Statistics

Values are given as means ± SEM. Comparisons between two groups were made by ANOVA with the *post hoc* Fisher test. Differences at $P < 0.05$ were accepted as statistically significant.

Results

Expression of GHS-Rs in fetal tissue

GHS-R1a mRNA expression was detected in various fetal tissues with a high density in the spinal cord from E14 until birth (Fig. 1A). GHS-R mRNA expression in the fetal pituitary was also detected at E19. To confirm the expression of GHS-R in fetal tissues at the protein level, we performed immunohistochemistry on E17 fetuses using an antibody specific for GHS-R. Positive cells were distributed extensively in fetal tissues; the skin, bone, intestine, tongue, and muscle being stained particularly strongly (Fig. 1, C–G). Immunoreactivity was not detected in sections that were incubated with GHS-R antiserum that had been preabsorbed with excess synthetic GHS-R (Fig. 1, D-2 and F-2). Although RT-PCR analysis demonstrated the expression of GHS-R mRNA in sections of the brain, pituitary, stomach, and lung, only relatively weak staining was observed in these organs.

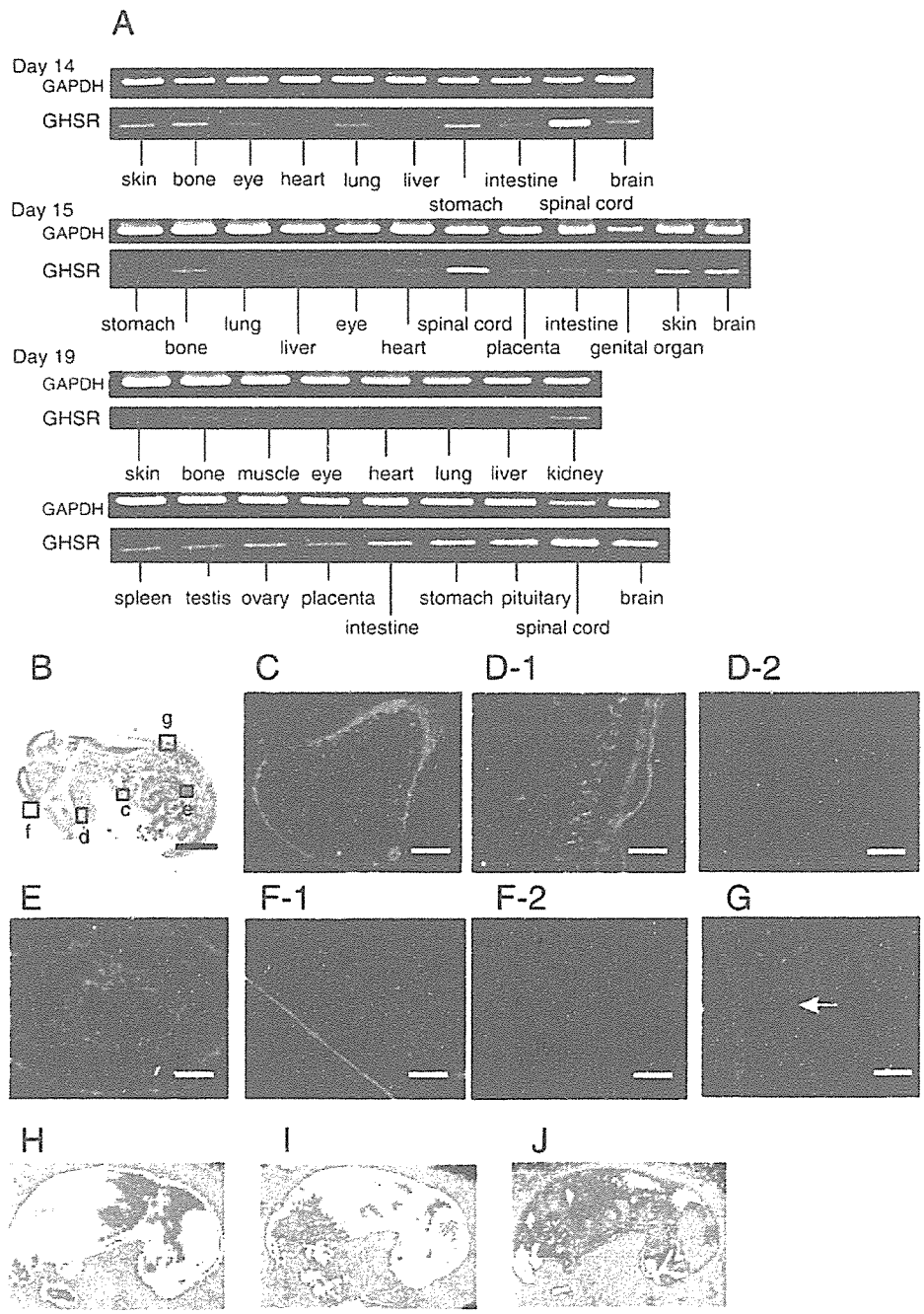

FIG. 1. A, GHS-R 1a mRNA expression in various fetal tissues. Fetal (E14, 15, 19) tissue cDNA fragments were amplified by PCR in the presence of oligonucleotide primers specific for GHS-R 1a. B, Sagittal section of E17 fetuses immunostained for GHS-R by avidin-biotin-peroxidase complexes (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) using a diaminobenzidine substrate kit (Vector Laboratories). C–G, Immunofluorescence staining for GHS-R in fetal ribs (C), tongue (D), intestine (E), skin (F), and the muscle between the two transverse processes of a thoracic vertebra (G). D-2 and F-2 represent the immunostaining using absorbed antiserum with excess of synthetic GHS-R. *Bar* scales are: 3.7 mm (B); 75 μm (C); 65 μm (D); 75 μm (E); 100 μm (F); 75 μm (G). H–J, [^{125}I]acyl ghrelin autoradiograph of a sagittally sectioned fetus (E17) (H). Replacement was examined in the presence of excess unlabeled acyl (I) or des-acyl (J) ghrelin. The *red color* indicates the highest binding state with [^{125}I]acyl ghrelin (high binding order: *red* > *yellow* > *green* > *blue colors*).

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[^{125}I]Acyl ghrelin autoradiography revealed dense binding to bone, skin, heart, and tongue (Fig. 1H); similar to the immunohistochemistry, the brain and digestive tract bound the isotope only weakly. In addition, excess unlabeled acyl ghrelin (Fig. 1I) and des-acyl ghrelin (Fig. 1J) could displace with [^{125}I]acyl ghrelin binding. More potent replacement was observed in excess unlabeled des-acyl ghrelin treatment (Fig. 1J).

Circulating ghrelin levels during late pregnancy

We measured the circulating levels of acyl and des-acyl ghrelin in pregnant rats and their fetuses, respectively. The levels of acyl ghrelin in maternal plasma exhibited a gradual but not significant decline in late pregnancy (Fig. 2A). In contrast, des-acyl ghrelin increased significantly during late pregnancy (Fig. 2A). Both ghrelin forms, acyl and des-acyl ghrelin, were also present in the fetal circulation; these levels decreased gradually as the time for delivery approached (Fig. 2B). We noticed a significant difference in des-acyl ghrelin levels when compared between the maternal and fetal plasma: the fetal levels of des-acyl ghrelin were 5- to 10-fold higher than the maternal levels (Fig. 2, A and B). On d 17 and 19 of pregnancy, we detected a large quantity of des-acyl ghrelin in the amniotic fluid (Fig. 2C). Acyl ghrelin levels increased rapidly in fetal blood within 5 min of administration of either 0.2 or 20 nmol acyl ghrelin (iv) into the mother (Fig. 2D). In the case of the 20-nmol dose, although maternal trunk ghrelin levels declined 30 min after injection, fetal trunk ghrelin levels were still increased at the sampling time.

