5. グレリンのその他の生理作用

グルカゴン抗体を用いた二重免疫染色により，グレリンは膵ランゲルハンス島の辺縁部に存在し，グルカゴンと共存していた[52]。膵ランゲルハンス島にグレリン受容体遺伝子は発現し，生理的濃度（10^{-12}～10^{-11}M）のグレリンは，高血糖下（8.3 mM）でラット単離膵β細胞の細胞内 Ca^{2+} 濃度を増加させ，インスリン分泌を促進する。一方，低血糖下（2.8 mM）では，グレリンはβ細胞内遊離 Ca^{2+} 濃度とインスリン分泌を変化させなかった[52]。グレリン受容体を発現している肝細胞腫瘍株 Hep G2 を用いた実験で，グレリン投与によりインスリン受容体を介さずに，insulin receptor substrate-1（IRS-1）のリン酸化が時間および濃度依存性に増加することも示されている[53]。グレリンは肝臓におけるインスリン作用を修飾し，糖代謝に関与している可能性がある。

グレリンをラットに静脈内投与すると，用量依存性に胃酸分泌と胃運動を亢進した[54]。この作用はアトロピン投与と迷走神経切除により阻止されたが，H_2 ブロッカーでは影響を受けないことから，迷走神経遠心路を介した作用である。グレリンは脳室内投与でも胃酸分泌を促進し，アトロピン前処置と迷走神経切除により，この作用は消失した。グレリンは延髄の迷走神経背側運動核を活性化して，これらの胃機能に作用している。摂食と消化管機能は深く連関しており，グレリンは中枢と末梢を介し，エネルギー同化作用に機能している[54,55]。

健常者へグレリンを静脈内投与すると，心拍数は変化させずに，有意な平均動脈圧の低下（−12 mmHg）と心拍出量の増加（+16%）を生じた[56]。グレリンの前腕動脈内投与は，血清 GH を増加させずに前腕血流量を用量依存性に増加させた。慢性心不全患者でカヘキシア（6か月以内に7.5% 以上の体重減少）がある症例では，血漿グレリン濃度は増加していた[57]。心筋梗塞後心不全モデルラットにグレリンを3週間皮下投与すると，血清 GH の上昇とともに左室駆出率の増加，左室リモデリング進展の抑制，カヘキシアの是正が認められ[58]，

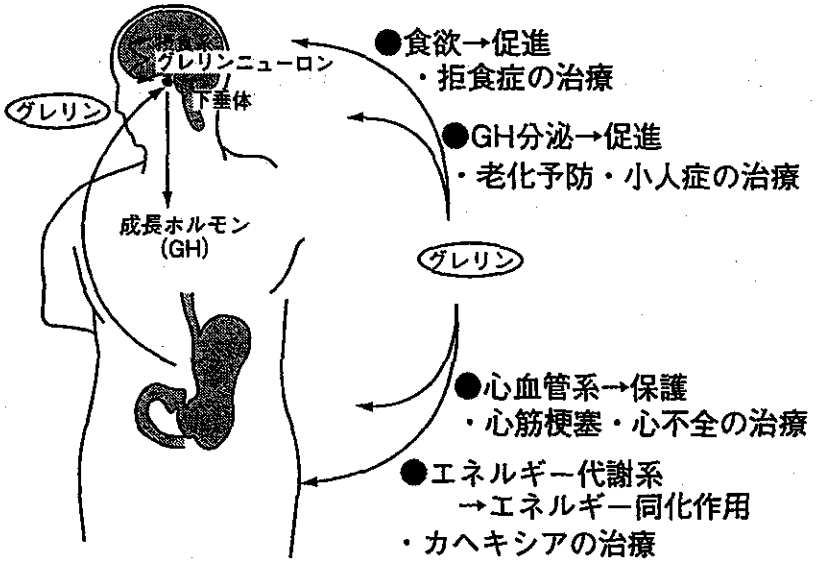

図5－7　グレリンの主たる生理作用と治療薬としての可能性

心機能改善および低栄養状態の是正によるグレリンの心不全治療薬としての有用性が示唆されている。慢性心不全患者へのグレリン投与でも心係数の増加や血行動態の改善が報告されている[59]。図5－7に，グレリンの治療薬として可能性が考えられている病態を示している。

6. おわりに

グレリンの発見により，胃が消化機能だけでなく，GHの分泌調節やエネルギー代謝調節にも機能していることが明らかになった。グレリンは，摂食亢進に作用することが初めて証明された消化管ペプチドであり，脂肪組織から分泌されるレプチンに拮抗して作動している。グレリンは脳と消化管で産生され，かつ両者を機能的に連係してエネルギー代謝を調節している。胃から分泌されるグレリンは，迷走神経を介して速やかに末梢空腹情報を中枢に伝達し，摂食促進に機能する。グレリンの自律神経系を介する摂食調節機構の解明は，肥満や摂食障害などの病因の究明に意義深い。カヘキシアに対する治療薬としてのグレリンの臨床研究もすでに始まっており，グレリンの持つ幅広い生理作用の解明や薬剤としての臨床応用研究が急速に進展しつつある。

文　献

1) Kojima M., Hosoda H., Date Y. et al : Ghrelin is a novel growth hormone releasing acylated peptide from stomach. Nature 1999 ; 402 ; 656-660.
2) Spiegelman B.M., Flier J.S. : Obesity and the regulation of energy balance. Cell 2001 ; 104 ; 531-543.
3) Ahima R.S., Osei S.Y. : Molecular regulation of eating behavior: new insights and prospects for therapeutic strategies. Trends Mol Med 2001 ; 7 ; 205-213.
4) Schwartz M.W., Woods S.C., Porte D. Jr. et al: Central nervous system control of food intake. Nature 2000 ; 404 ; 661-671.
5) Momany F.A., Bowers C.Y., Reynolds G.A. et al : Design, synthesis, and biological activity of peptides which release growth hormone *in vitro*. Endocrinology 1981 ; 108 ; 31-39.
6) Smith R.G., Van der Ploeg L.H., Howard A.D. et al : Peptidomimetic regulation of growth hormone secretion. Endocr Rev 1997 ; 18 ; 621-645.
7) Chapman I.M., Bach M.A., Van Cauter E. et al : Stimulation of the growth hormone (GH)-insulin-like growth factor I axis by daily oral administration of a GH secretogogue (MK-677) in healthy elderly subjects. J Clin Endocrinol Metab 1996 ; 81 ; 4249-4257.
8) Camanni F., Ghigo E., Arvat E. : Growth hormone-releasing peptides and their analogs. Front Neuroendocrinol 1998 ; 19 ; 47-72.
9) Casanueva F.F., Dieguez C. : Growth hormone secretagogues: Physiological role and clinical utility. Trends Endocrinol Metab 1999 ; 10 ; 30-38.
10) Smith R.G., Cheng K., Schoen W.R. et al : A nonpeptidyl growth hormone secretagogue. Science 1993 ; 260 ; 1640-1643.
11) Pong S.S., Chaung L.Y., Dean D.C. et al : Identification of a new G-protein-linked receptor for growth hormone secretagogues. Mol Endocrinol 1996 ; 10 ; 7-61.
12) Hosoda H., Kojima M., Matsuo H. et al : Purification and characterization of rat des-Gln14-ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. J Biol Chem 2000 ; 275 ; 21995-22000.
13) Kaiya H., Kojima M., Hosoda H. et al : Bullfrog ghrelin is modified by n-octanoic acid at its third threonine residue. J Biol Chem 2001 ; 276 ; 40441-40448.
14) Kaiya H., Van Der Geyten S., Kojima M, et al : Chicken ghrelin : purification, cDNA cloning, and biological activity. Endocrinology 2002 ; 143 ; 3454-3463.
15) Unniappan S., Lin X., Cervini L. et al : Goldfish ghrelin : molecular characterization of the complementary deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake. Endocrinology

2002；143；4143-4146.
16) Date Y., Kojima M., Hosoda H. et al：Ghrelin, a novel growth-hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 2000；141；4255-4261.
17) Hayashida T., Nakahara K., Mondal M.S. et al：Ghrelin in neonatal rats: distribution in stomach and its possible role. J Endocrinol 2002；173；239-245.
18) Lu S., Guan JL., Wang Q.P. et al：Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus. Neurosci Lett 2002；321；157-160.
19) McKee K.K., Palyha O.C., Feighner S.D. et al：Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors. Mol Endocrinol 2002；11；415-123.
20) Petersenn S., Rasch A.C., Penshorn M. et al：Genomic structure and transcriptional regulation of the human growth hormone secretagogue receptor. Endocrinology 2001；142；2649-2659.
21) Kaji H., Kishimoto M., Kirimura T. et al：Hormonal regulation of the human ghrelin receptor gene transcription. Biochem Biophys Res Commun 2001；284；660-666.
22) Nakazato M., Murakami N., Date Y. et al：A role for ghrelin in the central regulation of feeding. Nature 2001；409；194-198.
23) Tschöp M., Smiley D.L., Heiman M.L.：Ghrelin induces adiposity in rodents. Nature 2000；407；908-913.
24) Wren A.M, Seal L.J, Cohen M.A. et al：Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab 2001；86；5992-5995.
25) Stanley B.G., Kyrkouli S.E., Lampert S. et al：Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. Peptides 1986；7；1189-1192.
26) Billington C.J., Briggs JE., Grace M. et al：Effects of intracerebroventricular injection of neuropeptide Y on energy metabolism. Am J Physiol 1991；260；R321-R327.
27) Shutter J.R., Graham M., Kinsey A.C. et al：Hypothalamic expression of ART, a novel gene related to agouti, is up-regulated in obese and diabetic mutant mice. Genes Dev 1997；11；593-602.
28) Sakurai T., Amemiya A., Ishii M. et al：Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 1998；92；573-585.
29) Qu D., Ludwig D.S., Gammeltoft S. et al：A role for melanin-concentrating

hormone in the central regulation of feeding behaviour. Nature 1996 ; 380 ; 243-247.

30) Kalra S.P., Dube M.G., Pu S. et al : Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. Endocr Rev 1999 ; 20 ; 68-100.

31) Zhang Y., Proenca R., Maffei M. et al: Positional cloning of the mouse obese gene and its human homologue. Nature 1994 ; 372 ; 425-432.

32) Elmquist J.K. : Anatomic basis of leptin action in the hypothalamus. Front Horm Res 2000 ; 26 ; 21-41.

33) Hahn T.M., Breininger JF., Baskin D.G. et al : Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. Nat Neurosci 1998 ; 1 ; 271-272.

34) Willesen MG., Kristensen P., Romer J. : Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. Neuroendocrinology 1999 ; 70 ; 306-316.

35) Sagar SM., Sharp FR., Curran T. : Expression of c-*fos* protein in brain : metabolic mapping at the cellular level. Science 1988 ; 240 ; 1328-1331.

36) Fan W., Boston B.A., Kesterson R.A. et al : Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 1997 ; 385 ; 165-168.

37) Kristensen P., Judge ME., Thim L. et al : Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 1998 ; 393 ; 72-76.

38) Marks D.L., Cone R.D. : Central melanocortins and the regulation of weight during acute and chronic disease. Recent Prog Horm Res 2001 ; 56 ; 359-375.

39) Mountjoy K.G., Mortrud M.T., Low M.J. et al : Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol Endocrinol 1994 ; 8 ; 1298-1308.

40) Lin L., Faraco J., Li R. et al : The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell 1999 ; 98 ; 365-376.

41) Chemelli R.M., Willie J.T., Sinton C.M. et al : Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 1999 ; 98 ; 437-451.

42) Lu S., Guan J.L., Wang Q.P. et al : Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus. Neurosci Lett 2001 ; 321 ; 157-160.

