ABSTRACT
Ghrelin is a peptide that is secreted from the stomach and plays a role in appetite, weight gain, and skeletal muscle composition. Thus, compounded human ghrelin is a candidate drug for improving nutritional status after pancreatic surgery. However, in patients with pancreatic carcinomas, adverse influences on the occult tumor growth of ghrelin-induced secretion are a concern. The present study describes the effects of the administration of compounded human ghrelin on weight gain and pancreatic cancer cell growth in a mouse model. Changes in body weight and tumor growth in a subcutaneously transplanted pancreatic carcinoma cell line in vivo (5-week-old BALB/c-nu/nu mice) were examined with or without the administration of compounded human ghrelin. Compounded human ghrelin was administered at 44 days after post-transplantation. Changes in weight were not significantly different between the control and compounded human ghrelin groups 8 days after compounded human ghrelin administration, and no association between weight and concentration of compounded human ghrelin was identified. Tumor growth after the administration of compounded human ghrelin was significantly lower than that of the control group, with the magnitude of the decrease being associated with increasing compounded human ghrelin concentration (p<0.05). At 6 and 8 days after compounded human ghrelin administration, increases in tumor weights of the control groups (0.5±0.3 g and 0.9±0.2 g, respectively) were significantly greater than those observed for groups receiving 3, 15, and 30 nmol per kg of compounded human ghrelin (0.1 and 0.2, 0.2 and 0.3, and 0.2 and 0.3, respectively). There were no adverse effects of compounded human ghrelin administration. Plasma leptin levels were significantly lower in cancer cells compared with the control vehicle (p<0.05), which was decreased in mice receiving 30nmol per kg of compounded human ghrelin in comparison with those receiving vehicle (p<0.05). Although the administration of compounded human ghrelin did not influence weight gain, compounded human ghrelin significantly inhibited pancreatic cancer cell growth and might inhibit plasma leptin levels.

INTRODUCTION
In pancreatic cancer patients, weight loss and malnutrition are evident after pancreatic resection due to the advanced tumor stage and the invasiveness of radical surgery [1,2]. To promote early recovery after major surgery, nutritional and hormonal forms of support are necessary during the perioperative period. However, such effective supportive treatments have not been established to date.

Ghrelin was discovered as an intrinsic ligand for the growth hormone secretagogue receptor (GHSR) in 1999 by Kojima et al. and Kangawa et al. [3, 4]. Endogenous ghrelin is primarily produced in the stomach. Ghrelin has multiple functions, such as exerting orexigenic effects on the hypothalamus or gastrointestinal motility, stimulating growth hormone secretion, performing anti-inflammatory activities, and strengthening skeletal muscle, as well as various other metabolic functions [3-11]. In particular, ghrelin is a powerful gastrointestinal appetite-stimulating hormone, a function that is regulated by the circadian rhythm [12]. Recently, clinical trials of compounded human ghrelin (CHG) were undertaken to increase oral feeding and weight gain and induce early recovery and anti-inflammatory protection after invasive

Effects of Compounded Human Ghrelin in a Mouse Model of Pancreatic Carcinoma

Atsushi Nanashima1,2, Tomoaki Kodama3, Goushi Murakami2, Katsunori Takagi2, Junichi Arai2, Yorihisa Sumida2, Takeshi Nagayasu2

1Division of Hepato-biliary Pancreatic Surgery, Department of Surgery, University of Miyazaki Faculty of Medicine, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan
2Department of Surgical Oncology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
3Department of Health and Nutrition, Faculty of Health Management, Nagasaki International University, 2825-7 Hausutenbosu-machi, Sasebo 859-3243, Japan

Received August 11th, 2015 – Accepted September 28th, 2015

Keywords Body Weight; Ghrelin; Leptin; Pancreatic Carcinoma; tumor migration inhibition factor

Abbreviations BALB an albino, laboratory-bred strain of the house mouse; CHG compounded human ghrelin; MIA-PaCa2 pancreatic carcinoma cell line

Correspondence Atsushi Nanashima
Division of Hepato-biliary Pancreatic Surgery
Department of Surgery, University of Miyazaki Faculty of Medicine
5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan
Phone +81 985 85 2905
Fax +81 985 85 3780
E-mail a.nanashima@med.miyazaki-u.ac.jp

surguries, such as gastrectomy and esophagectomy, in cancer patients [13-16]. In the fields of biliary and pancreatic surgery, we also undertook a clinical trial for CHG in patients who underwent major hepatectomy and pancreatectomy (in press). However, in many of these patients, occult cancer cells may remain, even though a radical operation has been performed, leading to a higher rate of recurrence in comparison with that of patients with gastrointestinal carcinomas [17, 18]. Ghrelin has been used for the improvement of cancer- or chemotherapy-related cachexia [19, 20]; therefore, this peptide is predicted not to have adverse effects on cancer progression. However, the precise mechanism for this phenomenon has not been fully clarified to date. As ghrelin also has other functions, including stimulation of pancreatic secretion [21, 22], the influence of CHG administration on pancreatic tumor growth is concerning. We must clarify whether CHG stimulates cancer cell growth before using it in future clinical applications.