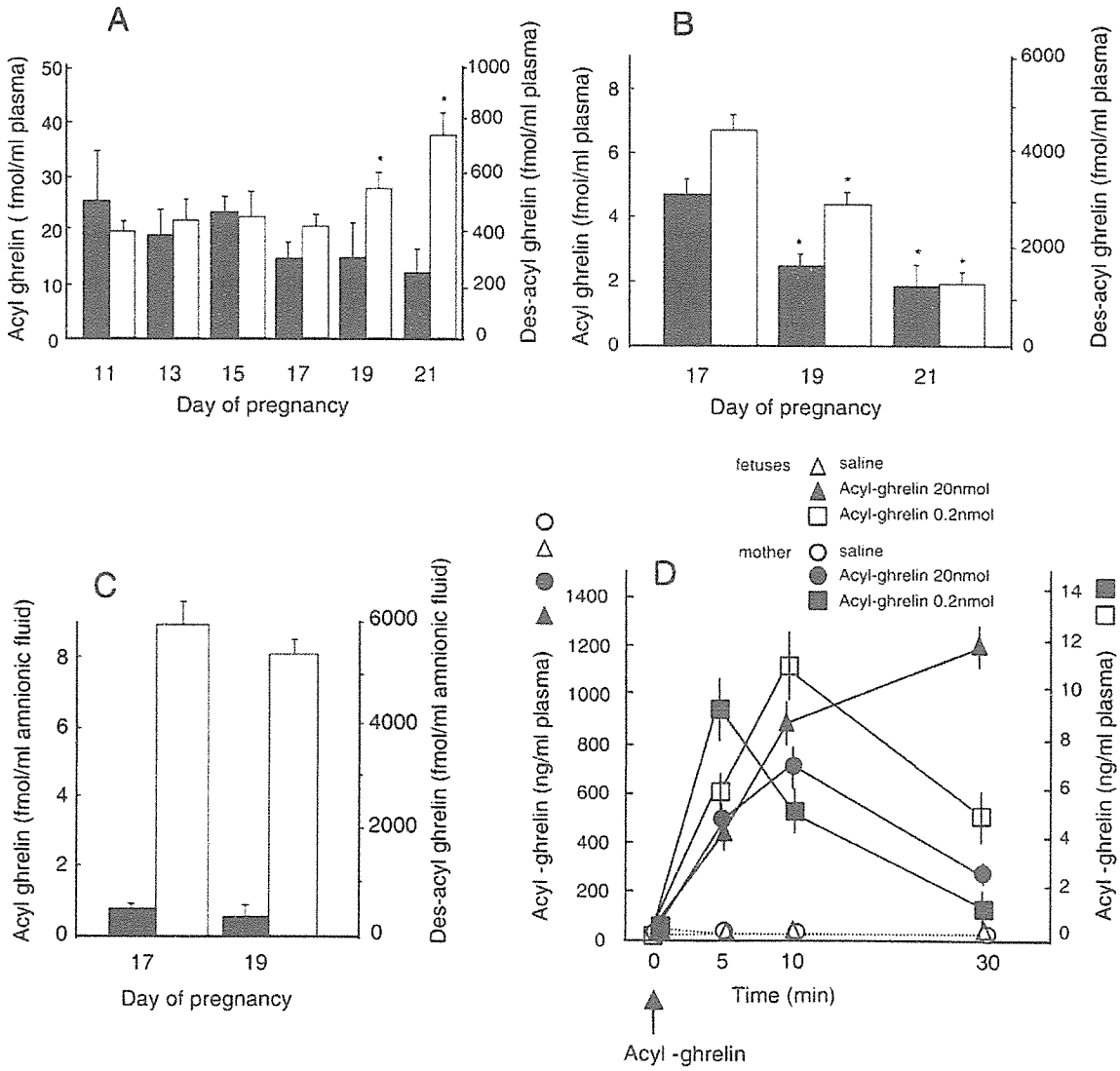

FIG. 2. A, Plasma acyl and des-acyl ghrelin levels in pregnant rats during late pregnancy. B, Fetal plasma levels of acyl and des-acyl ghrelin. C, Acyl and des-acyl ghrelin levels in amniotic fluid. All *black and white bars* represent the levels of acyl and des-acyl ghrelin, respectively. Each *bar* and *vertical line* represent the mean ± SEM (n = 8). *Asterisks* indicate significant differences (A, $P < 0.05$ *vs.* E11; B, $P < 0.05$ *vs.* E17). D, Maternal and fetal levels of circulating acyl ghrelin after iv injection of saline or 0.2 and 20 nmol acyl ghrelin into mothers at d 19 of pregnancy. The *right and left vertical scales* correspond to 0.2 and 20 nmol acyl ghrelin treatments, respectively.

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Effect of chronic ghrelin treatment on fetal body weight at birth

We examined the effect of prolonged maternal treatment with ghrelin, beginning at d 14 or 15 of pregnancy and lasting until delivery, on neonatal body weight at birth. Chronic treatment with acyl ghrelin, either by injection three times per day (Fig. 3A) or constant infusion through an osmotic mini-pump (Fig. 3B), significantly increased the average neonatal body weight at birth in comparison to that of neonates delivered by a saline-treated group. We observed more than a 10% body weight gain, and the increase was dose-dependent. No significant changes were observed after treatment with des-acyl ghrelin.

We investigated the effect of acyl ghrelin injection on food intake of pregnant females. Daily treatment with acyl ghrelin significantly increased daily maternal food intake (Fig. 3C). However, a paired feeding study demonstrated that even when pregnant females treated with acyl ghrelin consumed the same amount of food as saline-treated pregnant females, neonatal body weight was significantly greater in the ghrelin-treated group (Fig. 3D).

To examine the effect of endogenous maternal ghrelin on fetal development, we compared the birth weight of pups born to mothers passive-immunized against a complex of acyl ghrelin and mcKLH (carrier protein) with that of pups born to mothers passive-immunized against mcKLH. After six immunizations at 2-wk intervals beginning at 44 d after birth, rats were mated when the relative ghrelin binding titer was maximally increased (Fig. 3E). Although body weight gain was temporarily lower, it was not significantly so. The body weights of ghrelin-immunized females recovered gradually to normal levels at 104 d of age (Fig. 3E). The body weights of neonates born to mothers passive-immunized against acyl ghrelin were lower than those of neonates born to saline-treated mothers (Fig. 3F).

Effect of ghrelin on GH mRNA levels in fetal pituitary tissue, and IGF-I and corticosterone levels in fetal plasma

If GH, prolactin, or corticosterone secretions from fetal pituitary or adrenal tissues were stimulated by maternal ghrelin, the released hormone might stimulate fetal development. We examined the effect on fetal pituitary GH mRNA levels and fetal plasma IGF-I or corticosterone levels by administering acyl ghrelin to pregnant females. However, pituitary GH mRNA at E19 and E20 was not affected by this treatment (Fig. 4A). In addition, fetal plasma IGF-I and corticosterone concentrations at E19 and E20 were not affected by maternal ghrelin treatment (Fig. 4, B and C). We found no significant change in fetal prolactin levels (data not shown).

Effect of ghrelin on proliferation of cultured fetal skin cells

To examine a possibility of direct effect of circulating ghrelin on fetal development, we examined the fetal cell proliferation by ghrelin using [^{3}H]thymidine and BrdU incorporation. We used primary cultured fetal skin cells at E17, because abundant cells at this stage were easy to collect. Both [^{3}H]thymidine (Fig. 5A) and BrdU (Fig. 5, B–E) incorporation increased significantly after treatment with acyl ghrelin in a dose-dependent or time-dependent manner. Des-acyl ghrelin was more potent than acyl ghrelin at stimulating the proliferation of fetal skin cells (Fig. 5E). The GHS-R antagonist [D-Lys^3]-GHRP-6 inhibited acyl ghrelin- and des-acyl ghrelin-stimulated cell proliferation (Fig. 5E).

Calcium-imaging analysis revealed two types of fetal skin cells (Fig. 5F): one type responding to des-acyl ghrelin, but not to acyl ghrelin, and the other responding to acyl ghrelin, but not to des-acyl ghrelin. No. 21 and 23 cells were shown as examples, respectively.

Discussion

The present study clearly demonstrated that maternal ghrelin would play an important role in fetal development during pregnancy; first, exogenous chronic treatment of the mother with ghrelin increased fetal body weight at birth; second, mothers immunized against ghrelin delivered fetuses with a lower body weight; and third, proliferation of cultured fetal skin cells was stimulated by ghrelin. Both GHS-R1a mRNA expression and GHS-R protein were detected in various fetal tissues. Autoradiography using [^{125}I]acyl ghrelin also demonstrated dense binding to the bone, skin, heart, and tongue. This distribution of functional GHS-R throughout peripheral fetal tissues suggests that ghrelin acts on such fetal peripheral tissues. Surprisingly, excess unlabeled des-acyl ghrelin could displace completely with [^{125}I]acyl ghrelin binding, suggesting that the acyl modification is dispensable for ghrelin function in binding site of fetal tissues. Because des-acyl ghrelin does not bind to GHS-R (1), we presume that fetal tissues may express a GHS-R subtype for des-acyl ghrelin. In support of this supposition (28, 29), it has been shown that the increases in plasma glucose and decreases in insulin, but not increases in GH secretion, induced by acyl ghrelin administration can be counteracted by coadministration of des-acyl ghrelin (28). In addition, ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and phosphatidylinositol 3-kinase/AKTJ (30).

Plasma total ghrelin levels have been measured in pregnant women, rats, and human fetuses (31–34). In pregnant rats, plasma total ghrelin, determined with an antibody recognizing the C-terminal region, was shown to decrease at around the middle to late stage of pregnancy (31). Total ghrelin increases at around mid-gestation in human pregnancy (32, 33). Human fetuses exhibit levels of total ghrelin in umbilical venous blood that are not correlated with either gestational age or maternal ghrelin levels (34). In addition, ghrelin mRNA expression has been observed in the placenta and ovary of pregnant rats, and in the fetal pancreas (3, 35, 36). It has also been reported that ghrelin might play an important role in the regulation of blood pressure and the development of preimplantation embryos (37, 38). In the present study, both acyl and des-acyl ghrelin were present in the maternal and fetal circulations during the last half of pregnancy, and there was a significant difference in des-acyl ghrelin levels between the maternal and fetal plasma. The fetal levels of plasma des-acyl ghrelin were 5- to 10-fold higher than the maternal levels. In addition, we detected a large quantity of des-acyl ghrelin in the amniotic fluid. As

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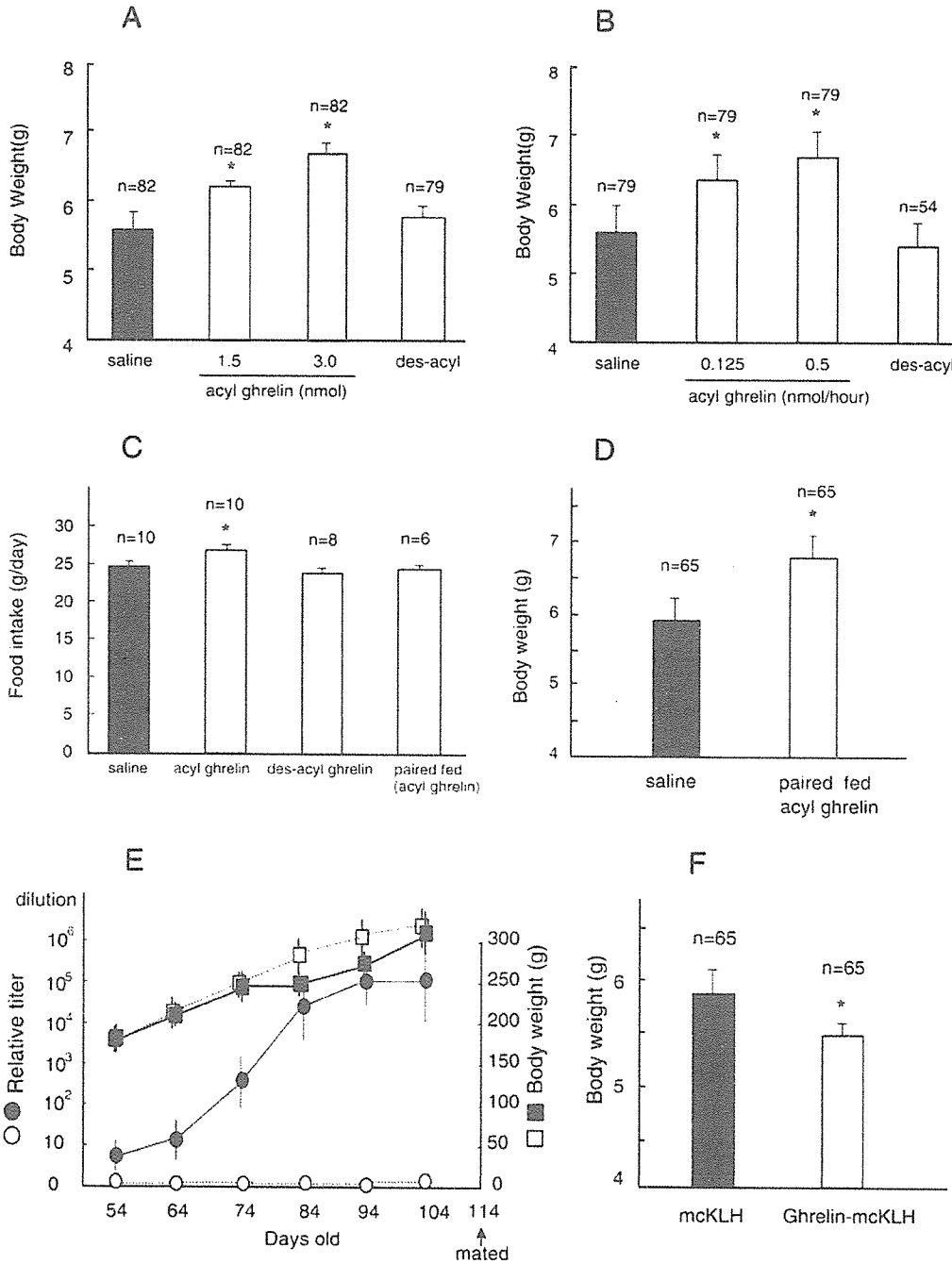