43) Horvath T.L., Diano S., van den Pol A.N. : Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: a novel circuit implicated in metabolic and endocrine regulations. J Neurosci 1999 ; 19 ; 1072-1087.

44) Date Y., Murakami N., Toshinai K. et al : The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion.

Gastroenterology 2002 ; 123 ; 1120-1128.

45) Wei J.Y., Wang Y.H. : Effect of CCK pretreatment on the CCK sensitivity of rat polymodal gastric vagal afferent *in vitro*. Am J Physiol Endocrinol Metab 2000 ; 279 ; E695-E706.

46) Shiiya T., Nakazato M., Mizuta M. et al : Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. J Clin Endocrinol Metab 2002 ; 87 ; 240-244.

47) Cummings D.E., Purnell J.Q., Frayo R.S. et al : A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 2001 ; 50 ; 1714-1719.

48) Tschöp M., Wawarta R., Riepl R.L. et al Post-prandial decrease of circulating human ghrelin levels. J Endocrinol Invest 2001 ; 24 ; RC19-21.

49) Toshinai K., Mondal M.S., Nakazato M. et al : Upregulation of ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. Biochem Biophys Res Commun 2001 ; 281 ; 1220-1225.

50) Hayashida T., Murakami K., Mogi K. et al : Ghrelin in domestic animals : distribution in stomach and its possible role. Domest Anim Endocrinol 2001 ; 21 ; 17-24.

51) Norrelund H., Hansen T.K., Orskov H. et al : Ghrelin immunoreactivity in human plasma is suppressed by somatostatin. Clin Endocrinol 2002 ; 57 ; 539-546.

52) Date Y., Nakazato M., Hashiguchi S. et al : Ghrelin is present in pancreatic α-cells of humans and rats and stimulates insulin secretion. Diabetes 2002 ; 51 ; 124-129.

53) Murata M., Okimura Y., Iida K. et al : Ghrelin modulates the downstream molecules of insulin signaling in hepatoma cells. J Biol Chem 2002 ; 277 ; 5667-5674.

54) Date Y., Nakazato M., Murakami N. et al : Ghrelin acts in the central nervous system to stimulate gastric acid secretion. Biochem Biophys Res Commun 2001 ; 280 ; 904-907.

55) Masuda Y., Tanaka T., Inomata N. et al : Ghrelin stimulates gastric acid secretion and motility in rats. Biochem Biophys Res Commun 2000 ; 276 ; 905-908.

56) Nagaya N., Kojima M., Uematsu M. et al : Hemodynamic and hormonal effects of human ghrelin in healthy volunteers. Am J Physiol Regul Integr Comp Physiol 2001 ; 280 ; R1483-R1487.

57) Nagaya N., Uematsu M., Kojima M. et al : Elevated circulating level of ghrelin

0021-972X/03/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 88(11):5473–5477

doi: 10.1210/jc.2002-021350

Ghrelin Concentration in Cord and Neonatal Blood: Relation to Fetal Growth and Energy Balance

SEIKO KITAMURA, ICHIRO YOKOTA, HIROSHI HOSODA, YUMIKO KOTANI, JUNKO MATSUDA, ETSUO NAITO, MICHINORI ITO, KENJI KANGAWA, AND YASUHIRO KURODA

Department of Pediatrics, University of Tokushima School of Medicine (S.K., I.Y., Y.Ko., J.M., E.N., M.I., Y.Ku.), Tokushima 770-8503, Japan; and Department of Biochemistry, National Cardiovascular Center Research Institute (H.H., K.K.), Osaka 565-8565, Japan

To investigate the relationship between ghrelin and both fetal and neonatal growth parameters and energy balance, we measured plasma ghrelin concentrations in 54 cord blood samples (male, n = 34; female, n = 20; gestational age, 37.0–41.6 wk; birth weight, 2206–4326 g) and 47 neonatal blood samples (male, n = 27; female, n = 20; postnatal d 3–8). The plasma ghrelin concentrations in cord blood ranged from 110.6–446.1 pmol/liter (median, 206.7 pmol/liter), which were equal to or higher than those in normal weight adults. These values were inversely correlated with birth weight (r = −0.40; $P = 0.002$), birth length (r = −0.36; $P = 0.007$), placental weight (r = −0.35; $P = 0.01$), and IGF-I concentration (r = −0.49; $P = 0.0002$), but were not significantly correlated with the GH concentration (r = 0.22; $P = 0.12$). The ghrelin concentrations in small for gestational age newborn were significantly higher than those in appropriate for gestational age newborns ($P = 0.0008$). The ghrelin concentrations in the vein were significantly higher than those in the artery in 8 cord blood samples ($P = 0.01$), which suggests that the placenta is an important source of fetal ghrelin. In neonates, the ghrelin concentrations ranged from 133.0–481.7 pmol/liter (median, 268.3 pmol/liter), which were significantly higher than those in cord blood ($P < 0.0001$). These results suggest that ghrelin may contribute to fetal and neonatal growth. (*J Clin Endocrinol Metab* 88: 5473–5477, 2003)

GHRELIN IS AN endogenous ligand for the GH secretagogue receptor that stimulates the release of GH (1, 2). In addition, ghrelin positively regulates the energy balance as an antagonist of leptin through hypothalamic nuclei (3, 4). These actions seem to be especially important during the growth stage in humans. In adults, the major source of circulating ghrelin in the blood comes from the neuroendocrine cells in the fundus of the stomach (5, 6), and the level of ghrelin rises during fasting and falls to a nadir after eating (7). Significantly decreased ghrelin levels have been observed in obese individuals (8, 9).

We previously reported changes in the leptin concentration during the fetal and neonatal periods (10–12). A relatively high level of leptin at birth and the expression of leptin in the placenta suggested that leptin may play a role during the perinatal period (10–13). Interestingly, ghrelin has also been reported to be expressed in the placenta (14). Therefore, it is important to examine changes in the ghrelin concentration during the fetal and neonatal periods to evaluate the relation between ghrelin and both the growth of the fetus and neonate and the regulation of feto-maternal and neonatal energy balance.

In the present study we measured ghrelin concentrations in cord and neonatal blood using a specific RIA system we developed (15), and examined its relationship to other growth-related hormones and clinical characteristics of the fetus and neonate.

Abbreviations: AGA, Appropriate for gestational age; IRI, immunoreactive insulin; LGA, large for gestational age; SGA, small for gestational age.

Subjects and Methods

Subjects

Venous cord blood samples were obtained from 54 full-term newborns (34 males and 20 females; gestational age, 37.0–41.6 wk; birth weight, 2206–4326 g; birth length, 44.0–54.5 cm). Their characteristics are shown in Table 1. Forty-four of the newborns were classified as appropriate for gestational age (AGA), 7 were small for gestational age (SGA), and 3 were large for gestational age (LGA). AGA was defined as a newborn whose birth weight was from −1.5 to +1.5 SD of the mean birth weight in each gestational age. SGA was defined as below −1.5 SD, and LGA was defined as over +1.5 SD. The mean birth weight and SD were calculated according to the Japanese population fetal growth curve published in 1994 by a study group of the Japanese Ministry of Health and Welfare. To estimate the source of ghrelin in the fetal circulation, both arterial and venous cord blood samples were collected from another 8 full-term newborns. Blood samples for ghrelin assay were collected in chilled tubes containing EDTA·2Na (1 mg/ml) and aprotinin (500 U/ml), and plasma was separated at 4 C immediately after birth. Serum was simultaneously separated for other hormone assays. Neonatal samples were obtained from 47 full-term healthy neonates (27 males and 20 females; postnatal d 3–8), whose characteristics are shown at Table 2. In 27 of these neonates, cord blood had already been collected, and the change in the ghrelin concentration between cord and neonatal blood was compared in this group. Each sample was collected at 0900 h, and plasma was separated immediately.

Plasma and serum samples were kept frozen at −80 C until analysis. All of the newborns and neonates were healthy, and their mothers had had no remarkable complications during pregnancy. The study protocol was approved by the ethical committee of University of Tokushima School of Medicine, and all parents of the newborns gave their written informed consent before enrollment.

Ghrelin and other hormone assays

Plasma ghrelin concentration was determined by RIA using polyclonal antibodies raised against the carboxyl-terminal fragment ghrelin-[13–28] (15). The value determined by this RIA system gives the total concentration of ghrelin. Serum GH and IGF-I were determined using

TABLE 1. Characteristics of the fetus and plasma ghrelin concentration in cord blood

	Total (n = 54)	Male (n = 34)	Female (n = 20)
Gestational wk	39.4 (37.0–41.6)	39.5 (37.0–41.4)	39.3 (37.0–41.6)
Birth body weight (g)	2994 (2206–4326)	3008 (2206–3778)	2884 (2318–4326)
Birth body length (cm)	48.5 (44.0–54.5)	48.5 (44.0–52.5)	48.3 (46.0–54.5)
Kaup index (g/cm^2 × 10)	12.6 (10.6–15.3)	12.8 (10.6–15.3)	12.5 (11.0–15.2)
Birth weight/birth length (g/cm)	60.9 (49.0–79.4)	61.5 (49.0–74.1)	60.6 (50.4–79.4)
Placental weight (g)	532 (322–804)	514 (325–804)	562 (322–783)
Plasma ghrelin (pmol/liter)	206.7 (110.6–446.1)	207.8 (110.6–404.2)	201.1 (119.2–446.1)

Values are medians, with the range in *parentheses*.

TABLE 2. Characteristics of the neonate and plasma ghrelin concentration in neonatal blood

	Total (n = 47)	Male (n = 27)	Female (n = 20)
Days after birth	5.0 (3.0–8.0)	5.0 (3.0–8.0)	5.0 (3.0–6.0)
Gestational wk	39.3 (37.0–41.4)	39.4 (37.0–41.4)	39.1 (37.0–40.6)
Birth body weight (g)	3030 (2324–4082)	3030 (2620–3994)	2961 (2324–4082)
Body weight (g)	2870 (2246–3816)	2966 (2530–3650)	2758 (2246–3816)
Body length (cm)	48.9 (44.0–52.0)	49.0 (44.0–51.5)	48.6 (45.0–52.0)
Mean calorie intake (kcal/kg·d)	66.5 (35.3–105.8)	57.1 (41.0–105.8)	72.4 (35.3–97.0)
Body weight loss for 5 d (%)	4.9 (0.5–24.3)	3.9 (0.5–24.3)	6.4 (1.7–9.7)
Body weight gain for 1 month (g/d)	46.5 (12.3–69.4)	47.7 (12.3–69.4)	45.7 (30.4–57.9)
Plasma ghrelin (pmol/liter)	268.3 (133.0–481.7)	272.0 (133.0–421.0)	265.0 (172.3–481.7)

Values are medians, with the range in *parentheses*.

immunoradiometric assay kits (Daiichi Radioisotope Laboratories, Tokyo, Japan). Serum IGF-II was determined using an ELISA kit (Diagnostic Systems Laboratories, Inc., Sinsheim, Germany). Serum immunoreactive insulin (IRI) was determined using an immunoradiometric assay kit (Eiken Chemical, Tokyo, Japan). Serum IGF-binding protein-3 was determined using an RIA kit (Cosmic Corp., Tokyo, Japan). Serum leptin was determined using an RIA kit (Linco Research, Inc., St. Charles, MO).

Statistical analysis

All quantitative data are presented as the median and range. Pearson's correlations were used to examine relationships among clinical growth-related parameters and hormone levels. Differences between groups were evaluated by Mann-Whitney *U test or* Wilcoxon's signed rank test. Significance was considered to be $P < 0.05$. The analysis was conducted with StatView software (version 5.0 for Windows, SAS Institute, Inc., Cary, NC) or SPSS software (version 11.0J for Windows, SPSS. Inc., Chicago, IL).