Thus, in the present study, we examined the changes in body weight and tumor growth associated with pancreatic carcinoma cell lines transplanted into mice and compared these variables between groups receiving either control vehicle or CHG in an in vivo mouse model.

**MATERIALS AND METHODS**

**Tumor Xenograft and CHG Administration Protocol**

Five-week-old BALB/c-nu/nu mice (body weight, 14-16 g; CLEA Japan, Inc., Tokyo, Japan) were used in this study. Mice were housed 2-3 per plastic cage at 23±2 °C under a 12-hour light-dark cycle. Water and food (CRF-1; Oriental Yeast Co., Tokyo, Japan) were freely available.

In these experiments, 1×10⁷ MIA-PaCa2 (pancreatic carcinoma) cells [23] suspended in 150 µL of Dulbecco’s Modified Eagle’s Medium were inoculated subcutaneously into the hemi-lateral abdomen of mice (n=24). In the control group, only Dulbecco’s Modified Eagle’s Medium was injected (n=24). The maximum and minimum axes of the produced tumors were precisely measured by the Vernier caliper, and due to difficulties in measuring tumor volume, the tumor weight was calculated using the following formula [24] because of difficulty of measuring tumor volume itself in each group:

\[
\text{tumor weight (g)} = \left[\left(\text{maximum axis (cm)}\right) \times \left(\text{minimum axis (cm)}\right)^2\right]/2
\]

Body weight and tumor growth (size or weight) at 6 and 8 days after CHG administration were measured.

CHG (Peptide Institute, Osaka, Japan) was injected intraperitoneally when the mean body weight of the mice began to decrease. Animals of the same weight were allocated to either a control group or one of four groups for CHG administration (at the time of CHG administration; 44 days after inoculation in experimental models). For the CHG groups, 0, 3, 15, or 30 nmol/kg CHG dissolved in 100 µL of saline was injected twice daily (at 10:00 h and 18:00 h) for 6 days. Forty-eight hours after the final injection, blood was taken from the vena cava under general anesthesia, and the heparinized blood was centrifuged at 3000 rpm for 15 minutes to obtain plasma. Plasma leptin concentrations were measured using an ELISA kit (Morinaga Institute of Biological Science Inc., Yokohama, Japan).

**Statistical Analysis**

Data are expressed as the mean ± standard deviation (SD). Statistical significance was determined by two-way repeated-measures ANOVA, one-way factorial ANOVA, unpaired t-test, and multiple comparison Tukey’s test using the statistical package SPSS Statistics 19 (IBM, NY, NY, USA). A P value of less than 0.05 was considered to be statistically significant.

**RESULTS**

During the in vivo experiments, 5 vehicle mice and 3 mice transplanted with MIA-PaCa2 cells exhibited dermatitis and were therefore excluded from the study. In total, 19 vehicle and 21 MIA-PaCa2 mice were used for the present study.

**Figure 1** shows the changes in body weight over the course of 44 days for the groups with and without inoculation of MIA-PaCa2 cell lines. There were no significant differences between the groups. Variability of calculated tumor weight was 0.4-4.3 g in non-CHG group, 0.7-1.7 g in the 3 nmol/kg group, 0.2-2.5 g in the 15 nmol/kg group and 0.3-2.6 g in the 30 nmol/kg group. There were no significant differences of tumor weight before CHG administration between groups. **Figure 2** shows the changes in body weight for 8 days after CHG injection; no changes in body weight were identified between the groups. No side effects of CHG were observed in any mice. **Figure 3** shows that the group that did not receive CHG had increasing tumor growth (size); in contrast, in the CHG-administered groups, this growth was significantly lower (p<0.05). Furthermore, the inhibition of tumor growth was more significant with increasing concentrations of CHG. The increase in tumor growth at days 6 and 8 was inhibited by all doses of CHG administered, as shown in **Figure 4**. Compared with the control (no treatment), the tumor weight for MIA-PaCa2 cells was significantly lower upon administration of 3, 15, and 30 nmol/kg CHG: for 3 nmol/kg (0.5±0.15 g), 15 nmol/kg (0.2±0.1 g), and 30 nmol/kg (0.2±0.15 g) at 6 days; and for 3 nmol/kg (0.9±0.2 g), 15 nmol/kg (0.3±0.15 g), and 30 nmol/kg (0.3±0.1%) at 8 days. There were no significant differences between the doses of CHG that were administered. In **Figure 5**, plasma leptin concentrations at day 8 are shown. With 0 nmol/kg CHG, leptin concentrations were significantly lower in vehicle mice than in mice inoculated with MIA-PaCa2 cells (p<0.05). Although leptin concentrations did not change with administration of CHG doses up to 15 nmol/kg, leptin concentrations were significantly decreased in tumor-bearing mice after administration of 30 nmol/kg CHG in comparison with mice receiving 0 nmol/kg CHG (p<0.05).
Figure 1. In vivo model. Five-week-old BALB/c-nu/nu mice were transplanted subcutaneously with MIA-PaCa2 cells leading to the formation of pancreatic tumors in the back. Comparison of changes in body weight after cell transplantation between the control group (n=19) (open circles) and transplanted mice (n=21) (closed circles).