FIG. 3. A and B, Effect on neonatal body weight at birth of daily sc injections of ghrelin (A) or continuous infusion of ghrelin with an osmotic minipump (B) into pregnant rats. Treatment with ghrelin was performed from d 14 until delivery. C, Daily food intake in pregnant rats (A) and in pair-fed pregnant rats treated with acyl ghrelin on the same schedule as A. D, The effect of daily sc injection of 3 nmol acyl ghrelin into pair-fed pregnant rats on neonatal body weight at birth. Acyl ghrelin was injected three times daily from d 14 until delivery. Paired feeding for the saline-treated group also begun on d 15 of pregnancy. E, Comparison of body weight and relative antibody titer between rats immunized against ghrelin-mcKLH complex (●, ■) or carrier protein alone (○, □). The relative titer (●, ○) was expressed as a dilution rate at 50% binding capacity. Each *bar* (A–E) and *symbol* (F) and *vertical line* represent the mean ± SEM. The *upper numbers* represent the total number of newborn rats compared in each group. *Asterisks* indicate significant differences ($P < 0.05$ *vs.* control). F, Comparison of the body weight of newborn rats delivered from rats passive-immunized with the ghrelin-mcKLH complex or with carrier protein alone (mcKLH).

demonstrated previously, ghrelin-positive cells were not evident in the fetal stomach until E19 by immunohistochemistry using an antibody recognizing the N-terminal of acyl ghrelin, suggesting that fetal plasma ghrelin originates from the maternal placenta and/or the maternal blood (3, 22). Indeed, acyl ghrelin levels in fetal plasma increased rapidly

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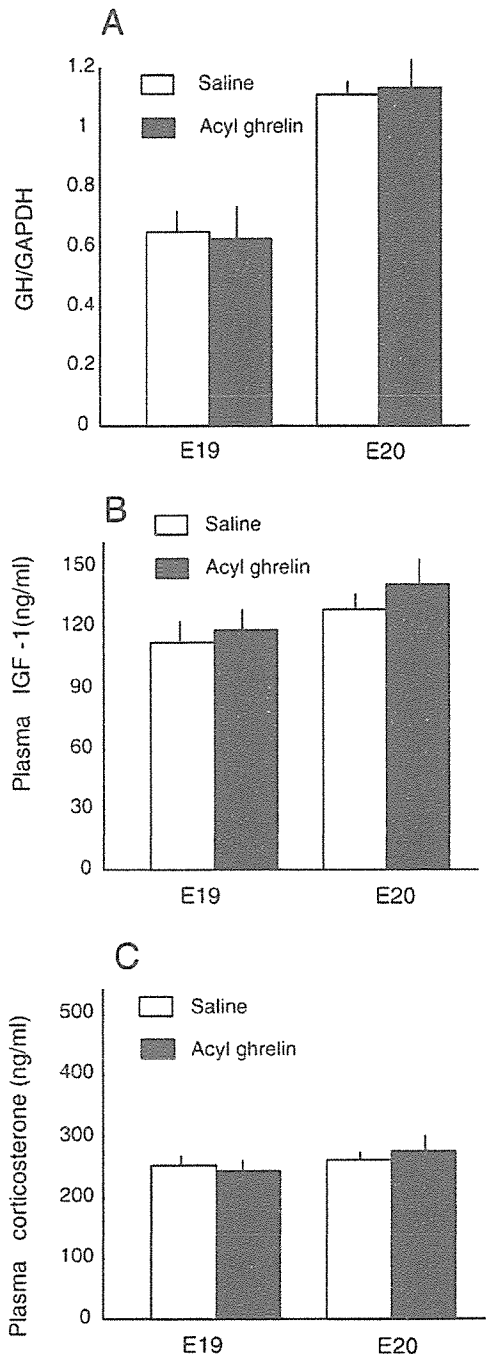

FIG. 4. A, The effect of continuous infusion of acyl ghrelin into pregnant rats on fetal pituitary GH mRNA levels (A) and fetal plasma IGF-I (B) and corticosterone levels (C). Acyl ghrelin was infused at a rate of 0.5 nmol per hour with an osmotic minipump beginning on d 14 and lasting until delivery. The fetal pituitary and plasma were collected at E19 and E20. Each *bar and vertical line* represent the mean ± SEM (n = 6).

within 5 min after administration of acyl ghrelin to the mother, indicating that maternal ghrelin easily transits to the fetal circulation. Although maternal trunk ghrelin levels declined 30 min after injection, fetal trunk ghrelin increased at the time, probably resulting from a longer half-life of ghrelin in fetuses than in adults, and high levels of des-acyl ghrelin might accumulate in the fetal circulation. The existence of GHS-R and an additional GHS-R subtype in fetal tissues, combined with both acyl ghrelin and large quantities of des-acyl ghrelin in the fetal circulation and amniotic fluid, supports the hypothesis that maternal ghrelin plays a critical role in fetal development.

Fetal growth is mainly influenced by the nutrition provided by the mother through the arteria umbilicalis (39, 40). Decreases in the amount of food given to pregnant mothers during the gestational period tend to decrease the size of their neonatal pups in comparison with pups born to mothers fed *ad libitum*. Daily treatment with acyl ghrelin significantly increased daily maternal food intake. The stimulation of fetal growth by maternal ghrelin injection would result from increased nutrition provided by the mother. However, a paired feeding study demonstrated that even when pregnant females treated with acyl ghrelin consumed the same amount of food as saline-treated pregnant females, neonatal body weight was significantly greater in the ghrelin-treated group. This result indicates that maternal ghrelin affected fetal development through a mechanism independent of increased nutrition.

In rats, a rapid increase in fetal body weight occurs during the last quarter of pregnancy. The somatotroph, a GH-secreting cell, appears in the fetal pituitary near E18 (41). Pituitary GH mRNA at E19 and E20 was not altered by ghrelin treatment, indicating that maternal ghrelin-induced fetal development is not due to increased release of fetal GH. The stimulation of maternal GH secretion by daily treatment of ghrelin, leading to the transition of maternal GH to fetal circulation, may stimulate fetal development. Garcia-Aragon and colleagues (42) provided evidence for the wide distribution of GH receptor in the mid-late gestation of rat fetus. The receptor expression markedly increased between E12 and E18; the receptor was present in all major organ systems at E18. Genetically manipulated model mice, Laron dwarfs, with inactivating GH receptor mutations, were shorter in length than normal at birth. Congenitally GH-deficient newborn babies are also much shorter (43, 44). In contrast, the fetuses of GH-deficient dwarf rats were proportionately smaller in size (45). However, we previously reported that continuous infusion of ghrelin to rats stimulated GH secretion for several days, but that the effect decreased after prolonged administration (26). Levels of GH mRNA within the pituitary were also decreased by these treatments (26), probably due to transcriptional down-regulation. In addition, fetal plasma IGF-I levels were not affected by maternal treatment with ghrelin. We found no significant change in fetal circulating levels of corticosterone and prolactin during maternal ghrelin administration. Therefore, the stimulation of fetal development by maternal ghrelin administration is probably not due to the maternal GH and fetal circulating IGF-I and corticosterone levels.