Results

In 54 cord blood samples, plasma ghrelin concentrations ranged from 110.6–446.1 pmol/liter (median, 206.7 pmol/liter). Ghrelin concentrations were inversely correlated with birth weight (r = −0.40; $P = 0.002$), birth length (r = −0.36; $P = 0.007$), placental weight (r = −0.35; $P = 0.01$), birth weight/birth length ratio (r = −0.38; $P = 0.004$), Kaup index (r = −0.28; $P = 0.04$), IGF-I concentration (r = −0.49; $P = 0.0002$), and IGF-binding protein-3 concentration (r = −0.30; $P = 0.03$). Ghrelin concentrations in SGA newborn were significantly higher than those in AGA and LGA newborns ($P = 0.0008$ and $P = 0.02$, respectively; Figs. 1 and 2). No significant correlation was observed between ghrelin and GH concentrations (r = 0.22; $P = 0.12$; Fig. 2 and Tables 1 and 3).

As some anthropometrical parameters and IGF-I concentrations were inversely correlated with ghrelin concentrations, we performed some partial correlation analyses to determine which factor was predominantly correlated with them. The partial correlation analysis calculates a correlation between two variables while controlling for the effect of other additional variables. The partial correlation between ghrelin and IGF-I, while controlling for birth weight, birth length, or placental weight, was still significant (r = −0.43, −0.47, and

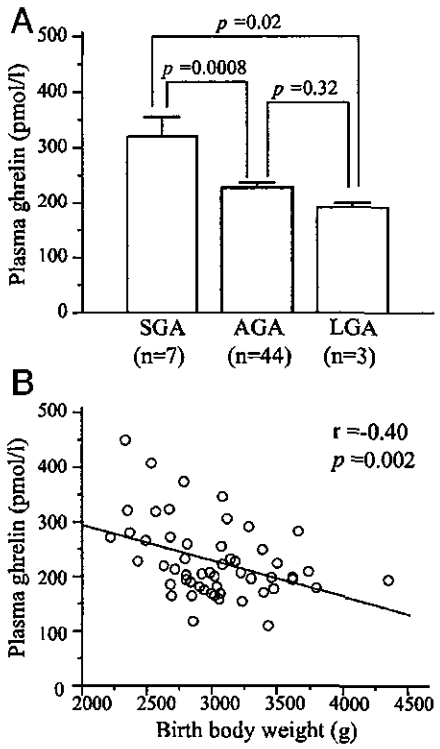

FIG. 1. A, Comparison of plasma ghrelin concentrations in SGA, AGA, and LGA newborns (mean ± SEM). Ghrelin concentrations in SGA newborns were significantly higher than those in AGA and LGA newborns. B, Inverse correlation between plasma ghrelin concentration and birth weight for the subjects in A. The *solid line* represents the regression line.

−0.39; $P = 0.002$, 0.001, and 0.006, respectively). On the other hand, the partial correlation between ghrelin and birth weight, birth length, or placental weight, while controlling for IGF-I, showed diminished statistical significance (r = −0.26, −0.28, and −0.19; $P = 0.07$, 0.05, and 0.20, respectively). These results suggest that IGF-I is a predominant factor that is correlated with the ghrelin concentration. The partial correlation between ghrelin and GH while controlling for each anthropometrical parameter or IGF-I confirmed the absence of a significant correlation between GH and ghrelin concentrations.

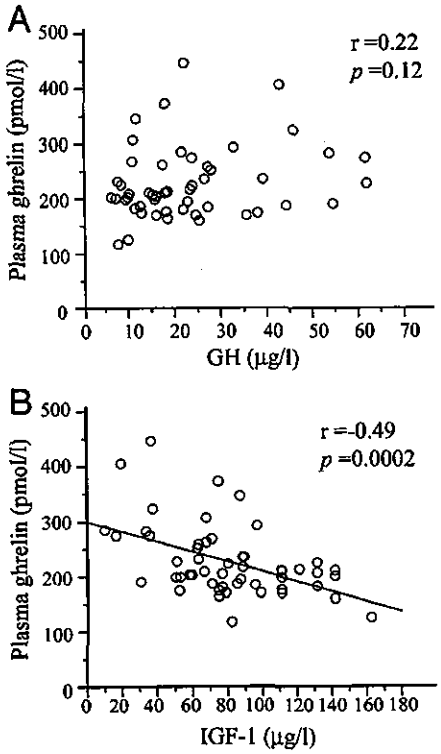

FIG. 2. A, Correlation between the ghrelin and GH concentrations in cord blood. No significant correlation was observed. B, A significant inverse correlation was seen between ghrelin and IGF-I concentrations in cord blood. The *solid line* represents the regression line.

In the eight newborns whose cord blood samples were collected from both the artery and vein, plasma ghrelin concentrations in the vein (median, 304.9 pmol/liter; range, 218.7–403.8 pmol/liter) were significantly higher than those in the artery (median, 287.5 pmol/liter; range, 181.9–392.4 pmol/liter; $P = 0.01$; Fig. 3).

In neonates, the plasma ghrelin concentrations ranged from 133.0–481.7 pmol/liter (median, 268.3 pmol/liter), which were significantly higher than those in cord blood ($P < 0.0001$). The sequential analysis of ghrelin concentrations after birth in 27 newborns confirmed that the ghrelin concentrations significantly increased during the early neonatal period ($P < 0.0001$; Fig. 4). The ghrelin concentrations in neonates did not significantly correlate with percentage body weight loss from birth to the sampling day (r = −0.13; $P = 0.37$), calorie intake per body weight on the sampling day (r = 0.04; $P = 0.80$), or the mean daily body weight gain during the first month of life (r = 0.23; $P = 0.13$; Table 4). No significant gender difference in ghrelin concentration was observed in either cord or neonatal blood (Tables 1 and 2).

Discussion

Our results showed that ghrelin is present in cord and neonatal blood at concentrations 1.5- to 2-fold higher than those found in normal weight adult (6, 9). The existence of ghrelin in cord blood is consistent with previous observations, although the use of nonextracted plasma is different compared with our study (16).

In adults, the plasma ghrelin concentration is mainly determined by the energy balance. Patients with anorexia nervosa and cachexia show elevated levels of ghrelin (6, 17), whereas obese subjects have reduced levels (8, 9). Thus, it is interesting that ghrelin concentrations were inversely correlated with several growth-related anthropometric parameters and the IGF-I concentration in the fetus. This result

TABLE 3A. Correlation coefficients between cord blood levels and size at birth

	Ghrelin	GH	IGF-I	IGF-II	IGFBP-3	Leptin	Insulin
Birth weight	−0.40[a]	−0.38[b]	0.30[b]	−0.05	0.23	0.53[c]	0.34[b]
Birth length	−0.36[b]	−0.41[a]	0.18	−0.23	0.15	0.25	0.24
Kaup index	−0.28[b]	−0.19	0.30[b]	0.19	0.23	0.54[c]	0.27
Body weight/body length	−0.38[a]	−0.32[b]	0.32[b]	0.05	0.25	0.56[c]	0.33[b]
Placental weight	−0.35[b]	−0.21	0.33[b]	−0.23	0.12	0.27	0.08

TABLE 3B. Correlation coefficients between cord blood levels

	Median (range)	Ghrelin	GH	IGF-I	IGF-II	IGFBP-3	Leptin
GH (μg/liter)	17.8 (5.7–61.0)	0.22					
IGF-I (μg/liter)	77 (12–160)	−0.49[d]	−0.47[d]				
IGF-II (μg/liter)	310 (181–481)	−0.13	0.08	0.02			
IGFBP-3 (mg/liter)	0.76 (0.45–1.28)	−0.30[e]	−0.40[f]	0.72[d]	0.22		
Leptin (μg/liter)	3.5 (0.8–13.8)	−0.12	−0.06	0.14	0.30[e]	0.28[e]	
Insulin (pmol/liter)	11.4 (6.0–100.2)	−0.17	−0.26	0.25	−0.15	0.20	0.01

IGFBP-3, IGF binding protein-3.
[a] $P < 0.005$.
[b] $P < 0.05$.
[c] $P < 0.0005$.
[d] $P < 0.0005$.
[e] $P < 0.05$.
[f] $P < 0.005$.

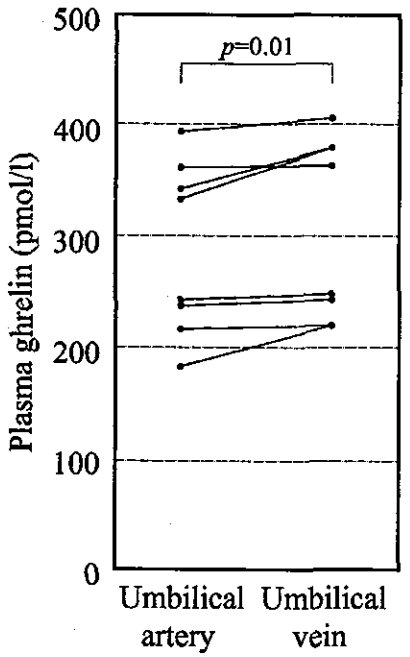

FIG. 3. Comparison of plasma ghrelin concentrations in arterial and venous cord blood. The plasma ghrelin concentrations in arterial and venous cord blood were compared in eight full-term newborns. Each *bar* links the arterial and venous ghrelin concentrations in each newborn. The plasma ghrelin concentrations in the vein were significantly higher than those in the artery ($P = 0.01$).

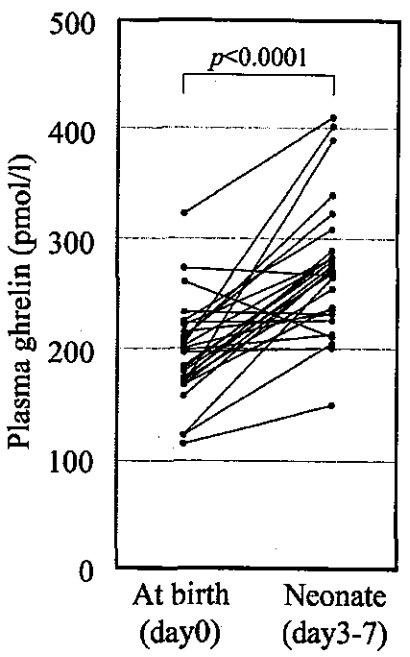

FIG. 4. Change in plasma ghrelin concentration after birth. The plasma ghrelin concentrations at birth (cord blood) and during the early neonatal period (postnatal d 3–7) were compared in 27 newborns (male, n = 18; female, n = 9). Each *bar* represents the change in the ghrelin concentration in each newborn. Ghrelin concentrations during the early neonatal period were significantly higher than those in cord blood ($P < 0.0001$).

suggests that the ghrelin concentration might be mainly regulated in a fetal growth-related manner *in utero*, as if its role was to accelerate fetal growth. In an experimental study, a significant level of GH secretagogue receptor expression was observed in the fetal rat hypothalamus, pituitary, and brainstem (18). However, fetal GH may not contribute to fetal growth itself to a large extent, as patients with Pit-1 gene or GH-1 gene deficiency, which are congenital disorders of pituitary GH synthesis, show nearly normal fetal growth. Fetal growth essentially depends on the energy transport from the mother. A positive correlation between size at birth and cord blood IGF-I concentration has been reported (19, 20). Considering that the ghrelin concentration correlated not with GH but with the IGF-I concentration, and the remarkable elevation of the ghrelin concentration in SGA newborns, negative feedback regulation between fetal growth and the ghrelin concentration may not originate in the fetal GH axis, but, rather, in feto-maternal energy transport, which would affect fetal growth and the IGF-I level.

