD 180 and 270 and slightly increased thereafter (TABLE 4).

DISCUSSION

Ghrelin plays an important role in regulating feeding behavior in rodents [11]. It is predominantly derived from the stomach and targets neuroendocrine networks within the central nervous system to regulate energy homeostasis [2]. Endocrine ghrelin cells are located in the lower regions of the oxyntic glands in the fundus and body of the stomach [13]. We found that the endogenous levels of ghrelin mRNA in the gastric body were highest in the stomach, followed by the

fundus. Kim et al. [14] investigated the expression of ghrelin mRNA and peptide in samples of stomach tissues using RT-PCR and immunohistochemistry. They found that ghrelin mRNA expression and immunoreactivity were highest at the upper portion of the stomach [14]. Zub-Pokrowiecka et al. [13] reported that the reduced fasting plasma ghrelin levels in patients with gastric cancer appear to depend upon the extent and proliferation of cancer in the stomach and H. pylori-induced atrophic gastritis. They also found lower fasting and postprandial ghrelin levels in patients with gastric cancer of the fundus than of the distal stomach. Jeon et al. [15] found that postoperative ghrelin levels increased more slowly in patients after resection of the proximal stomach, than after fundus-preserving resection, and that the preserved part of the stomach produced compensatory ghrelin. These and the present findings suggest that preservation of the fundus is more important than that of the distal stomach for ghrelin production after gastrectomy.

The average blood ghrelin concentrations were significantly higher in the DG and PPG, than in the TG

group until six months after surgery. However, these values did not significantly differ between the DG and TG groups at nine months and at one year after surgery, indicating that ghrelin is released from extra-stomach tissues at these time points. Jeon et al. [15] found that serum ghrelin levels fell after complete stomach resection to about 30% of preoperative levels and they did not find compensatory postoperative production. Takachi et al. [16] also found that ghrelin concentrations decreased by up to 12% of preoperative levels on POD 3 and 7 after total gastrectomy. The patients who had been treated by total gastrectomy had very low levels of ghrelin at a mean of 41 months after surgery. By contrast, hormone levels in patients treated by distal gastrectomy decreased to 39% on POD 3 and increased to 57% of preoperative levels on POD 7. In our study, plasma ghrelin concentrations were lower in the TG and PG than in the DG and PPG groups. Moreover, the recovery of plasma ghrelin concentrations was delayed in the TG and PG, compared with the DG and PPG groups. These results supported the notion that the cells producing ghrelin are mainly located in the body of the stomach and the fundus.

Other tissues only partially compensated for ghrelin production after resection of the stomach [15]. Hosoda et al. [17] showed that serum ghrelin concentrations tend to gradually normalize due to production by other tissues after complete gastric resection. Kamiji et al. [18] reported that ghrelin secreted by sources other than the stomach is likely to function in the long-term regulation of body weight after total gastrectomy. However, Ariyasu et al. [19] found that plasma ghrelin levels in patients at 1-8 years after total gastrectomy reached about 35% of the preoperative level and were comparable with control values. Takachi et al. [16] demonstrated very low levels of ghrelin during long-term follow up after total gastrectomy. Koizumi et al. [20] found that ghrelin is upregulated in extra-stomach tissue such as the duodenum and pancreas, and that plasma ghrelin levels are physiologically regulated in rats at 12 weeks after gastrectomy. After 270 POD, the concentration of plasma des-acyl ghrelin recovered and did not differ from those of the other groups. This finding suggested that extra-stomach tissue produced ghrelin as Koizumi et al. reported. However, plasma acyl ghrelin did not recover in the present study. The ratio of plasma des-acyl ghrelin to total plasma ghrelin was > 90% [21,22]. Yoshimoto et al. [22] reported that the antibodies used in their study to measure ghrelin concentrations in human blood could not distinguish ghrelin from desacyl ghrelin. Therefore, we speculated that the sensitivity of the ELISA kit was too low to

detect acyl ghrelin at 360 POD.

This time-related recovery of ghrelin production might provide a strategy for promoting feeding, increasing body weight, and improving the physical condition of gastrectomized patients. Frühbeck et al. [23] reported that the reduced circulating ghrelin concentrations in patients at six months after a Roux-Y gastric bypass are not determined by active weight loss or improved insulin sensitivity, but rather depend on a surgically induced bypass of the ghrelin-producing cells of the fundus. Dornonville de la Cour et al. [24] administered ghrelin to gastrectomized mice for eight weeks. They concluded that ghrelin is mainly associated with the control of fat metabolism and that ghrelin replacement therapy might alleviate the weight loss associated with gastrectomy. Adachi et al. [25] performed a prospective, randomized, placebo-controlled phase II study to investigate the effects of exogenous ghrelin administration in patients with gastric cancer after total gastrectomy. They concluded that short-term ghrelin administration was safe, ameliorated postoperative body weight loss and improved appetite and food intake. Patients administered ghrelin lost significantly more fat mass, whereas a placebo group tended to lose lean body mass and had a lower basal metabolic rate; however, the difference did not reach statistical significance. The overall positive effects of ghrelin such as body weight gain and caloric intake increase were consistent in all clinical trials. Here, we found that plasma des-acyl ghrelin concentrations did not significantly differ between the DG and TG groups from POD 270. Moreover, the BMI of the TG group decreased from POD 180 to 270, and increased slightly thereafter. These results suggest that ghrelin secreted by sources other than the stomach plays a role in the long-term regulation of body weight after total gastrectomy. Therefore, evaluating the nutritional status and weight of patients at nine months after total gastrectomy would help such patients enjoy a better QOL.

The Ethics Committee of Kurume University approved this study on May 15, 2006 (No. 06023).

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