Both [^{3}H]thymidine and BrdU incorporation increased significantly after treatment with acyl ghrelin in a dose-dependent and time-dependent manner. Interestingly, des-acyl ghrelin stimulated proliferation more potently than acyl ghrelin. The GHS-R antagonist [D-Lys^3]-GHRP-6 inhibited

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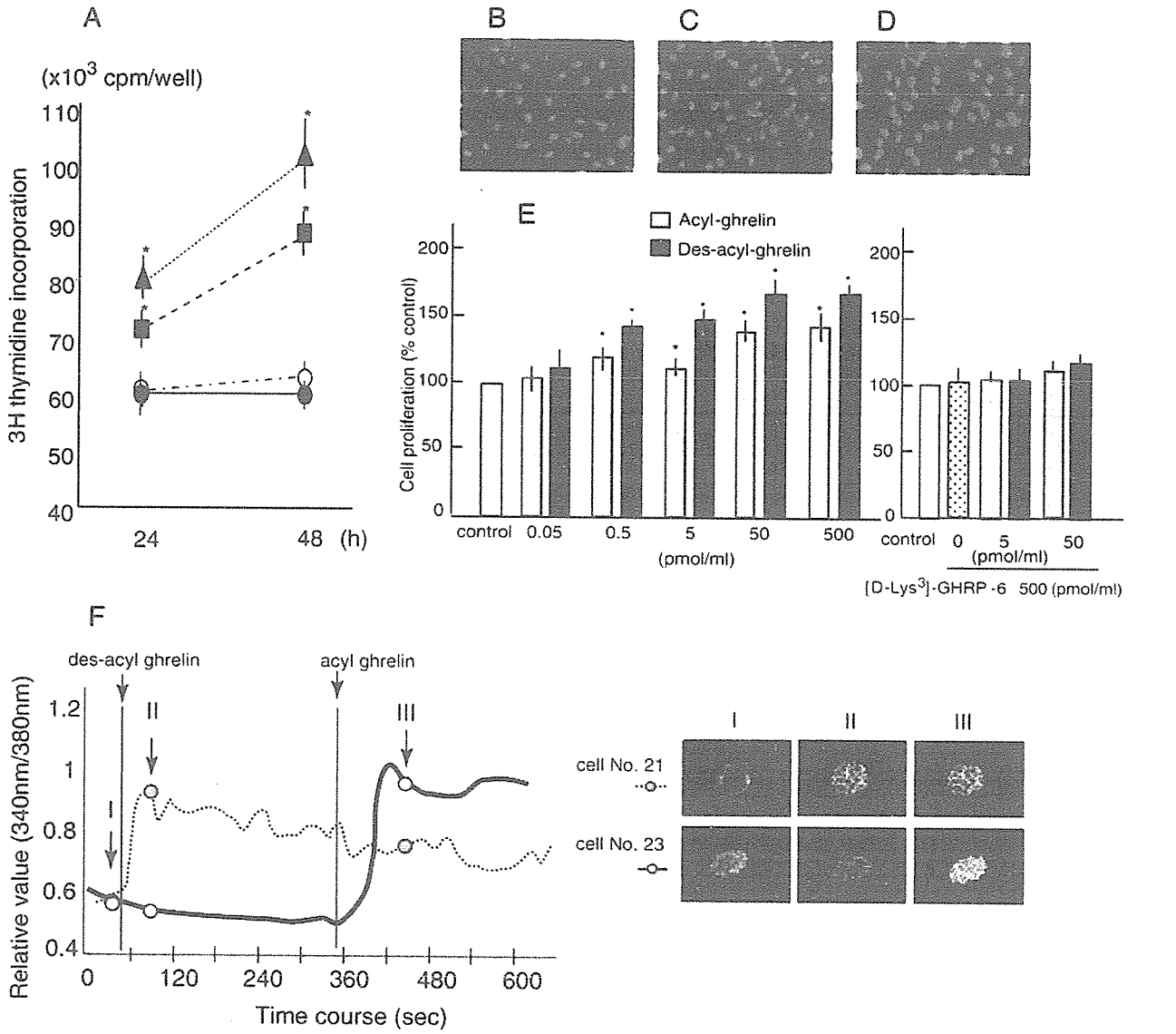

FIG. 5. A, The effect of acyl ghrelin on [^{3}H]thymidine incorporation by cultured fetal (E17) skin cells. One day after initiation of culture, acyl ghrelin (●, 0 pmol; ○, 0.5 pmol; ■, 5 pmol; ▲, 50 pmol/ml) and [^{3}H]thymidine (2 μCi/ml) were added for a 24- or 48-h incubation period. Each *symbol* and *vertical line* represents the mean ± SEM (n = 16 wells). *Asterisks* indicate significant differences ($P < 0.05$ *vs.* ●). B–D, Example of BrdU incorporation (shown in E) into the nucleus of cultured fetal (E17) skin cells incubated for 24 h with 50 pmol/ml acyl ghrelin (C), 50 pmol/ml des-acyl ghrelin (D), or without ghrelin (B). E (*left*), The effect of various doses of acyl and des-acyl ghrelin on BrdU incorporation by cultured fetal skin cells. Fetal (E17) skin cells were cultured for 24 h with or without ghrelin. E (*right*), Effect of [D-Lys3]-GHRP-6, a GHS-R antagonist, on the ghrelin-stimulated proliferation of fetal skin cells. Various doses of acyl or des-acyl ghrelin were added to the culture medium together with 500 pmol [D-Lys3]-GHRP-6. F, Calcium imaging analysis of fetal skin cells. The skin cells were prepared from E17 fetuses. Cell no. 21 responded to des-acyl ghrelin, but not to acyl ghrelin. In contrast, cell no. 23 responded to acyl ghrelin, but not to des-acyl ghrelin. Photographs on the *right* represent the calcium response in cells at points I, II, and III. The *red and yellow color spots* indicate high intracellular Ca^{2-} concentration. The *blue color* shows the basal condition. Des-acyl ghrelin and acyl ghrelin were added to the culture medium at the point indicated by the *arrow*. The *graph on the left* represents the transition of the relative value of Ca^{2+} concentration analyzed by an imaging scanner.

acyl ghrelin- and des-acyl ghrelin-stimulated cell proliferation. These results clearly indicate that both acyl ghrelin and des-acyl ghrelin stimulate proliferation of fetal skin cells. Acyl ghrelin induces neurogenesis in the dorsal motor nucleus (46) and stimulates bone formation (47). During pregnancy, maternal ghrelin is likely transferred to the fetal circulation, and then would prompt fetal growth through stimulation of cell proliferation. Calcium-imaging analysis revealed that two types of cells exist in cultured fetal skin cells: one responds only to des-acyl ghrelin, and the other one responds only to acyl ghrelin. These results strongly suggest that fetal skin cells have different type of receptors: one is a classical receptor for acyl ghrelin, GHS-R 1a, and the other is a novel receptor for des-acyl ghrelin that mediates intracellular calcium mobilization.

In this study, we detected high levels of des-acyl ghrelin

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in the fetal circulation and amniotic fluid. These findings suggest that amniotic fluid serves, in part, as an incubation medium to provide des-acyl ghrelin to the fetus. In this way, des-acyl ghrelin may act on fetal development by direct stimulation of proliferation. If this is true, however, the lack of an effect of des-acyl ghrelin treatment on neonatal body weight at birth (Fig. 3) remains to be explained. We speculate that, late in pregnancy, high endogenous quantities of des-acyl ghrelin in the fetal circulation and amniotic fluid saturate the GHS-R 1a subtype des-acyl ghrelin receptors, effectively preventing the exogenous des-acyl ghrelin from exerting an effect. It has been reported that ghrelin knockout mice do not exhibit any changes in development (probably including fetal development) (13). We do not know the reason for the discrepancy of neonatal body weights between mothers passive-immunized against acyl ghrelin and ghrelin knockout mice. Further studies are required to elucidate this discrepancy.

In conclusion, the present study has demonstrated that maternal ghrelin is easily transferred to the fetal circulation, and then prompts fetal growth through stimulation of cell proliferation during the late half of pregnancy. Recent reports that ghrelin directly stimulates bone formation (47) also supports this hypothesis. These findings may have implications for the clinical application of ghrelin for pregnant subjects.

Acknowledgments

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Effects of ghrelin and des-acyl ghrelin on neurogenesis of the rat fetal spinal cord

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Abstract

Expressions of the growth hormone secretagogue receptor (GHS-R) mRNA and its protein were confirmed in rat fetal spinal cord tissues by RT-PCR and immunohistochemistry. *In vitro*, over 3 nM ghrelin and des-acyl ghrelin induced significant proliferation of primary cultured cells from the fetal spinal cord. The proliferating cells were then double-stained using antibodies against the neuronal precursor marker, nestin, and the cell proliferation marker, 5-bromo-2′-deoxyuridine (BrdU), and the nestin-positive cells were also found to be co-stained with antibody against GHS-R. Furthermore, binding studies using [^{125}I]des-acyl ghrelin indicated the presence of a specific binding site for des-acyl ghrelin, and confirmed that the binding was displaced with unlabeled des-acyl ghrelin or ghrelin. These results indicate that ghrelin and des-acyl ghrelin induce proliferation of neuronal precursor cells that is both dependent and independent of GHS-R, suggesting that both ghrelin and des-acyl ghrelin are involved in neurogenesis of the fetal spinal cord.
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Keywords: Ghrelin; Des-acyl ghrelin; GHS-R; Neurogenesis; Spinal cord; Fetal development

Ghrelin, a peptide hormone secreted from the stomach, has been identified as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R), through which ghrelin stimulates GH release in the pituitary [1]. Two types of GHS-R, type1a and 1b (GHS-R1a and 1b, respectively), have so far been found, and only the former is able to activate signal transduction of the receptor downstream linking to phospholipase C, resulting in an increase of intracellular calcium [2]. Ghrelin consists of 28 amino acids and is characterized by esterified modification with octanoic acid on serine 3, which is essential for activation of GHSR-1a, although the modification mechanism remains unknown. On the other hand, the level of des-acyl ghrelin, which is inactive on GHS-R1a because of a lack of octanoic acid, is 4 times as high as that of ghrelin in the blood [3].

Many studies have reported that ghrelin has multiple effects other than GH secretion, including regulation of food intake [4] and energy metabolism [5], and gastrointestinal coordination [6,7], as well as facilitation of cell survival, and/or inhibition of apoptosis [8–15]. Although these multiple functions of ghrelin would account for the very wide distribution of GHS-R1a, it is debatable whether GHS-R1a contributes to all of the actions of ghrelin, i.e. that ghrelin may act as a ligand for other types of receptors [16]. So far, however, this possibility remains uninvestigated, and no such alternative receptor has been identified.

We have previously demonstrated that rat fetal growth was increased by treatment of the mother with exogenous ghrelin, and that the effect of ghrelin on fetal growth is diminished by immunization against ghrelin *in vivo* [17]. In addition, we have found that amniotic fluid contains a

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large quantity of des-acyl ghrelin, and that proliferation of cells from rat fetal skin is also stimulated by treatment with both ghrelin and des-acyl ghrelin *in vitro*. On the basis of these findings, we speculate that both ghrelin and des-acyl ghrelin play a crucial role in fetal growth, both peptides acting to facilitate fetal growth not only by enhancement of maternal appetite but also via direct stimulation of fetal cell proliferation by transfer of maternal ghrelin to the fetus.