Figure 2. Changes in body weight of the control and transplanted groups receiving various concentrations of CHG for 8 days after the final injection.

DISCUSSION

Although the role of ghrelin as an orexigenic hormone has long been documented, its mechanism is still debated. The role of ghrelin in cancer induction has not been disputed, but its characterization as a selective inhibitor of tumor growth is controversial. This study attempted to solve the mystery behind this hormone. Ghrelin induces pancreatic endocrine and exocrine secretion via the brain-gut axis system [21, 22, 25], as well as pancreatic cellular proliferation and growth hormone secretion [3-11, 25]. Thus, the stimulation of occult cancer cell proliferation by ghrelin administration is a clinical concern. Before using ghrelin to enhance a patient’s nutritional recovery after pancreatic resection for malignant diseases, in the present study, we undertook to examine the adverse effects of CHG on pancreatic cancer cells. Previous studies have reported that ghrelin was used to improve appetite loss in patients with cachexia who underwent treatment with anti-cancer drugs; however, its influences on tumor progression or tumor inhibition have not been discussed to date.
With respect to tumor progression, investigators have reported that ghrelin increased cancer progression or affected cancer invasiveness in neuroendocrine tumors or carcinomas of the kidney, colorectum, esophagus, stomach, prostate, mammary gland, uterus, ovary, pancreas, and thyroid [26-42]. Among these reports, Duxbury et al. described that ghrelin promoted cellular proliferation and invasion of a pancreatic carcinoma cell line [39]. This cell line expresses the ghrelin receptor. This manuscript indicated that pancreatic carcinoma is a ghrelin-responsive malignancy. Conversely, other investigators demonstrated that ghrelin inhibited tumor progression or prevented carcinogenesis in the colorectum, breast, ovary, esophagus, stomach, and kidney [43-54] or did not affect cancer progression [55-57]. Therefore, the mechanism by which ghrelin administration influences cancer progression or invasion remains controversial. The effects of growth hormone and its modulation by ghrelin might be associated with tumor growth [58], while on the other hand, ghrelin might produce anti-inflammatory cytokine responses [59]. Kawaguchi et al. clarified the effect of tumor suppression by anti-inflammatory responses in inflammation-based colon carcinoma [43]. Therefore, the effect of CHG in various carcinomas is most likely different. Our results in the present study show that CHG significantly suppressed the growth of pancreatic cancer cells (MIA-PaCa2) in a concentration-dependent manner. Therefore, in the case of pancreatic cancer patients undergoing chemotherapy or upon the appearance of occult pancreatic cancer cells after surgery, the administration of CHG may not produce adverse effects that induce cancer progression. Therefore, CHG is a clinically useful drug that not only improves cachexia and nutrition but also inhibits cancer growth. To clarify such effects, the model used for this study needs to be examined further with respect to additional variables.

Leptin is a regulator peptide that controls appetite and its effects are opposite to those of ghrelin [60]. In the present study, the plasma leptin levels in the cancer model were significantly lower in comparison with those in the control. Previous reports suggest that leptin might...
be increased in some cancers [61-63]. Although each peptide may influence assimilation or catabolism [64], direct ghrelin-induced effects on leptin have not been reported to date. Administration of low levels of CHG did not change plasma leptin levels. However, leptin levels were decreased in the cancer model by high concentration of CHG. Potential reasons for this include the following: 1) Ghrelin might affect adipose cells, leading to inhibition of leptin secretion. 2) Ghrelin also may affect cancer cells, leading to inhibition of tumor progression, which results in blocking of leptin production in cancer cells. 3) Ghrelin may also affect the immune system by decreasing the production of TNF-α TNF- α, which inhibits leptin section [65-68]. Thus, ghrelin might decrease leptin levels, as previous reports suggest [69]. The relationship between these peptides remains controversial; therefore, further study is necessary to clarify the mechanisms of these peptides’ effects on cancer growth.

In addition, we expected to observe an increase in body weight upon CHG administration in the pancreatic cancer model in the present study. As described above, previous reports suggested that ghrelin improved appetite or weight gain in patients with advanced-stage cancers [3-11, 21, 22, 25]. In the present model, differences in weight change between the control group and the pancreatic cancer model were not significant. Thus, the use of a more advanced cachexia model is necessary. In addition, the effect of CHG on body weight was also not significant. To optimize the effect of CHG on body weight, a severe cachexia model of pancreatic cancer will be a necessary next step. Controlled study models need to elucidate the cause-effect relationship in the future step.

In conclusion, tumor progression of pancreatic cancer was significantly inhibited by ghrelin in vivo. The use of ghrelin as treatment for nutritional improvement in patients with malnutrition is thus expected not to promote pancreatic cancer progression.

Grant support
This was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare of Japan (#10103853), between 2012 and March 2014

Conflict of Interest
The authors have no conflict of interest to declare

References