In the rat, ghrelin mRNA is expressed at a very low level in the fetal stomach (21, 22), whereas significant expression is observed in the placenta (14). In humans, ghrelin mRNA is also expressed in the placenta (14). Therefore, it is possible that some of the ghrelin in the fetal circulation might originate from the placenta, like leptin, and regulate feto-maternal energy transport locally, as ghrelin concentrations in the umbilical vein were significantly higher than those in the artery. In addition, immunohistochemical studies of the human fetus showed that ghrelin-immunoreactive cells were fairly well represented in the stomach, duodenum, pancreas, and lung from wk 10 of gestation (23, 24). The contributions of these peripheral organs may also be taken into consideration.

TABLE 4. Correlation coefficients between neonatal ghrelin levels and energy balance

	Ghrelin (*P*)
Birth weight	−0.21 (0.16)
Body weight	−0.17 (0.26)
Body length	−0.22 (0.14)
Kaup index	−0.10 (0.49)
Mean calorie intake[a]	0.04 (0.80)
Body weight loss[b]	−0.13 (0.37)
Body weight gain[c]	0.23 (0.13)

[a] Calorie intake per body weight on sampling day (kilocalories per kilogram).
[b] Percentage body weight loss from birth to sampling day (percentage).
[c] Mean daily body weight gain during first month of life (grams per day).

In our previous study the leptin concentration rapidly decreased after birth and remained at a low level during the early neonatal period (11). In contrast, plasma ghrelin concentrations at approximately 5 d after birth were significantly higher than those in cord blood. After birth, the energy supply through the placenta is interrupted, and a newborn needs to start taking milk for growth. Thus, in contrast to leptin, which reduces energy intake, it is reasonable for the ghrelin concentration in neonates to increase to stimulate appetite and give a positive energy balance. However, in rat stomach, the expression of ghrelin mRNA in neonates is lower than

that in adults (21, 22). The origin of the high concentration of ghrelin in neonates may need further investigation, with regard not only to its synthesis and secretion, but also to its degradation and clearance during the neonatal period.

In summary, this study demonstrates the existence of ghrelin in fetal and neonatal blood at rather high concentrations, an inverse correlation between the cord blood ghrelin concentrations and fetal growth-related parameters, and a significant elevation of the ghrelin concentration during the early neonatal period. Further study of ghrelin concentrations in fetuses and neonates with pathological status, such as premature delivery or severe intrauterine growth retardation, may provide useful information about regulation of the ghrelin concentration and its role during the fetal and neonatal periods.

Acknowledgments

We thank Dr. K. Maeda and Prof. M. Irahara (Department of Obstetrics and Gynecology, University of Tokushima School of Medicine) for their support with the sample collection.

Received August 22, 2002. Accepted July 31, 2003.

Address all correspondence and requests for reprints to: Ichiro Yokota, M.D., Department of Pediatrics, University of Tokushima School of Medicine, 3-Kuramoto cho, Tokushima 770-8503, Japan. E-mail: yichiro@clin.med.tokushima-u.ac.jp.

This work was supported in part by a grant from the Foundation for Growth Science (Tokyo, Japan).

References

1. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K** 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656–660
2. **Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K** 2000 Ghrelin strongly stimulates growth hormone release in humans. J Clin Endocrinol Metab 85:4908–4911
3. **Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S** 2001 A role for ghrelin in the central regulation of feeding. Nature 409:194–198
4. **Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I** 2000 Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression. Endocrinology 141:4797–4800
5. **Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M** 2000 Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 141: 4255–4261
6. **Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nakao K** 2001 Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. J Clin Endocrinol Metab 86:4753–4758
7. **Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS** 2001 A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50:1714–1719
8. **Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML** 2001 Circulating ghrelin levels are decreased in human obesity. Diabetes 50: 707–709
9. **Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozoe S, Hosoda H, Kangawa K, Matsukura S** 2002 Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. J Clin Endocrinol Metab 87:240–244
10. **Matsuda J, Yokota I, Iida M, Murakami T, Naito E, Ito M, Shima K, Kuroda Y** 1997 Serum leptin concentration in cord blood: relationship to birth weight and gender. J Clin Endocrinol Metab 82:1642–1644
11. **Matsuda J, Yokota I, Iida M, Murakami T, Yamada M, Saijo T, Naito E, Ito M, Shima K, Kuroda Y** 1999 Dynamic changes in serum leptin concentrations during the fetal and neonatal periods. Pediatr Res 45:71–75
12. **Matsuda J, Yokota I, Tsuruo Y, Murakami T, Ishimura K, Shima K, Kuroda Y** 1999 Developmental changes in long-form leptin receptor expression and localization in rat brain. Endocrinology 140:5233–5238
13. **Masuzaki H, Ogawa Y, Sagawa N, Hosoda K, Matsumoto T, Mise H, Nishimura H, Yoshimasa Y, Tanaka I, Mori T, Nakao K** 1997 Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. Nat Med 3:1029–1033
14. **Gualillo O, Caminos J, Blanco M, Garcia-Caballero T, Kojima M, Kangawa K, Dieguez C, Casanueva F** 2001 Ghrelin, a novel placental-derived hormone. Endocrinology 142:788–794
15. **Hosoda H, Kojima M, Matsuo H, Kangawa K** 2000 Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun 279:909–913
16. **Chanoine JP, Yeung LP, Wong AC, Birmingham CL** 2002 Immunoreactive ghrelin in human cord blood: relation to anthropometry, leptin, and growth hormone. J Pediatr Gastroenterol Nutr 35:282–286
17. **Nagaya N, Uematsu M, Kojima M, Date Y, Nakazato M, Okumura H, Hosoda H, Shimizu W, Yamagishi M, Oya H, Koh H, Yutani C, Kangawa K** 2001 Elevated circulating level of ghrelin in cachexia associated with chronic heart failure: relationships between ghrelin and anabolic/catabolic factors. Circulation 104:2034–2038
18. **Katayama M, Nogami H, Nishiyama J, Kawase T, Kawamura K** 2000 Developmentally and regionally regulated expression of growth hormone secretagogue receptor mRNA in rat brain and pituitary gland. Neuroendocrinology 72:333–340
19. **Klauwer D, Blum WF, Hanitsch S, Rascher W, Lee PD, Kiess W** 1997 IGF-I, IGF-II, free IGF-I and IGFBP-1, -2 and -3 levels in venous cord blood: relationship to birthweight, length and gestational age in healthy newborns. Acta Paediatr 86:826–833
20. **Ong K, Kratzsch J, Kiess W, Costello M, Scott C, Dunger D** 2000 Size at birth and cord blood levels of insulin, insulin-like growth factor I (IGF-I), IGF-II, IGF-binding protein-1 (IGFBP-1), IGFBP-3, and the soluble IGF-II/mannose-6-phosphate receptor in term human infants. The ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood. J Clin Endocrinol Metab 85:4266–4269
21. **Lee HM, Wang G, Englander EW, Kojima M, Greeley GH Jr** 2002 Ghrelin, a new gastrointestinal endocrine peptide that stimulates insulin secretion: enteric distribution, ontogeny, influence of endocrine, and dietary manipulations. Endocrinology 143:185–190
22. **Hayashida T, Nakahara K, Mondal MS, Date Y, Nakazato M, Kojima M, Kangawa K, Murakami N** 2002 Ghrelin in neonatal rats: distribution in stomach and its possible role. J Endocrinol 173:239–245
23. **Rindi G, Necchi V, Savio A, Torsello A, Zoli M, Locatelli V, Raimondo F, Cocchi D, Solcia E** 2002 Characterization of gastric ghrelin cells in man and other mammals: studies in adult and fetal tissues. Histochem Cell Biol 117: 511–519
24. **Volante M, Fulcheri E, Allia E, Cerrato M, Pucci A, Papotti M** 2002 Ghrelin expression in fetal, infant, and adult human lung. J Histochem Cytochem 50:1013–1021

ELSEVIER

Neuroscience Letters 342 (2003) 183–186

Neuroscience Letters

www.elsevier.com/locate/neulet

Growth hormone secretagogue receptor expression in the cells of the stomach-projected afferent nerve in the rat nodose ganglion

Ichiro Sakata[a], Mami Yamazaki[a], Kinji Inoue[a], Yujiro Hayashi[b], Kenji Kangawa[c], Takafumi Sakai[a,*]

[a]*Department of Regulation Biology, Faculty of Science, Saitama University, 255 Shimo-ohkubo, Saitama 338-8570, Japan*
[b]*Suntory Institute of Medicine Research Development, 2716-1 Kurakake, Akaiwa, Chiyoda, Gunma 370-0503, Japan*
[c]*Department of Biochemistry, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan*

Received 29 January 2003; received in revised form 24 February 2003; accepted 27 February 2003

Abstract

Growth hormone secretagogue receptor (GHS-R) is widely expressed in various regions of the body, such as the brain, pituitary gland, heart and gastrointestinal tract. Recently, ghrelin, an endogenous ligand for GHS-R, was found in the rat stomach, and several studies have suggested that ghrelin acts via the vagal afferent nerve. In this study, we studied the expression of GHS-R mRNA in the rat nodose ganglion by reverse transcriptase-polymerase chain reaction and in situ hybridization, the results of which clearly demonstrated the presence of GHS-R mRNA and GHS-R producing cells in the rat nodose ganglion. We also studied the retrograde tracing of nodose ganglion cells to the stomach and found that some GHS-R mRNA-expressing cells contain the retrograde rebelling. Our results provide direct morphological evidence that GHS-R is produced in afferent neurons of the nodose ganglion and suggest that ghrelin signals from the stomach are transmitted to the brain via vagal afferent nerves.

Keywords: Growth hormone secretagogue receptor; Nodose ganglion; Rat; Reverse transcriptase-polymerase chain reaction; In situ hybridization; Retrograde tracing; Fluorogold; Stomach

Growth hormone secretagogue (GHS) is a synthetic peptidyl or non-peptidyl compound, and its physiological effects such as GH release and food intake are mediated by the growth hormone secretagogue receptor (GHS-R) [4]. Ghrelin, a novel endogenous ligand for GHS-R, was recently discovered in the rat stomach and found to consist of 28 amino acids, the third serine residue of which is *n*-octanoylated [13]. It is well known that ghrelin and GHS-R are widely expressed in central and peripheral tissues [9,10, 17,18] and that circulating ghrelin is mainly produced in the stomach [1]. Peripheral intravenous administration of ghrelin stimulates not only GH release but also food intake [19], gastric acid secretion and gastric motor activity [8,14]. Interestingly, it has been reported that most of these effects are abolished by either atropine treatment or vagotomy [7,8, 14].

On the other hand, visceral sensory neurons of the nodose ganglion innervate many organs such as cardiovascular, respiratory and gastrointestinal organs [3,21]. It has also been shown that they have peripheral axons in the vagus nerve and contain many receptors, i.e. cardiac receptors, chemoreceptors and gastric receptors [3,21]. Moreover, it has been demonstrated that many gastrointestinal peptides regulate short-term food intake via vagal afferent neurons [3,21]. For example, it has been shown that leptin and cholecystokinin (CCK) receptors are expressed in the nodose ganglion and transmit leptin or CCK signals from the gastrointestinal tract to the central nervous system [5,6, 16].

It has been shown that ghrelin suppressed firing of the vagal afferent nerve [2,7]. Therefore, in order to determine whether gastric ghrelin signals act through vagal afferent neurons, we studied the existence of GHS-R mRNA in the nodose ganglion by RT-PCR and in situ hybridization, and the projection to the stomach was determined by retrograde tracing with Fluorogold (FG).

Male Wistar rats (weighing 200–240 g) were purchased

* Corresponding author. Tel.: +81-48-858-3422; fax: +81-48-858-3422.
E-mail address: tsakai@seitai.saitama-u.ac.jp (T. Sakai).