In the present study, we demonstrated that both ghrelin and des-acyl ghrelin facilitate neural cell proliferation in cultured cells from the fetal spinal cord, which express both the GHS-R gene and its protein, and identified these proliferating cells as neuronal precursor cells. Furthermore, in binding studies using [^{125}I]des-acyl ghrelin, we clarified that des-acyl ghrelin has at least one binding site in the membrane fraction from fetal spinal cord. These results suggest that ghrelin and des-acyl ghrelin can facilitate neurogenesis in the rat fetal spinal cord through both the GHS-R and also an unidentified GHS-R-independent alternative pathway.

Materials and methods

Primary culture of embryonic spinal cord cells. Embryonic spinal cords were obtained from a pregnant rat at day 17. The uterus usually contained 10–14 embryos, 10 of which were utilized for primary culture. The whole spinal cords were mechanically and enzymatically dissociated in papain solution, and the digestion was stopped by addition of culture medium. Cells were passed though a strainer, then centrifuged at 1000 rpm at 4 °C for 10 min and resuspended in DMEM supplemented with $NaHCO_3$, antibiotics (penicillin, streptomycin; Sigma, MO), and 5% fetal calf serum, followed by plating onto laminin-coated 96-well plates at 10^5 cells per well.

Ghrelin and des-acyl ghrelin treatment and cell proliferation assay. Cell proliferation was measured by Cell Proliferation ELISA with BrdU (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions with some optimization for the present cell conditions as follows. Briefly, after incubation for four days, the cells were treated with ghrelin or des-acyl ghrelin at a final concentration of 0.003–300 nM for 12 h. Subsequently, BrdU was added to the cells to label newly synthesized DNA, followed by further incubation for 6 h. After incubation, the cells were fixed and denatured, and incubated with anti-BrdU antibody for 90 min. Each well was washed out and reacted with substrate solution until color development. The absorbance of the reaction was measured by an immunoreader. Data were expressed as means ± SEM. The significance of differences between the control and treated cells was analyzed by Student's t test. Differences at $P < 0.05$ were considered statistically significant.

Immunohistochemistry. Frozen sections of the embryonic spinal cord 14 μm thick were prepared from embryos at embryonic day (ED) 17 and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min. After washing with 0.1 M phosphate buffer, the preparations were incubated with 2% normal goat serum in PBS for 30 min at room temperature, washed with PBS three times, and incubated overnight at 4 °C with each of the following primary antibodies: Polyclonal rabbit anti-microtubule-associated protein 2 (Map2; 1:1000, Chemicon International, Inc., CA), anti-neurofilament H (NF-H; 1:1000, Chemicon International), and anti-GHS-R and monoclonal mouse anti-nestin (1:10,000, Chemicon International). Subsequently, all the sections were washed in PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG, except the sections that were incubated with the anti-nestin antibody, for which FITC-conjugated goat anti-mouse IgG (1:200, Chemicon International) was used as the secondary antibody. After washing out the residual antibodies and embedding of the sections, they were observed using a light microscope.

Cultured spinal cord cells, which had incorporated BrdU after incubation for 4 days during the ELISA preparation procedure, were fixed with methanol and glacial acetic acid at −20 °C for 20 min. After DNA denaturation with 2 M HCl and blocking with 2% normal goat serum in PBS for 30 min at room temperature, the fixed cells were incubated overnight at 4 °C with either anti-Map2 or anti-nestin as the primary antibody. Afterwards, the cells were incubated at RT for 1 h with the same secondary antibodies as those used for staining the frozen sections. After the washing step, the cells were further incubated with rat anti-BrdU monoclonal antibody (1:1000, Abcam, Cambridge, UK) as a primary antibody for double staining, followed by incubation with Cy™3-conjugated donkey anti-rat IgG polyclonal antibody (1:1000, Jackson Immuno Research Laboratories, Inc., PA) as the secondary antibody. For double staining of the GHS-R for either Map2 or nestin, cells fixed with 4% paraformaldehyde in 0.1 M phosphate buffer were first incubated with either mouse anti-Map2 or anti-nestin primary antibody, and then with rabbit anti-GHS-R antibody.

RT-PCR. Total RNA was isolated from the spinal cord of embryos at ED 13, 15, 17, 19, and postnatal day (PD) 0 using Trizol Reagent (Life Technologies, Inc., Gaithersburg, MD). Single-strand DNA was generated from 1 μg of total RNA with the use of Superscript 3 preamplification reagent (Life Technologies, Inc., Bethesda, MD) according to the manufacturer's instructions. PCR was carried out using a BD advantage™ 2 PCR Enzyme System (BD Science, CA). The PCR primers specific for GHS-R1a were 5′-GATACCTCTTTTCCAAGTCCTTCGAGCC-3′ for sense and 5′-TTGAACACTGCCACCCGGTACTTCT-3′ for antisense (nucleotides 842–869 and 1001–1025; Accession No. AB001982, GenBank), and those specific for GAPDH were 5′-CGGCAAGTTCA ACGGCACA-3′ for sense and 5′-AGACGCCAGTAGACTCCACGA CA-3′ for antisense (nucleotides 1002–1020 and 1125–1147; Accession No. AF106860, GenBank).

Des-acyl ghrelin binding assay. Binding of des-acyl ghrelin to tissue membranes of fetal spinal cord was studied using [^{125}I]des-acyl ghrelin as a radioligand. Membrane fractions (30,000g pellet) were isolated from fetal spinal cord tissue as described previously [18–20]. Membranes with a protein content of 10 μg, as determined by the Lowry method, were incubated at 4 °C for 1 h with increasing concentrations (0.13–16.64 nM) of [^{125}I]des-acyl ghrelin in a final volume of 0.5 ml assay buffer (50 mM Tris–HCl, 2.5 mM EGTA, 0.1% BSA, and protease inhibitor cocktail (Sigma, MO), pH 7.4). Parallel incubations in the presence of 1.0 μM unlabeled des-acyl ghrelin were used to determine nonspecific binding, which was subtracted from total binding to yield specific binding values. For competition assay, tissue membranes were incubated with 0.1 nM labeled des-acyl ghrelin and either unlabeled des-acyl ghrelin or ghrelin at 4 °C for 1 h. After incubation, the reaction solution was filtered through Whatman GF/B filters, which were then rinsed three times with assay buffer. The radioactivity of the membranes on the filter was measured with a gamma counter. Saturation isotherms were transformed using the method of Scatchard and the maximal number of binding sites (B_{max}) and the dissociation constant (K_d) were calculated using the GraphPAD Prism 4 program (GraphPAD Software, CA).

Results

GHS-R mRNA and protein expression in fetal spinal cord

Using RT-PCR, we examined GHS-R mRNA expression in spinal cords obtained from rat fetuses at ED 13, 15, 17, 19, and PD 0 (Fig. 1a). Abundant levels of GHS-R mRNA were expressed in the spinal cord in fetuses at all ages examined, as we have reported previously [17]. We then investigated GHS-R expression at the protein level by immunohistochemistry. At the same time, we detected neuronal cells by using antibodies against the neuron-specific markers Map2 and NF-H, as well as nestin for

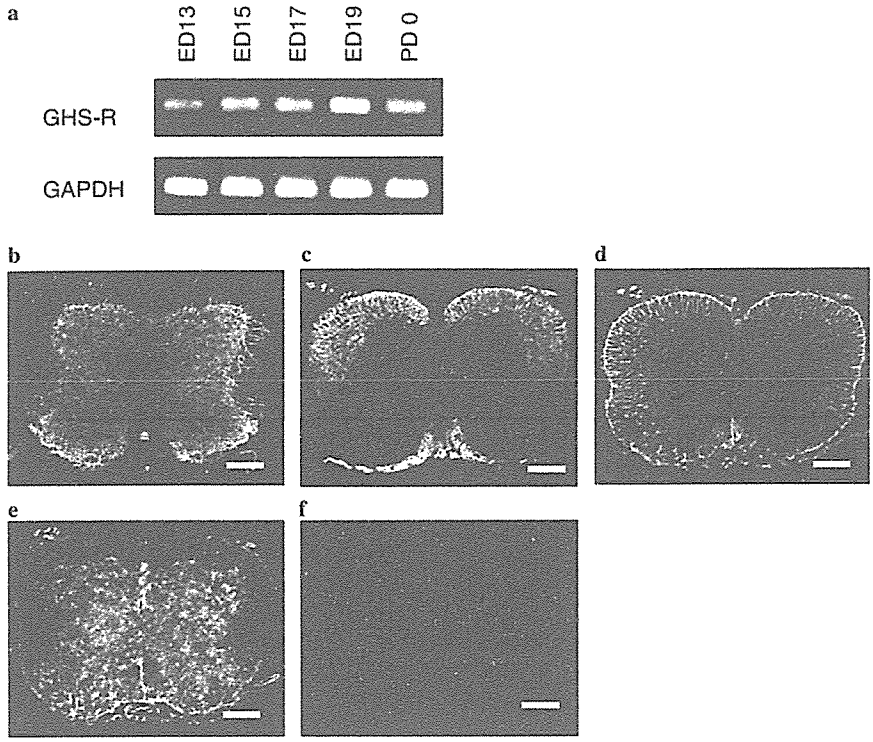

Fig. 1. (a) Detection of GHS-R mRNA in the rat fetal spinal cord by RT-PCR. The PCR product amplified with primers specific for GHS-R1a was detected from embryonic day (ED) 13 to postnatal day (PD) 0. GAPDH mRNA was also detected as an internal control. (b–f) Immunofluorescence staining with antibodies against neuron marker and GHS-R proteins in the rat fetal spinal cord. Map2-positive (b) and Neurofilament H-positive cells (c) were localized in the gray and white matters, respectively. Immunoreactivity of the neuronal precursor cell marker, nestin, was found throughout the spinal cord but with strongest staining in the white matter (d) GHS-R immunoreactivity was localized in the gray matter (e) and was not observed in sections that had been exposed to the preadsorbing antibody (f). Bars, 200 μm.

neuroprogenitor cells. Cells immunoreactive for Map2 were located in the core of the spinal cord, the so-called gray matter (Fig. 1b), while NF-H immunoreactivity was confirmed in the white matter (Fig. 1c). Nestin-positive cells were located in all regions, but the most intense staining was observed in the white matter (Fig. 1d). GHS-R-positive staining was located in the gray matter of the spinal cord (Fig. 1e), and the staining was abolished by pre-absorption of the antibody (Fig. 1f).