0304-3940/03/$ - see front matter
doi:10.1016/S0304-3940(03)00294-5

from Japan Laboratory Animals, Inc. (Tokyo, Japan) and were housed under controlled conditions (23 °C, lights on from 08:00 to 20:00 h). The rats were also provided with standard rat chow and water ad libitum. All procedures used in this study were performed in accordance with institutional guidelines for animal care at Saitama University.

Animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused first with 10 mM phosphate-buffered saline (PBS, pH 7.5), and then with 4% paraformaldehyde in 50 mM phosphate buffer (PB, pH 7.4). Nodose ganglia were quickly removed from the rats and postfixed with a microwave for 15 s as described elsewhere [12]. The tissues were dehydrated with an ascending ethanol series and then immersed in xylene and embedded in Paraplast (Oxford Labware, MO, USA). Serial sections (8 μm thick) were mounted on silane (ShinEtsu Chemicals, Tokyo, Japan)-coated slides.

Total RNA was extracted from the rat nodose ganglion using an RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Trace DNA contamination was removed by DNase digestion. RT-PCR was performed using a one-step RT-PCR kit (QIAGEN, Germany) according to the manufacturer's instructions. The following primers were designed to amplify GHS-R (fragment size: 728 bp): sense primer, TCACTATGCTGGTGGTGTCC; antisense primer, CAGGTTGCAGTACTGGCTGA. The following primers were used to amplify the rat glyceraldehydes 3-phosphatase dehydrogenase (GAPDH) as an internal control (fragment size: 457 bp): sense primer, CCATCACCATCTTCCAGGAG; antisense primer, TTCAGCTCTGGGATGACCTT. Reactions were performed using an *i*-Cycler (Bio Rad, MA, USA). The cycle profile was programmed as follows: 30 min at 60 °C (reverse transcription), 15 min at 95 °C (initial PCR activation step), 1 min at 94 °C (denaturation), 1 min at 60 °C (annealing) and 1 min at 72 °C (extension). Forty cycles of the profile were run, and the final extension step was increased to 10 min at 72 °C. To examine the specificity of PCR reactions, the PCR products of GHS-R were treated with the restriction enzyme *Sma*I for 1 h at 37 °C.

For retrograde tracing, 1 μl of 1% water-diluted solution of the fluorescent tracer Fluorogold (FG, Fluorochrom, CO, USA) was injected into five to seven points of the gastric wall. After 5 days, rats were killed and nodose ganglia were removed for preparation of frozen sections.

For *in situ* hybridization, we used digoxigenin (DIG)-labeled GHS-R anti-sense and sense RNA probes (positions 185–790, 2826–2934, GenBank # U94321) synthesized from pituitary cDNA using a labeling kit (Boehringer Mannheim, GmbH, Germany). GHS-R antisense and sense probes performed alkaline hydrolysis to 150 bp. The sections were deparaffinized with xylene, rehydrated through descending concentrations of ethanol, and washed in PBS for 15 s twice. The sections were then washed in PBS, treated with 8.0 μg/ml proteinase K for 30 min at 37 °C, and fixed with 4% paraformaldehyde in 50 mM PB (pH 7.4). After washing in PBS for 1 min, the sections were incubated with 0.2 M HCl in water and then washed in PBS for 1 min. The sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, washed in PBS for 1 min, and then immersed in a graded ethanol series (70%, 80%, 90%) for 15 s each. Next, the sections were treated with 100% ethanol for 15 s twice and dried for 20 min. Finally, 2 ng/μl of each DIG-labeled RNA probe in a hybridization buffer containing 50% formamide, 3 × SSC, 0.12 M PB, 1 × Denhardt's solution, 10% dextrane sulfate, 125 μg/ml yeast tRNA and 0.1 mg/ml salmon sperm DNA was dropped onto the section, and the section was covered with Parafilm (American Can Company, CT, USA) and incubated for 16 h at 47 °C in a humidity chamber. The covers were removed by soaking the slides in 5 × SSC, and the slides were immersed in 2 × SSC containing 50% formamide for 30 min. The sections were then treated with TNE (10 mM Tris–HCl, pH 7.6, 500 mM NaCl, 1 mM EDTA, pH 8.0) for 10 min and next with RNase A (50 μg/ml in TNE) for 30 min at 37 °C. The sections were immersed in TNE for 10 min at 37 °C and washed with 2 × SSC for 20 min at 55 °C and then with 0.2 × SSC for 20 min, twice each. The sections were incubated for 5 min in buffer-1 (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.01% Tween 20), immersed in 1.5% blocking reagent (Boehringer Mannheim, GmbH, Germany) in buffer-1 for 1 h at 37 °C, and then washed in buffer-1 for 5 min. After washing, the sections were incubated with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics Corporation, IN, USA) diluted 1:2000 in buffer-1 for 16 h at 4 °C. This anti-DIG antibody was absorbed with extracted powder of the brain before use. The sections were washed in buffer-1 for 15 min twice and in buffer-2 (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 3 min. A chromagen solution (337 μg/ml NBT, 175 μg/ml BCIP in buffer-2) was added, and the sections were incubated until a visible signal was detected. The sections were then washed with distilled water, dehydrated with a methanol series, cleared in xylene, mounted with Entellan, and viewed under a light microscope (BX60, Olympus, Tokyo, Japan). In this study, instead of DEPC-treated water we used Gengard water (Gradient A10, Millipore, Tokyo, Japan) as an RNase-free water.

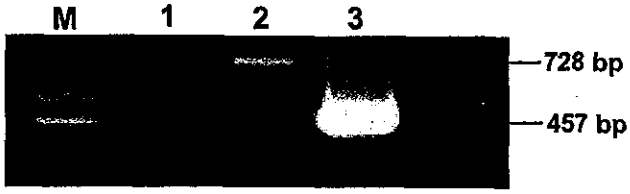

Fig. 1. Determination of expression of GHS-R 1a mRNA by one-step RT-PCR analysis in the rat nodose ganglion. Agarose gel (2%) electrophoresis of RT-PCR products using appropriate primers. GHS-R mRNA was found in the rat nodose ganglion. GAPDH was used as an internal control. In the negative control experiment, PCR was performed without primers. M, DNA molecular weight; 1, negative control; 2, GHS-R 1a; 3, GAPDH.

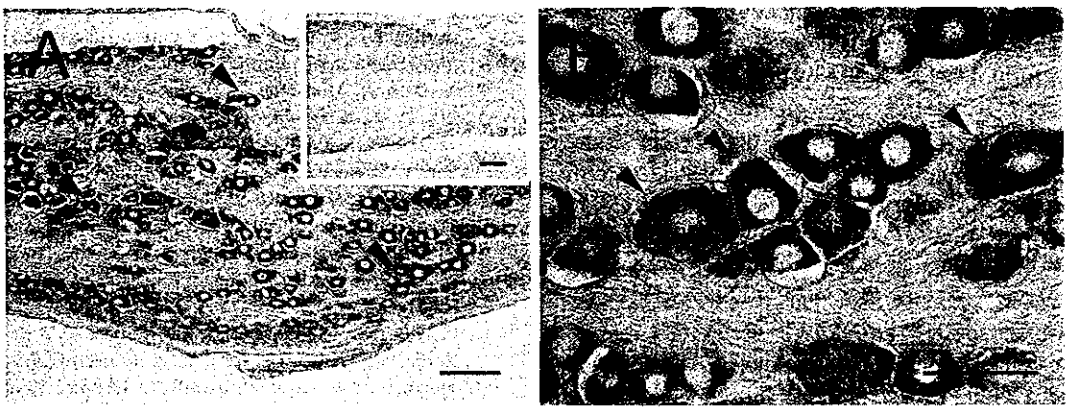

Fig. 2. Microphotographs of GHS-R mRNA-expressing cells in the nodose ganglion. (A) GHS-R mRNA-expressing cells (arrowheads) were found throughout the nodose ganglion. No positive cells were found when the sense probe was used (inset). (B) High magnification of the image of GHS-R mRNA-expressing cells (arrowheads). Bar: 200 μm (A), 50 μm (B).

RT-PCR analysis revealed that GHS-R (728 bp) mRNA was expressed in the rat nodose ganglion (Fig. 1). The specificity of the PCR for GHS-R was determined by digesting with the restriction enzyme *Sma*I, and the predicted sizes of the PCR product were confirmed (data not shown).

GHS-R mRNA-expressing cells were found throughout the caudal regions of the nodose ganglion toward the petrosal ganglion, and two kinds of stained cells, weakly and strongly stained cells, were scattered throughout the nodose ganglion (Fig. 2). The sizes of GHS-R mRNA-expressing cells varied from 25 to 40 μm in diameter. No positive cells were found when the sense probe was used, indicating the specificity of this reaction (Fig. 2A, inset). Results of retrograde tracing studies showed the coexistence of FG-labeled cells and GHS-mRNA-expressing cells in the nodose ganglion. However, the number of FG-labeled cells was much less than that of GHS-R mRNA-expressing cells (Fig. 3), and all of the FG-labeled cells expressed GHS-R mRNA (data not shown).

In this study, we used primers corresponding to GHS-R type 1a but not type 1b. It has been reported that GHS-R type 1a consists of seven transmembrane (TM) domains and three intracellular and three extracellular loops, but that GHS-R type 1b lacks TM 6 and 7, indicating that GHS-R type 1a is a bioactive form [11,15]. Therefore, the results obtained in this study suggest that functional GHS-Rs are produced in the rat nodose ganglion.

It has been reported that intravenous administration of ghrelin increased gastric acid secretion and gastric motor activity in both amplitude and frequency in a dose-dependent manner [14]. However, lateral cervical vagotomy and atropine treatment completely abolished the intravenously administered ghrelin-induced gastric acid secretion and gastric motor activity [14]. Furthermore, truncal vagotomy eliminated the effect of intraperitoneally administered ghrelin-induced food intake [2]. The results of the present study and previous studies suggest that at least a part of the ghrelin action is mediated through vagal afferent nerves in vivo. Further studies are needed to determine how peripheral ghrelin information is transmitted to the brain and regulates food intake, gastric acid secretion and gastric motor activity.

Ghrelin that is *n*-octanoylated at the third amino acid residue is biologically unstable [1]. The half-life of physiologically active ghrelin in blood is relatively short

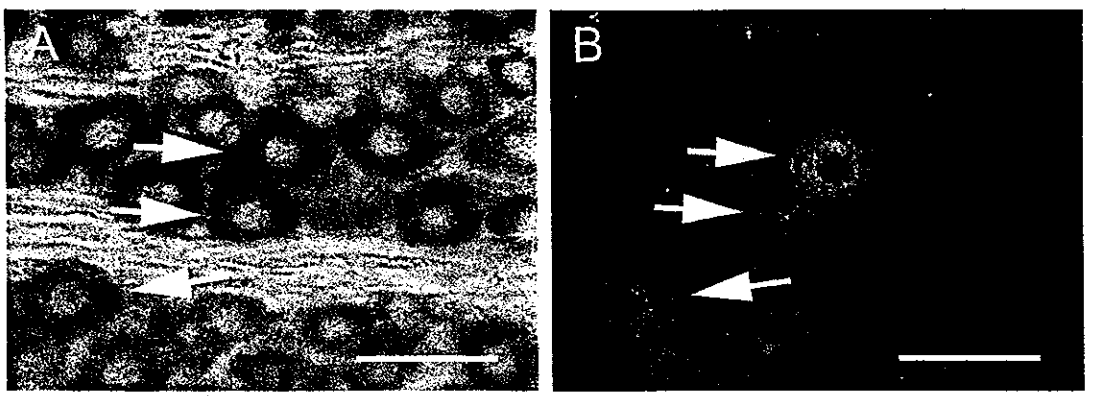

Fig. 3. Double visualization of FG-labeled cells and GHS-R mRNA-expressing cells in the same section. Some the GHS-R mRNA-expressing cells (B) were labeled by Fluorogold (FG), a fluorescent tracer. FG-labeled cells were scattered throughout the nodose ganglion. Bar: 50 μm (A,B).

because *n*-octanoylated ghrelin is easily degraded by plasma esterase and becomes des-*n*-octanoyl ghrelin, a non-physiological form [1]. It is reasonable to assume that ghrelin secreted from the stomach rapidly becomes biological inactive by passing from the portal vein to the liver. Therefore, it is questionable whether gastric ghrelin directly stimulates GH release from the pituitary gland. Moreover, using an isolated anterior pituitary cell perifusion system, we demonstrated that ghrelin stimulates GH release in vitro in a weaker manner than that in vivo [20]. These results suggest that gastric ghrelin stimulates GH release from pituitary somatotropes by an indirect route. Our results taken together with the results of these studies suggest that ghrelin also stimulates GH release via vagal afferent nerves.