Proliferation of spinal cord cells upon treatment with ghrelin and des-acyl ghrelin

Primary culture of spinal cord cells from rat fetus at ED17 was performed. The cells were cultured with BrdU for 6 h after initial incubation for 4 days, and then treated with ghrelin and des-acyl ghrelin for a further 12 h. BrdU is incorporated into DNA when cells synthesize DNA during the S phase of the cell cycle and can be immunodetected using anti-BrdU antibody. BrdU-positive cells were detected under all conditions, irrespective of treatment, although BrdU positivity was more abundant in cells that had been cultured with ghrelin and des-acyl ghrelin than in non-treated cells. To quantify the increase in the number of cells positive for BrdU, we measured cell proliferation by BrdU ELISA. Treatment with both ghrelin (Fig. 2a) and des-acyl ghrelin (Fig. 2b) at over 3 nM significantly increased the incorporation of BrdU.

Identification of the proliferative cell type and cell type expressing GHS-R

Immunofluorescence double staining of cultured cells treated with ghrelin that had incorporated BrdU into their DNA was performed to identify proliferating cells among cultured rat fetal spinal cord cells. Cells with Map2 positivity showed a typical neuron-like shape with extended dendrites and did not show BrdU positivity in their nuclei (Fig. 3a). Cells with nestin positivity were pleomorphic and showed BrdU positivity in their nuclei, i.e., neuronal precursor cells (Fig. 3b). In addition, BrdU positivity was also found in cells that were unstained by antibodies against both Map2 and nestin (data not shown). GHS-R-expressing cells were then examined by immunofluorescence double staining, as was the case for cultured cells without BrdU treatment. GHS-R immunoreactivity was observed in the nestin-positive cells (Fig. 3c).

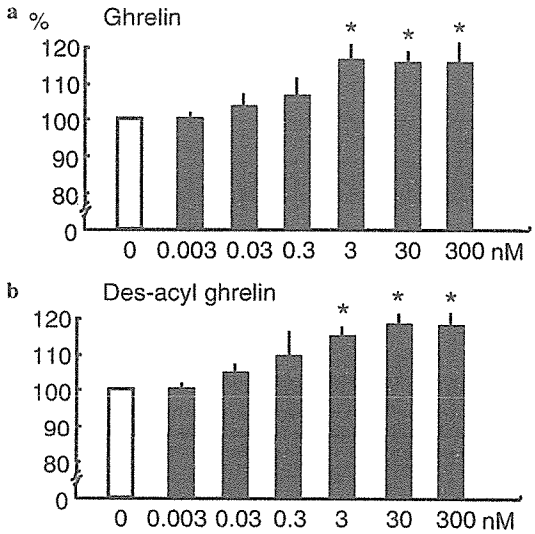

Fig. 2. Cell proliferation effects of ghrelin and des-acyl ghrelin on cultured fetal spinal cells. Proliferative cells were quantified by BrdU ELISA. Significant proliferation was indicated in cells treated with over 3 nM ghrelin (a) and des-acyl ghrelin (b). Values are presented by means + SEM ($^*P < 0.05$).

Binding assay

To identify the presence of the binding site of des-acyl ghrelin, [^{125}I]des-acyl ghrelin binding to membranes from fetal spinal cord was assayed. Specific, high affinity and saturable binding of labeled ghrelin were observed ($K_d = 3.467$, $B_{max} = 1.061$ fmol/mg protein) (Fig. 4a). The binding of labeled des-acyl ghrelin was displaced by unlabeled des-acyl ghrelin and ghrelin (Fig. 4b). The IC_{50} values for des-acyl ghrelin and ghrelin were 23.52 and 41.60 nM, respectively.

Discussion

Our previous study showed that ghrelin, as well as des-acyl ghrelin, play important roles in fetal growth, and that GHS-R mRNA is abundantly expressed in the spinal cord of rat fetus compared with other tissues [17]. Therefore, we reasoned that these ligands and their receptor might exert important actions during neurogenesis of the embryonic spinal cord. In the present study, in fact, ghrelin and des-acyl ghrelin both facilitated the proliferation of cells from fetal spinal cord. In addition, GHS-R mRNA and

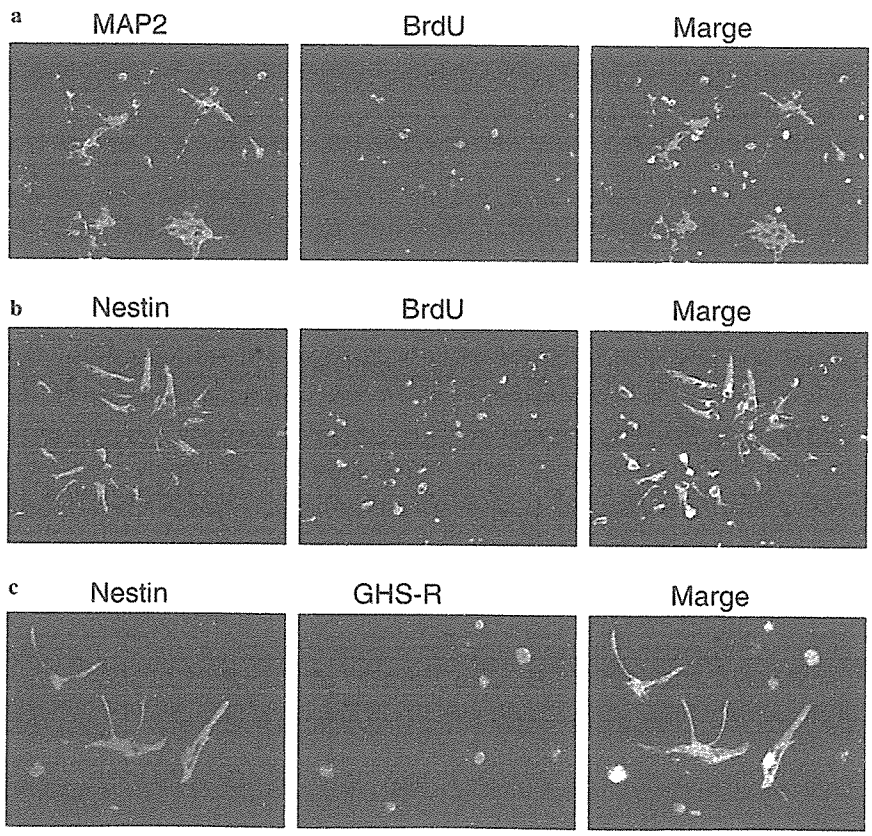

Fig. 3. Identification of proliferative cells in the rat fetal spinal cord. Double immunofluorescence staining demonstrated immunoreactivity for both Map2 and BrdU in distinct cells (a), and co-localization of nestin and BrdU in the same cells (b) Co-localization of nestin and GHS-R was also observed in neuron precursor cells (c).

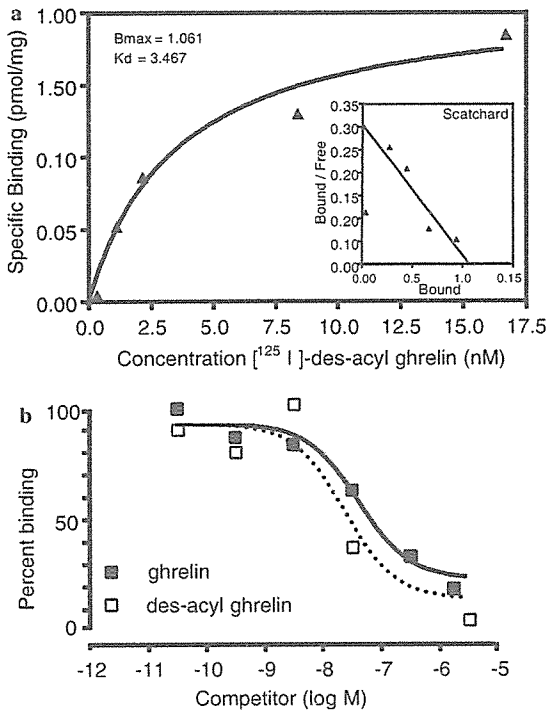

Fig. 4. (a) Representative saturation curve and the Scatchard plot of [^{125}I]des-acyl ghrelin binding to membranes from the fetal spinal cord. Binding assay was performed by incubating a fixed amount of membranes with increasing concentrations (0.13–16.64 nM) of the radioligand. Specific binding values were obtained by subtracting non-specific binding from total binding. (b) Displacement curve of [^{125}I]des-acyl ghrelin binding in the presence of unlabeled des-acyl ghrelin or ghrelin. Binding assay was performed by incubation of fixed amounts of membranes and labeled ligand with increasing concentrations of either of the unlabeled ligands.

GHS-R protein were detected in spinal cord tissue, and neuronal precursor cells in primary culture possessed GHS-R immunoreactivity, indicating that ghrelin stimulated the proliferation of neuronal precursor cells through GHS-R. Although the receptor recognizing des-acyl ghrelin has not yet been characterized, the present binding study indicated that there was at least one binding site specific for des-acyl ghrelin in membranes from fetal spinal cord tissue. Several recent studies have reported that not only ghrelin but also des-acyl ghrelin exert a biological effect even in tissues or cells that do not express GHS-R, suggesting that these reactions would not require octanoic acid modification and could be achieved without GHS-R [11,17,21,22]. Interestingly, many of the effects induced by both peptides at the cellular level are associated with cell fate, such as cell survival and/or apoptosis as well as cell proliferation, although activation or inhibition of the cell survival and proliferation pathways appear to be independent of cell type [11]. Thus, it is assumed that the effect of ghrelin and des-acyl ghrelin on spinal cord cells observed in the present study could be induced through both the GHS-R and another unknown pathway.

Recently, Zhang et al. demonstrated that ghrelin acted directly on dorsal motor nucleus of the vagus neurons to stimulate neurogenesis, and concluded that neuronal proliferation would result from an increase of calcium concentration associated with cellular depolarization through activation of GHSR-1a by ghrelin [15]. In the present study, however, diltiazem, a blocker of L-type voltage-dependent calcium channels, did not inhibit proliferation of spinal cord neuronal cells, inconsistent with dorsal motor nucleus of the vagus neurons, suggesting that the proliferation effect was likely mediated via a pathway other than the calcium increase caused by depolarization of L-type calcium channels (data not shown). Some studies of the molecular mechanism involved in the induction of cell proliferation and adhesion by ghrelin have suggested cascades of intracellular events, such as the MAPK and/or PI3 K/Akt pathways. In hepatoma cells expressing GHS-R, ghrelin has been shown to activate the IRS-1-GRB2-MAPK pathway, downstream from the insulin receptor, but to inhibit Akt activity [23]. Also in cardiomyocytes and endothelial cells, ghrelin induces phosphorylation of tyrosine, and both ghrelin and des-acyl ghrelin activate the MAPK and Akt pathways [9]. In addition, activation of the MAPK pathway by ghrelin has also been reported in a rat pituitary somatotroph cell line [13] and human adrenal zona glomerulosa cells [12]. Nanzer has explained the possible pathways leading to MAPK activation, resulting from stimulation of phospholipase C and PKC, or transactivation of tyrosine kinase receptors via the beta and gamma subunits of the G protein. Similarly, it is assumed that the cell proliferation effect of ghrelin and des-acyl ghrelin in the rat fetal spinal cord might involve activation of MAPK and/or PI3K/AKt.

In this study, not only neuronal precursor cells but also neurons seemed to possess GHS-R protein, because the localization of Map-2-positive cells was consistent with that of GHS-R-positive cells in sections of spinal cord tissue. These results suggested that ghrelin would play an unidentified role via GHS-R in neurons of the rat fetus, for instance during formation of the neuronal network. Although there is no evidence of any abnormality in GHS-R-knockout mouse fetus [24], this function as well as the cell proliferative effect may be concealed by compensating actions of growth factors such as nerve growth factor.

In summary, we have demonstrated that both the GHS-R gene and protein are expressed in the rat fetal spinal cord from ED 13 to PD 0. In primary cultures of fetal spinal cord cells, ghrelin and des-acyl ghrelin induced cell proliferation effects, whereby neuronal precursor cells possessing GHS-R protein were increased. Moreover, a binding study using labeled des-acyl ghrelin showed that specific binding to des-acyl ghrelin could be displaced by unlabeled ghrelin and des-acyl ghrelin in membranes from fetal spinal cord. Taken together, our findings suggest that in the rat fetal spinal cord, ghrelin and des-acyl ghrelin are involved in neurogenesis via both GHS-R and an unidentified receptor

for des-acyl ghrelin. Further examinations to identify this unknown receptor for des-acyl ghrelin are warranted.

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Purification and characterization of feline ghrelin and its possible role

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Abstract

Ghrelin, a novel 28-amino acid peptide with an *n*-octanoyl modification at Ser^3, has been isolated from rat and human stomach as an endogenous ligand for the growth hormone secretagogue receptor. Here, we purified feline ghrelin and examined its possible physiological role in cats. The major active form of feline ghrelin is a 28-amino acid peptide octanoylated (C8:0) at Ser^3; except for one amino acid residue replacement, this structure is identical to those of rat and human ghrelins. However, much structural divergence in peptide length and fatty acid modification was observed in feline ghrelin: peptides consisting of 27 or 26 amino acids lacking Gln^{14} and/or Arg^{28} were found, and the third serine residue was modified by octanoic acid (C8:0), decanoic acid (10:0), or unsaturated fatty acids (C8:1, C10:1 and C10:2). In agreement with the structural divergence, two kinds of cDNA with different lengths were isolated. Administration of synthetic rat ghrelin increased plasma growth hormone levels in cats, with a potency similar to that in rat or human. Plasma levels of ghrelin in cats increased approximately 2.5-fold after fasting. The present study indicates the existence of structural

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divergence in feline ghrelin and suggests that, as in other animals, ghrelin may play important roles in GH release and feeding in cats.
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Keywords: Ghrelin; Cat; Growth hormone; Feeding behavior

1. Introduction

Ghrelin, a novel 28-amino acid peptide, was originally isolated from rat stomach as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) [1]. A unique feature of the structure of ghrelin is modification of the Ser^3 residue by *n*-octanoic acid. This octanoyl modification is essential for receptor binding and subsequent expression of biological activity, such as growth hormone (GH) release. Structural divergence has been observed in rat and human ghrelins. For example, ghrelins lacking Gln^{14} or Arg^{28} have been isolated from rat and human, respectively [2,3]. In addition to octanoylated (C8:0) ghrelin, decanoylated (C10:0) and decanoylated (C10:1) ghrelins were also found in human stomach [3]. These structural divergences of peptide length and fatty acid modification have also been reported in non-mammalian (rainbow trout, chicken and bullfrog) ghrelins [4–6]. Although GH release induced by the cognate ghrelin has been confirmed in various animals, potency differs according to peptide length and fatty acid modification [2–6].

In addition to stimulation of GH secretion in vivo and in vitro, ghrelin has been reported to stimulate food intake, body weight gain and adiposity when administered peripherally or centrally to rodents, and these activities are independent of GH secretion [7,8]. The effect of peripheral ghrelin on appetite is mediated via the gastric afferent vagal nerve [9]. On the other hand, its central effect is thought to occur via neuropeptide Y and agouti-related peptide secretion from the arcuate nucleus in the hypothalamus [8]. These results suggest that ghrelin plays important roles in the regulation of food intake and energy expenditure.

Obesity and anorexia have become serious problems in humans. Administration of ghrelin increases food intake in cancer patients with anorexia [10]. Human gastrectomy reduces plasma ghrelin levels by one-half, after which levels gradually increase as a result of compensation by other tissues [3]. Increasing ghrelin levels might restore food intake in patients, and research on the clinical application of ghrelin for anorexia is now in progress. In the veterinary field, obesity and anorexia have also become serious problems in companion animals, especially dogs and cats [11–13]. Cats have long been used as an important model to study the regulation of feeding, since lesions in the ventromedial hypothalamus produce rapid hyperphagia and abnormal body weight gain that persist for a long time [14]. In addition, diabetes in cats closely resembles type 2 diabetes in humans [15]. Recently, it has been reported that plasma levels of ghrelin change in rats with hyperphagia induced by streptozotocin-induced diabetes [16], and that a ghrelin $Arg^{51}Gln$ mutation is a risk factor for type 2 diabetes and hypertension in middle-aged humans [17]. Therefore, it appears important to determine the structure of feline ghrelin and the physiological role of ghrelin in cats. In the present study, we purified feline ghrelin from the stomach. During the course of purification, we found several minor peptides with ghrelin-like activity but with charac-

teristics different from those of standard ghrelin. We identified these stomach peptides as ghrelin-derived molecules and calculated the amount of these substances. In addition, we examined in cats whether ghrelin affects plasma GH after intravenous (i.v.) injection and whether satiety signals affect plasma ghrelin levels.

2. Materials and methods

2.1. Purification of feline ghrelin

During the purification process, ghrelin activity was followed by measuring changes in intracellular calcium concentrations ($[Ca^{2+}]_i$) with a fluorometric imaging plate reader (FLIPR) system (Molecular Devices, CA, USA) in a cell line stably expressing rat GHS-R [Chinese hamster ovary (CHO)-GHSR62], as described previously [1]. Feline stomach was obtained from Miyazaki veterinary hospital. Frozen stomach (15 g) was used as the starting material. The basic peptide fraction (SP-III) was prepared as described previously [1]. The SP-III fraction was subjected to carboxymethyl (CM) ion-exchange high-performance liquid chromatography (HPLC) on a column of TSK CM-2SW (4.6 mm × 250 mm; Tosoh, Tokyo, Japan) with an ammonium acetate ($HCOONH_4$) (pH 6.5) a liner gradient of 10 mM to 0.6 M in the presence of 10% acetonitrile (ACN) at a flow rate of 1 ml/min from 16 min to 136 min. Active fractions were separated by reverse-phase (RP)-HPLC with a μBondasphere C18 column (3.9 mm × 150 mm, Waters, MA, USA) at a flow rate of 1 ml/min of a linear gradient from 10% to 60% ACN/0.1% trifluoroacetic acid (TFA) for 80 min. Active fractions were further purified by RP-HPLC using a Chemcosorb 3ODS-H column (2.1 mm × 75 mm; Chemco, Osaka, Japan) for 80 min under a linear gradient from 10% to 60% ACN/0.1% TFA at a flow rate of 0.2 ml/min. Fractions corresponding to absorption peaks were collected, and an aliquot of each fraction (1 g tissue equivalent) was assayed by the FLIPR system. Approximately 20 pmol of the final purified peptide from the main activity fraction was analyzed with a protein sequencer (model 494; Applied Biosystems, CA, USA). Approximately 1 pmol of each active fraction was used for molecular weight determination by matrix-assisted laser desorption–ionization time of flight (MALDI-TOF) mass spectrometry with a Voyager-DE PRO instrument (Applied Biosystems).