Moreover, during the course of our studies, it was recently reported by Date et al. that ghrelin-induced feeding and GH secretion were abolished by vagotomy or perivagal application of capsaicin, and the presence of the ghrelin receptors in vagal afferent neurons was revealed by using RT-PCR and in situ hybridization histochemistry [7]. The results of our study showing the existence of GHS-Rs in the stomach-projected afferent nerve support the findings of Date et al.

In this study, we clearly demonstrated the presence of GHS-Rs in the afferent nerve in the nodose ganglion projected to the stomach, and this finding suggests that some gastric ghrelin acts through a neural pathway.

Acknowledgements

We are grateful to Dr H. Ichikawa (Okayama University Dental School, Okayama) for his valuable discussion and technical suggestions.

References

[1] H. Ariyasu, K. Takaya, T. Tagami, Y. Ogawa, K. Hosoda, T. Akamizu, M. Suda, T. Koh, K. Natsui, S. Toyooka, G. Shirakami, T. Usui, A. Shimatsu, K. Doi, H. Hosoda, M. Kojima, K. Kangawa, K. Nakao, Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans, J. Clin. Endocrinol. Metab. 86 (2001) 4753–4758.

[2] A. Asakawa, A. Inui, T. Kaga, H. Yuzuriha, T. Nagata, N. Ueno, S. Makino, M. Fujimiya, A. Niijima, M.A. Fujino, M. Kasuga, Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin, Gastroenterology 120 (2001) 337–345.

[3] H.R. Berthoud, W.L. Neuhuber, Functional and chemical anatomy of the afferent vagal system, Auton. Neurosci. 85 (2000) 1–17.

[4] C.Y. Bowers, Growth hormone-releasing peptide (GHRP), Cell. Mol. Life Sci. 54 (1998) 1316–1329.

[5] C. Broberger, K. Holmberg, T.J. Shi, G. Dockray, T. Hokfelt, Expression and regulation of cholecystokinin and cholecystokinin receptors in rat nodose and dorsal root ganglia, Brain Res. 903 (2001) 128–140.

[6] M. Buyse, M.L. Ovesjo, H. Goiot, S. Guilmeau, G. Peranzi, L. Moizo, F. Walker, M.J. Lewin, B. Meister, A. Bado, Expression and regulation of leptin receptor proteins in afferent and efferent neurons of the vagus nerve, Eur. J. Neurosci. 14 (2001) 64–72.

[7] Y. Date, N. Murakami, K. Toshinai, S. Matsukura, A. Niijima, H. Matsuo, K. Kangawa, M. Nakazato, The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats, Gastroenterology 123 (2002) 1120–1128.

[8] Y. Date, M. Nakazato, N. Murakami, M. Kojima, K. Kangawa, S. Matsukura, Ghrelin acts in the central nervous system to stimulate gastric acid secretion, Biochem. Biophys. Res. Commun. 280 (2001) 904–907.

[9] S. Gnanapavan, B. Kola, S.A. Bustin, D.G. Morris, P. McGee, P. Fairclough, S. Bhattacharya, R. Carpenter, A.B. Grossman, M. Korbonits, The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans, J. Clin. Endocrinol. Metab. 87 (2002) 2988.

[10] H. Hosoda, M. Kojima, H. Matsuo, K. Kangawa, Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue, Biochem. Biophys. Res. Commun. 279 (2000) 909–913.

[11] A.D. Howard, S.D. Feighner, D.F. Cully, J.P. Arena, P.A. Liberator, C.I. Rosenblum, M. Hamelin, D.L. Hreniuk, O.C. Palyha, J. Anderson, P.S. Paress, C. Diaz, M. Chou, K.K. Liu, K.K. McKee, S.S. Pong, L.Y. Chaung, A. Elbrecht, M. Dashkevicz, R. Heavens, M. Rigby, D.J. Sirinathsinghji, D.C. Dean, D.G. Melillo, A.A. Patchett, R. Nargund, P.R. Griffin, J.A. Demartino, S.K. Gupta, J.M. Schaeffer, R.G. Smith, L.H. Van der Ploeg, A receptor in pituitary and hypothalamus that functions in growth hormone release, Science 273 (1996) 974–977.

[12] H. Ichikawa, C.J. Helke, Coexistence of s100beta and putative transmitter agents in vagal and glossopharyngeal sensory neurons of the rat, Brain Res. 800 (1998) 312–318.

[13] M. Kojima, H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, K. Kangawa, Ghrelin is a growth hormone-releasing acylated peptide from stomach, Nature 402 (1999) 656–660.

[14] Y. Masuda, T. Tanaka, N. Inomata, N. Ohnuma, S. Tanaka, Z. Itoh, H. Hosoda, M. Kojima, K. Kangawa, Ghrelin stimulates gastric acid secretion and motility in rats, Biochem. Biophys. Res. Commun. 276 (2000) 905–908.

[15] K.K. McKee, O.C. Palyha, S.D. Feighner, D.L. Hreniuk, C.P. Tan, M.S. Phillips, R.G. Smith, L.H. Van der Ploeg, A.D. Howard, Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors, Mol. Endocrinol. 11 (1997) 415–423.

[16] C. Peiser, J. Springer, D.A. Groneberg, G.P. McGregor, A. Fischer, R.E. Lang, Leptin receptor expression in nodose ganglion cells projecting to the rat gastric fundus, Neurosci. Lett. 320 (2002) 41–44.

[17] I. Sakata, K. Nakamura, M. Yamazaki, M. Matsubara, Y. Hayashi, K. Kangawa, T. Sakai, Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract, Peptides 23 (2002) 531–536.

[18] I. Sakata, T. Tanaka, M. Matsubara, M. Yamazaki, S. Tani, Y. Hayashi, K. Kangawa, T. Sakai, Postnatal changes in ghrelin mRNA expression and in ghrelin-producing cells in the rat stomach, J. Endocrinol. 174 (2002) 463–471.

[19] A.M. Wren, C.J. Small, H.L. Ward, K.G. Murphy, C.L. Dakin, S. Taheri, A.R. Kennedy, G.H. Roberts, D.G.A. Morgan, M.A. Ghatei, S.R. Bloom, The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion, Endocrinology 141 (2000) 4325–4328.

[20] M. Yamazaki, K. Nakamura, H. Kobayashi, M. Matsubara, Y. Hayashi, K. Kangawa, T. Sakai, Regulational effect of ghrelin on growth hormone secretion from perifused rat anterior pituitary cells, J. Neuroendocrinol. 14 (2002) 156–162.

[21] H. Zhuo, H. Ichikawa, C.J. Helke, Neurochemistry of the nodose ganglion, Prog. Neurobiol. 52 (1997) 79–107.

Increased Plasma Ghrelin Level in Lung Cancer Cachexia[1]

Yoshito Shimizu, Noritoshi Nagaya,[2] Takeshi Isobe, Michinori Imazu, Hiroyuki Okumura, Hiroshi Hosoda, Masayasu Kojima, Kenji Kangawa, and Nobuoki Kohno

The Second Department of Internal Medicine, Hiroshima University School of Medicine, Hiroshima [Y. S., T. I., M. I., N. K.], and Departments of Internal Medicine [N. N., H. O.] and Biochemistry [H. H., M. K., K. K.], National Cardiovascular Center, Research Institute, Osaka 565-8565, Japan

ABSTRACT

Purpose: **Ghrelin, a novel growth hormone-releasing peptide, has been shown to cause a positive energy balance by stimulating food intake and inducing adiposity. We sought to investigate the pathophysiology of ghrelin in cachexia associated with lung cancer.**

Experimental design: **Plasma ghrelin level was measured in 43 patients with lung cancer and 21 control subjects. Patients with lung cancer were divided into two groups: patients with cachexia (n = 21) and those without cachexia (n = 22).**

Results: **Plasma ghrelin level did not significantly differ between all patients with lung cancer and controls (157 ± 10 *versus* 132 ± 8 fmol/ml, P = 0.1). However, plasma ghrelin level was significantly higher in patients with cachexia than in those without cachexia (180 ± 17 *versus* 135 ± 10 fmol/ml, P = 0.011). Furthermore, plasma ghrelin level increased significantly in patients with decreased food intake after chemotherapy (from 136 ± 11 fmol/ml to 170 ± 16 fmol/ml on day 8, 179 ± 20 fmol/ml on day 21 after start of chemotherapy), although plasma ghrelin level did not significantly change in those without decreased food intake.**

Conclusions: **Baseline plasma ghrelin level was elevated in cachectic patients with lung cancer, and follow-up plasma ghrelin level increased in patients with anorexia after chemotherapy. Considering the positive energy effects induced by ghrelin, increased ghrelin may represent a compensatory mechanism under catabolic–anabolic imbalance in cachectic patients with lung cancer.**

Received 1/10/02; revised 9/19/02; accepted 10/3/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the Research Grant for Cardiovascular Disease (12C-2) from the Ministry of Health, Labour, and Welfare and the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

[2] To whom requests for reprints should be addressed, at Department of Internal Medicine, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Phone: 81-6-6833-5012; Fax: 81-6-6833-9865; E-mail: nagayann@hsp.ncvc.go.jp.

INTRODUCTION

Cancer cachexia, which is a catabolic state characterized by weight loss and muscle wasting, occurs in some patients with advanced lung cancer (1, 2) and is a strong independent risk factor for mortality in such patients (3). Furthermore, chemotherapy may cause anorexia because of its toxicity (4). Thus, impaired energy balance is one of the most important problems in patients with lung cancer.

Ghrelin is a novel GH[3]-releasing peptide, isolated from the stomach, which is identified as an endogenous ligand for growth hormone secretagogues receptor (5). Recently, ghrelin has been shown to cause a positive energy balance by decreasing fat utilization through GH-independent mechanisms (6). In addition, both intracerebroventricular and peripheral administration of ghrelin have been shown to elicit potent, long-lasting stimulation of food intake via activation of neuropeptide Y neurons in the hypothalamic arcuate nucleus (7–9). These findings raise the possibility that ghrelin plays an important role in the regulation of metabolic balance. However, little information is available regarding the pathophysiology of ghrelin in cachexia associated with lung cancer. In addition, the relationship between plasma ghrelin and anorexia induced by chemotherapy remains unknown.

Thus, the purposes of this study were: (*a*) to investigate whether plasma ghrelin level is associated with cachexia and disease severity in patients with lung cancer; and (*b*) to examine whether plasma ghrelin level is influenced by anorexia after chemotherapy.