2.2. Cloning of feline ghrelin cDNA

The rapid amplification of cDNA ends (RACE) PCR method was used for cDNA cloning. Total RNA was extracted from a stomach with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and poly(A)$^+$ RNA was isolated with an mRNA purification kit (TaKaRa Bio Inc., Kyoto, Japan). For 3′-RACE PCR, first-strand cDNAs were synthesized from 200 ng of poly(A)$^+$ RNA by using an adaptor primer supplied with the 3′-RACE system (Invitrogen) and the SuperScript II reverse transcriptase (RT) (Invitrogen). One-tenth of the cDNA was used as template. Primary PCR was performed as described previously [6] with four degenerate primers based on the N-terminal seven-amino acid sequence of human ghrelin (GSSFLSP): GRL-S7, 5′-GGGTCGAGYTTCTTRTCNCC-3′; GRL-S8,

5′-GGGTCGAGYTTCTTRAGYCC-3′; GRL-S9, 5′-GGGTCGAGYTTCCTNTCNCC-3′; and GRL-S10, 5′-GGGTCGAGYTTCCTNAGYCC-3′. Amplification was performed as follows: 94 °C for 1 min, 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, and a final extension for 3 min at 72 °C. Amplified products were purified with a Wizard PCR Preps DNA purification system (Promega, Madison, WI). For second-round nested PCR, a nested sense primer for feline ghrelin-(7-13) (5′-CCNGARCAYCARAARGTNCARC-3′) was used. The amplification reaction was 94 °C for 1 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension for 3 min at 72 °C. The candidate ghrelin cDNA fragment was subcloned with a TOPO TA cloning kit (pCR II-TOPO vector, Invitrogen) and sequenced. For 5′-RACE PCR, first-strand cDNAs were synthesized from 200 ng of poly(A)$^+$ RNA with oligo-dT$_{12-18}$ primer and the SuperScript II RT at 42 °C for 1 h. One-fifth of the purified cDNA was subjected to a TdT-tailing reaction of the 5′-ends of the first-strand cDNA with deoxy CTP according to the manufacturer's protocol (Invitrogen). The resultant dC-tailed cDNAs were used as template. A gene-specific primer was designed on the basis of the sequence of the feline ghrelin cDNA as determined by 3′-RACE PCR: Fel GRL-AS2, 5′-GTGGATCAAGCCTTCCAGAG-3′; Fel GRL-AS3, 5′-GACAGCTTGATTCCAACATC-3′. Primary PCR was performed with Fel GRL-AS3, an abridged anchor primer supplied with the 5′-RACE kit, and Ex *Taq* DNA polymerase under the following reaction conditions: 94 °C for 1 min, 35 cycles at 94 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min, and a final extension for 3 min at 72 °C. The resulting product was purified with Wizard PCR Preps, and the second-round nested PCR was performed with Fel GRL-AS2 and an abridged universal amplification primer. The amplification reaction was 94 °C for 1 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension for 3 min at 72 °C. The candidate PCR product was subcloned into the pCR-II TOPO vector and sequenced. The nucleotide sequence of the isolated cDNA fragment was determined by automated sequencing (DNA sequencer: model 3100, Applied Biosystems) according to the protocol for the BigDye terminator cycle sequencing kit (Applied Biosystems).

2.3. *GH-releasing activity in cats*

Healthy adult male ($n = 5$) and female ($n = 5$) cats, 1.5–4 years old and weighing approximately 4–6 kg, were used for this study. These animals had been kept by students of the Miyazaki University veterinary course, and all procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care. We evaluated plasma GH levels after i.v. administration of synthetic rat ghrelin. The experiments were performed on four groups: saline control, and doses of 0.05, 0.5 or 2.5 μg/kg (body weight) of rat ghrelin (Peptide Institute, Inc., Osaka, Japan) by i.v. bolus injection. Each dose of ghrelin was administered to five cats and two times a cat at random groups 1-month following after first injection. Blood was collected from the cephalic vein before and 10, 20, 40 and 80 min after injection into tubes containing EDTA-2Na (1 mg/ml blood) (Sigma, St. Louis, USA). Blood collection (500 μl per sample) and i.v. injection were performed through a 24-SWG catheter (19 mm long, Insyte 24 GA; Becton Dickinson, Sandy, UT, USA) introduced into the cephalic vein without tranquilizer. Plasma was separated by centrifugation (9100 × g, 10 min at 4 °C) and was kept at −80 °C until measurement of GH. Plasma GH concentration

was measured by a heterologous canine radioimmunoassay (RIA) that has previously been validated for feline GH [18]. The canine GH RIA kit was supplied by National Hormone and Peptide Program (CA, USA). Radioiodination was performed by the chloramine-T method. The second antibody was a goat anti-monkey IgG serum (HAC-MKA2-02GTP88) supplied by the Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University. The assay procedure was performed according to the method described previously [19]. The intra- and interassay coefficients of variation were 6.4% and 3.9%, respectively.

2.4. Plasma ghrelin level and feeding state

To examine the effect of fasting on plasma ghrelin levels in cats, blood was collected from the cephalic vein through a 21 *G* needle (38 mm long, Terumo, Tokyo, Japan) without tranquilizer before and after overnight fasting ($n = 8$ cats). Blood was collected in chilled tubes containing EDTA-2Na (1 mg/ml blood) and aprotinin (500 U/ml blood) (Sigma, St. Louis, USA). Plasma was separated by immediate centrifugation (9100 × *g*, 10 min at 4 °C), and was acidified with 1/10 volume of 1N HCl. Samples were stored at −80 °C until determination of ghrelin levels. Plasma ghrelin levels were measured with a human active ghrelin ELISA kit (Mitsubishi Kagaku latron, Inc., Tokyo, Japan). This active ghrelin ELISA kit measures human ghrelin-(1-28)(C8:0) on the principle of a two-site sandwich enzyme-linked immunosorbent assay. It can detect not only human ghrelin but also rat and mouse ghrelin. A 50 μl sample was added to a 96-well ELISA plate coated with anti-N-terminal ghrelin monoclonal antibody. After incubation, the plate was washed and horseradish peroxidase-conjugated anti-C-terminal ghrelin monoclonal antibody was added to each well. After incubation, the plate was washed and substrate solution was added to each well. Then, the absorbance of each well was determined at 450 nm. In addition, a fasting plasma sample was loaded onto a Sep-Pak plus C18 cartridge (Waters) and the eluate was separated by RP-HPLC on a μBondasphere C18 column at a flow rate of 1 ml/min of a linear gradient from 10% to 60% ACN/0.1% TFA over 40 min. The eluate was collected in 0.5 ml fractions and ghrelin levels were measured with the active ghrelin ELISA kit.

2.5. Statistical analysis

Values are expressed as means ± S.E.M. The GH data were analyzed statistically by repeated measures ANOVA followed by the Student–Newman–Keuls test. Comparison of ghrelin before and after fasting was performed by paired Student's *t*-test. Differences with a value of $P < 0.05$ were considered significant.

3. Results

3.1. Purification of feline ghrelin

Nine groups of ghrelin activity were identified by CM ion-exchange HPLC (pH 6.5) of the SP-III fraction (Fig. 1A). Each active group was purified by two different rounds of RP-

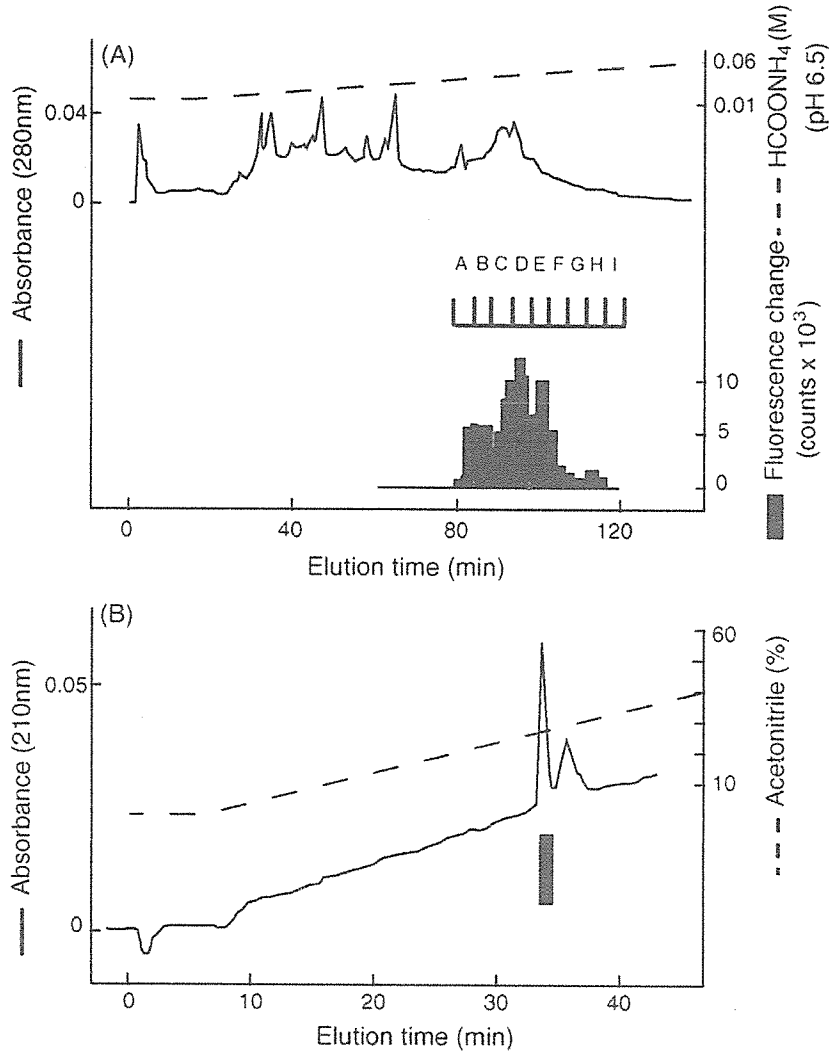

Fig. 1. Purification of feline ghrelin from stomach extract. *Black bars* indicate the fluorescence changes in $[Ca^{2+}]_i$ in CHO-GHSR62 cells. (A) CM ion-exchange HPLC (pH 6.5) of the SP-III fraction of stomach extract. Each active fraction (A–I) was subjected to two steps of RP-HPLC. (B) Final purification of active fraction D from CM-HPLC by RP-HPLC (Table 1, peak 13).

HPLC. Fig. 1B shows the final isolation of the major feline ghrelin from group D in Fig. 1A. We were able to isolate 25 active peptides from the nine groups in CM-HPLC (Table 1). The complete amino acid sequence of the main activity from group D was determined by protein sequencing to be GSXFLSPEHQKVQQRKESKKPPAKLQPR (X was unidentified by the sequencer because of acyl modification). From comparison of sequence homology with other ghrelin, we determined the isolated peptide to be feline ghrelin.

3.2. Cloning of feline ghrelin cDNA

cDNA encoding prepro-ghrelin was isolated from stomach mRNA. cDNAs of two different lengths were isolated; both were identical in the lengths of their 5′-untranslated