PATIENTS AND METHODS

Patient Characteristics. Forty-three patients with cytologically or histologically established lung cancer at Hiroshima University Hospital between January and July 2001 were included in this study. Patients with one or more of the following conditions were excluded: (*a*) recent antineoplastic therapy such as chemotherapy, radiation therapy, or surgery; (*b*) the presence of active infection or gastric ulcer; (*c*) other primary cachectic states such as thyroid disease or severe liver disease; and (*d*) chronic renal impairment (serum creatinine level ≥ 1.5 mg/dl). None of the patients was receiving parenteral or tube feeding or had undergone gasterectomy. ECOG performance status was used as an index of the patients' general condition. Food intake was measured using a self-assessment numerical rating scale ranging from 0 to 10, where 0 indicates absolutely no appetite, and 10 indicates an extremely good appetite. The study included 21 healthy control subjects (17 men and 4 women, mean age = 63, range = 33–78 years). Healthy control subjects were healthy hospital personnel without recent body

[3] The abbreviations used are: GH, growth hormone; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; RIA, radioimmunoassay; CI, confidence interval.

weight loss or gain, and they had no acute or chronic disease and no regular medication. All patients gave informed and written consent, and the study was approved by the local committee and conducted in accordance with good clinical practice guidelines.

Patients were divided into two groups by the presence or absence of cachexia. Cachexia was defined clinically as documented nonintentional dry weight loss of >5 kg (all >10% of their previous normal weight) at least in ≤3 months (10). All cachectic patients also complained of weight loss. Consequently, 21 of 43 patients with lung cancer were defined as having cancer cachexia.

Blood Sampling and Assay for Plasma Ghrelin. Blood samples were taken from the antecubital vein between 7 and 8 a.m. after an overnight fast. The blood was immediately transferred into a chilled glass tube containing disodium EDTA (1 mg/ml) and aprotinin (500 units/ml) and centrifuged immediately at 4°C. Before RIA, plasma samples were extracted using Sep-Pak C18 cartridges (Waters, Milford, MA; Ref. 11). RIA for plasma ghrelin was performed as described previously (11). Briefly, a polyclonal antibody was raised against the COOH-terminal fragment [13–28] of rat ghrelin in a rabbit. A maleimide-activated mariculture keyhole limpet hemocyanin-[Cys 0]-ghrelin [13–28] conjugate was used for immunization. The RIA incubation mixture consisted of 100 μl of standard ghrelin or unknown sample, normal rabbit serum, and 200 μl of antiserum at a dilution of 1:10,000. After 12-h incubation at 4°C, 100 μl of ^{125}I-labeled ligand (15,000 cpm) were added to the mixture. After 36-h incubation at 4°C, 100 μl of goat antirabbit IgG antiserum were added. Free and bound tracers were separated by centrifugation at 3,000 rpm for 30 min after incubation for 24 h at 4°C. Pellet radioactivity was quantified using a gamma counter. This assay for plasma ghrelin was performed in one batch run and in a blinded fashion. The antiserum exhibited 100% cross-reactivity with rat and human ghrelin [13–28]. Intraobserver variability of ghrelin measurement was <6%, and its interobserver variability was <9%.

Change in Plasma Ghrelin Level in Patients with and without Decreased Food Intake after Chemotherapy. We examined the time course of plasma ghrelin level in 17 patients with inoperable clinical stage III or IV who underwent single agent chemotherapy or combination therapy. Patients undergoing chemotherapy were divided into two groups by the presence or absence of decreased food intake. Anorexia including decreased food intake was evaluated by the toxicity grading criteria of the National Cancer Institute-Common Toxicity Criteria. Decreased food intake was defined as toxicity at least grade 2 of the grading criteria and was observed in 10 of the 17 patients on day 8 after chemotherapy. Thus, blood sampling for ghrelin measurement was performed in patients with decreased food intake (n = 10) and those without (n = 7) at baseline and on days 8 and 21 after the start of the first chemotherapy course. The patients were required to have measurable disease, ECOG performance status of 0–2, adequate bone marrow, and adequate renal and liver function. Changes in body weight after chemotherapy were determined with the following formula: % changes in body weight = (body weight on days 8 or 21 − baseline body weight)/baseline body weight × 100.

There was no significant difference in age, sex, body mass index, tumor stage, histology, or ECOG performance status between patients with and without decreased food intake. Chemotherapy regimens were decided by the attending physicians *ad libitum* (Table 1). Platinum-containing chemotherapy was more frequently used in patients with decreased food intake (n = 8) than in those without (n = 2).

Table 1 Medication list of antineoplastic agents used in chemotherapy

Platinum-containing chemotherapy	(n)	Nonplatinum-containing chemotherapy	(n)
Cisplatin			
+ Adriamycin + etoposide	2	Gemcitabine	3
+ Etoposide	2	Paclitaxel	2
+ Gemcitabine	2	Docetaxel	1
+ Vinorelbine	1	Irinotecan	1
Carboplatin			
+ Paclitaxel	2		
+ Etoposide	1		

Statistical Analysis. The original accrual goal of this study was 40 patients. This sample size was based on 80% power so one could detect a 30% elevation in plasma ghrelin level of patients with cachexia. All data were expressed as mean ± SE unless otherwise indicated. Comparisons of parameters between the two groups were made by Fisher's exact test or unpaired Student's t test. Comparisons of parameters among the three groups were made using one-way ANOVA, followed by Newman-Keuls test. Comparisons of the time course of parameters between the two groups were made by two-way ANOVA for repeated measures, followed by Newman-Keuls test. 95% CIs for the differences between the two groups were calculated. Multiple regression analysis was applied to determine independent relations of clinical parameters with plasma ghrelin level. $P < 0.05$ was considered statistically significant.

RESULTS

Patient Characteristics According to Cachexia. The clinical characteristics of patients with and without cachexia and control subjects are summarized in Table 2. There was no significant difference in age or sex among the three groups. Neither tumor stage nor histology differed between patients with and without cachexia. Body mass index was significantly lower in cachectic patients than in noncachectic patients and in control subjects. ECOG performance status, a score of severity of general condition, was significantly higher in cachectic patients with lung cancer than in noncachectic patients. Numerical rating scale, a marker for food intake, was significantly lower in cachectic patients. Serum albumin level was significantly lower, and LDH was significantly higher in cachectic patients than in noncachectic patients and control subjects. There was no significant difference among the three groups in serum total cholesterol, triglyceride, fasting glucose, or creatinine.

Plasma Ghrelin Level in Cachexia. Plasma ghrelin level did not significantly differ between all patients with lung cancer and control subjects (157 ± 10 *versus* 132 ± 8 fmol/ml, P = 0.1). However, plasma ghrelin level was significantly higher in cachectic patients (180 ± 17 fmol/ml) than in noncachectic patients (135 ± 10 fmol/ml) and control subjects

Table 2 Baseline characteristics according to cachexia in patients with lung cancer[a]

	Control (n = 21)	Noncachexia (n = 22)	Cachexia (n = 21)
Age (yr)	64 ± 3	70 ± 2	63 ± 2
Sex, male/female	14/7	17/5	16/5
Body mass index (kg/m^2)	23.4 ± 0.5	23.0 ± 0.5	18.5 ± 0.5[b,c]
Tumor stage	NA		
Non-small cell (I/II/III/IV)		3/1/3/9	1/0/2/10
Small cell (limited/extensive)		0/6	3/5
Histology (ad/sq/sm)[d]	NA	12/4/6	11/2/8
ECOG performance status (0/1/2/3)	NA	(16/3/2/1)	(7/5/4/5)[e]
Numerical rating scale (0–10)	NA	9.7 ± 0.2	5.8 ± 0.7[c]
Albumin (g/dl)	4.1 ± 0.1	4.1 ± 0.1	3.5 ± 0.1[c,f]
Total cholesterol (mg/dl)	193 ± 3	191 ± 7	183 ± 10
Triglyceride (mg/dl)	116 ± 4	119 ± 10	114 ± 8
Fasting glucose (mg/dl)	92 ± 1	98 ± 3	116 ± 15
Creatinine (mg/dl)	0.77 ± 0.02	0.86 ± 0.06	0.75 ± 0.04
LDH (IU/liter)	335 ± 11	410 ± 24	706 ± 151[e,f]

[a] Data are mean ± SE.
[b] $P < 0.01$ *versus* control.
[c] $P < 0.01$ *versus* noncachexia.
[d] ad, adenocarcinoma; sq, squamous cell carcinoma; sm, small cell carcinoma.
[e] $P < 0.05$ *versus* noncachexia.
[f] $P < 0.05$ *versus* control.

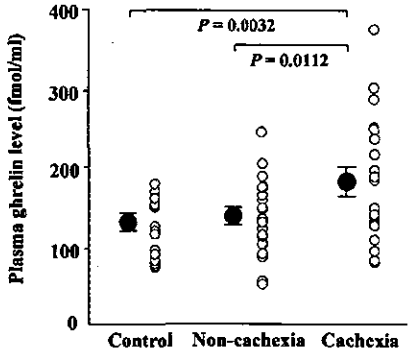

Fig. 1 Plasma ghrelin level in control subjects (*Control*), noncachectic patients with lung cancer (*Non-cachexia*), and cachectic patients with lung cancer (*Cachexia*).

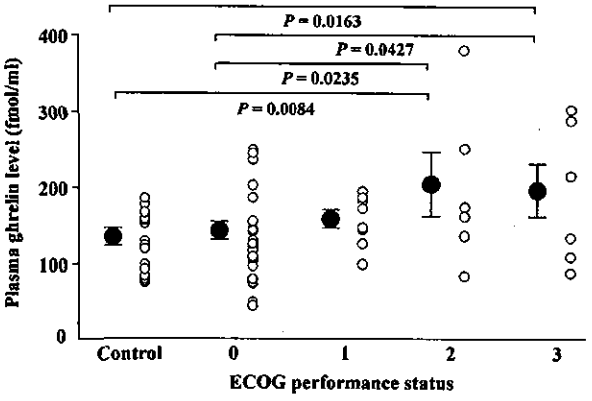

Fig. 2 Plasma ghrelin level in patients with lung cancer according to ECOG performance status.

(Fig. 1). In addition, plasma ghrelin level tended to increase with the severity of ECOG performance status (Fig. 2). In particular, plasma ghrelin level was significantly higher in patients both with ECOG performance status 2 and 3 than in controls. Multiple regression analysis (R^2 = 0.39; P = 0.013) also demonstrated cachexia and ECOG performance status were independently correlated with plasma ghrelin level in patients with lung cancer (Table 3). There were no significant correlations between plasma ghrelin level and biochemical markers, such as plasma albumin, total cholesterol, fasting glucose, and creatinine.

Changes in Plasma Ghrelin Level in Anorexia after Chemotherapy. Decreased food intake was observed in 10 of the 17 patients after chemotherapy. There was no significant difference in baseline clinical characteristics between patients with and without decreased food intake (Table 4). In addition, baseline plasma ghrelin level did not significantly differ between the two groups (Fig. 3). Interestingly, plasma ghrelin level significantly increased in patients with decreased food intake after chemotherapy (from 136 ± 11 fmol/ml to 170 ± 16 fmol/ml on day 8, 179 ± 20 fmol/ml on day 21 after start of chemotherapy), whereas the ghrelin level did not significantly change in patients without decreased food intake (Fig. 3). The

Table 3 Multiple regression analysis of variables associated with plasma ghrelin level in patients with lung cancer

Variables	Standardized coefficient	P
Age	−0.106	0.5401
Male sex	−0.201	0.1891
Body mass index	0.454	0.0587
Sm histology[a]	−0.107	0.5055
Cachexia	0.579	0.0300
ECOG performance status	0.391	0.0251

[a] Sm, small cell carcinoma.

Table 4 Alteration of data during chemotherapy[a]

	Baseline[b]	Day 8	Day 21
Numerical rating scale (0–10)			
Decreased food intake			
(+)[c]	7.9 ± 0.9	5.5 ± 1.1[d]	6.5 ± 0.9[e]
(−)	8.7 ± 1.0	8.1 ± 1.0	9.0 ± 0.9
% Change in body weight (%)			
Decreased food intake			
(+)		−1.51 ± 0.41[d,e]	−1.21 ± 0.61
(−)		0.65 ± 0.69	0.32 ± 0.77
Serum albumin (g/dl)			
Decreased food intake			
(+)	4.0 ± 0.2	3.6 ± 0.6[e,f]	3.8 ± 0.5
(−)	4.2 ± 0.2	4.0 ± 0.1	3.9 ± 0.4
Serum sodium (meq/liter)			
Decreased food intake			
(+)	139 ± 1.1	136 ± 0.9	140 ± 1.0
(−)	139 ± 0.9	138 ± 1.3	139 ± 1.4
Neutrophil count ($\times 10^3/mm^3$)			
Decreased food intake			
(+)	5.57 ± 1.20	3.60 ± 0.73	5.22 ± 1.61
(−)	3.33 ± 0.38	2.82 ± 0.58	3.75 ± 0.92
Serum creatinine (mg/dl)			
Decreased food intake			
(+)	0.94 ± 0.05	1.03 ± 0.07	0.99 ± 0.05
(−)	0.78 ± 0.05	0.85 ± 0.09	0.80 ± 0.08

[a] Data are mean ± SE.
[b] Baseline, before chemotherapy.
[c] +, patients with decreased food intake; −, patients without decreased food intake.
[d] $P < 0.01$ *versus* baseline.
[e] $P < 0.05$ *versus* patients without decreased food intake.
[f] $P < 0.05$ *versus* baseline.

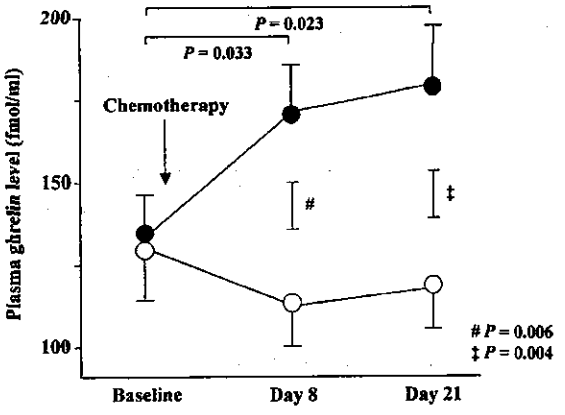

Fig. 3 Change in plasma ghrelin level in lung cancer patients with decreased food intake (●) and those without decreased food intake (○) after chemotherapy. *Baseline*, before chemotherapy.

differences between the two groups were 7 fmol/ml (95% CI, 0–13 fmol/ml) at baseline, 58 fmol/ml (95% CI, 50–65 fmol/ml) on day 8, and 61 fmol/ml (95% CI, 54–68 fmol/ml) on day 21. Numerical rating scale, body weight, and serum albumin level significantly decreased in patients with decreased food intake after chemotherapy, whereas these parameters did not significantly change in those without. No significant differences in serum sodium, neutrophil count, or serum creatinine between the two groups were seen after chemotherapy (Table 4). There was no significant difference in response rate for chemotherapy based on the WHO criteria (12) between patients with and without decreased food intake ($P = 0.15$).

DISCUSSION

In the present study, we demonstrated that: (*a*) baseline plasma ghrelin level was significantly higher in cachectic patients with lung cancer than in noncachectic patients and control subjects; and (*b*) follow-up plasma ghrelin level increased in the presence of anorexia after chemotherapy.

Severe weight and appetite loss associated with cachexia is an important problem in several types of cancer, including lung cancer (1, 2). In the present study, a marked decrease in body mass index, appetite loss, and low serum albumin level was observed in cachectic patients with lung cancer. These results suggest the presence of impaired energy balance in cachectic patients with lung cancer. In addition, both ECOG performance status and serum LDH level were significantly higher in cachectic patients with lung cancer than in noncachectic patients. These results suggest that a cachectic condition is associated with the severity of lung cancer. Interestingly, plasma ghrelin level was significantly higher in cachectic patients with lung cancer than in noncachectic patients and control subjects. Multiple regression analysis demonstrated that cachexia and ECOG performance status were independently correlated with plasma ghrelin level. Recently, peripheral administration of ghrelin has been reported to induce a positive energy balance and weight gain by decreasing fat utilization and increasing carbohydrate utilization, through a GH-independent mechanism (6). These results raise the possibility that increased plasma ghrelin

may represent a compensatory mechanism under conditions of anabolic/catabolic imbalance in cachectic patients with lung cancer. In the present study, plasma ghrelin level increased in proportion to ECOG performance status, which is associated with the severity and mortality of patients with lung cancer (13). Taken together, measurement of plasma ghrelin may be a simple, noninvasive, and relatively inexpensive method to assess the development of cachexia and disease severity in patients with lung cancer.

A recent animal study has demonstrated that endogenous production of ghrelin is enhanced by fasting (14). Therefore, in the present study, ghrelin measurement was repeated after chemotherapy to examine whether plasma ghrelin level is influenced by anorexia. After chemotherapy, numerical rating scale, body weight, and serum albumin level were significantly decreased in patients with anorexia, reflecting deterioration of nutrition. Expectedly, plasma ghrelin level was significantly increased in patients with decreased food intake on days 8 and 21 after the start of chemotherapy, although the ghrelin level did not significantly change in patients without decreased food intake. These results suggest that anorexia may influence plasma ghrelin level in patients with lung cancer. Ghrelin has been shown to elicit potent, long-lasting stimulation of food intake via activation of neuropeptide Y neurons in the hypothalamic arcuate nucleus (7–9). Thus, ghrelin synthesis may be up-regulated as a compensatory mechanism by chemotherapy-induced anorexia. It should be noted that plasma ghrelin level appears to be easily influenced by short-term appetite loss. This result is consistent with earlier findings that ghrelin is rapidly influenced by fasting and refeeding (6). Appetite loss is known to be one of the most important adverse effects of chemotherapy (15). Thus, measurement of plasma ghrelin may serve as a marker for the severity of chemotherapy-induced toxicity.

A number of studies has examined the effects of medical treatment on cancer anorexia (10, 16). Nevertheless, some cases are refractory to those treatments. Ghrelin may have beneficial effects on energy metabolism not only through GH-dependent mechanisms but also through GH-independent mechanisms. Thus, it would be interesting to investigate whether supplementation of ghrelin would attenuate the development of cachexia in the most severe forms of lung cancer.

This study included some study limitations: (*a*) this study is a cross-sectional observational study, and the patient numbers are small; additional investigation in a large scale study would be needed; (*b*) platinum-containing chemotherapy was more frequently used in patients with decreased food intake than in those without; nonetheless, platinum does not have direct biological effects on the stomach which produces ghrelin; and (*c*) renal dysfunction induced by platinum was not observed in the present study; thus, elevated plasma level of ghrelin is unlikely to be attributable to administration of platinum.

In conclusion, baseline plasma ghrelin level was elevated in cachectic patients with lung cancer, and follow-up ghrelin level increased in patients with anorexia after chemotherapy. Considering the positive energy effects induced by ghrelin, increased ghrelin may represent a compensatory mechanism under catabolic–anabolic imbalance in cachectic patients with lung cancer.

ACKNOWLEDGMENTS

We thank Drs. Keiichi Kondo, Yoshinori Haruta, Tetsuya Oguri, and Kazunori Fujitaka for referral of patients and helpful advice.

REFERENCES

1. Dewys, W. D., Begg, C., Lavin, P. T., Band, P. R., Bennett, J. M., Bertino, J. R., Cohen, M. H., Douglass, H. O., Jr., Engstrom, P. F., Ezdinli, E. Z., Horton, J., Johnson, G. J., Moertel, C. G., Oken, M. M., Perlia, C., Rosenbaum, C., Silverstein, M. N., Skeel, R. T., Sponzo, R. W., and Tormey, D. C. Prognostic effect of weight loss prior to chemotherapy in cancer patients. Eastern Cooperative Oncology Group. Am. J. Med., *69:* 491–497, 1980.

2. Nixon, D. W., Heymsfield, S. B., Cohen, A. E., Kutner, M. H., Ansley, J., Lawson, D. H., and Rudman, D. Protein-calorie undernutrition in hospitalized cancer patients. Am. J. Med., *68:* 683–690, 1980.

3. Lanzotti, V. J., Thomas, D. R., Boyle, L. E., Smith, T. L., Gehan, E. A., and Samuels, M. L. Survival with inoperable lung cancer: an integration of prognostic variables based on simple clinical criteria. Cancer (Phila.), *39:* 303–313, 1977.

4. Donaldson, S. S., and Lenon, R. A. Alterations of nutritional status: impact of chemotherapy and radiation therapy. Cancer (Phila.), *43:* 2036–2052, 1979.

5. Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature, *402:* 656–660, 1999.

6. Tschop, M., Smiley, D. L., and Heiman, M. L. Ghrelin induces adiposity in rodents. Nature (Lond.), *407:* 908–913, 2000.

7. Wren, A. M., Small, C. J., Ward, H. L., Murphy, K. G., Dakin, C. L., Taheri, S., Kennedy, A. R., Roberts, G. H., Morgan, D. G., Ghatei, M. A., and Bloom, S. R. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology, *141:* 4325–4328, 2000.

8. Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., and Matsukura, S. A role for ghrelin in the central regulation of feeding. Nature (Lond.), *409:* 194–198, 2001.

9. Shintani, M., Ogawa, Y., Ebihara, K., Aizawa-Abe, M., Miyanaga, F., Takaya, K., Hayashi, T., Inoue, G., Hosoda, K., Kojima, M., Kangawa, K., and Nakao, K. Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes, *50:* 227–232, 2001.

10. Rowland, K. M., Jr., Loprinzi, C. L., Shaw, E. G., Maksymiuk, A. W., Kuross, S. A., Jung, S. H., Kugler, J. W., Tschetter, L. K., Ghosh, C., Schaefer, P. L., Owen, D., Washburn, J. H., Jr., Webb, T. A., Mailliard, J. A., and Jett, J. R. Randomized double-blind placebo-controlled trial of cisplatin and etoposide plus megestrol acetate/placebo in extensive-stage small-cell lung cancer: a North Central Cancer Treatment Group study. J. Clin. Oncol., *14:* 135–141, 1996.

11. Hosoda, H., Kojima, M., Matsuo, H., and Kangawa, K. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem. Biophys. Res. Commun., *279:* 909–913, 2000.

12. Miller, A. B., Hoogstraten, B., Staquet, M., and Winkler, A. Reporting results of cancer treatment. Cancer (Phila.), *47:* 207–214, 1981.

13. Buccheri, G., Ferrigno, D., and Tamburini, M. Karnofsky and ECOG performance status scoring in lung cancer: a prospective, longitudinal study of 536 patients from a single institution. Eur. J. Cancer, *32A:* 1135–1141, 1996.

14. Toshinai, K., Mondal, M. S., Nakazato, M., Date, Y., Murakami, N., Kojima, M., Kangawa, K., and Matsukura, S. Upregulation of Ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. Biochem. Biophys. Res. Commun., *281:* 1220–1225, 2001.

15. Copeland, E. M., III, Daly, J. M., and Dudrick, S. J. Nutrition as an adjunct to cancer treatment in the adult. Cancer Res., *37:* 2451–2456, 1977.

16. Goldberg, R. M., Loprinzi, C. L., Mailliard, J. A., O'Fallon, J. R., Krook, J. E., Ghosh, C., Hestorff, R. D., Chong, S. F., Reuter, N. F., and Shanahan, T. G. Pentoxifylline for treatment of cancer anorexia and cachexia? A randomized, double-blind, placebo-controlled trial. J. Clin. Oncol., *13:* 2856–2859, 1995.