Localization of Ghrelin-Producing Cells in the Stomach of the Rainbow Trout (Oncorhynchus mykiss)

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Localization of Ghrelin-Producing Cells in the Stomach of the Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT—Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (GHS-R), was isolated from the rat stomach and determined to be n-octanoylated 28-amino-acid peptide. In this study, we studied the distribution of ghrelin-producing cells (ghrelin cells) in the gastrointestinal tract of male and female rainbow trout (*Oncorhynchus mykiss*) by immunohistochemistry using N-terminal region-recognizing antibody and also by *in situ* hybridization using a trout ghrelin-specific cRNA probe. Ghrelin cells were found in the mucosal layer of the stomach but not in the myenteric plexus, and no ghrelin cells were observed in other regions of the gastrointestinal tract. Ghrelin cells could be classified into two types: closed- and opened-type cells. The density of ghrelin cells increased gradually in the direction from the cardiac to pyloric portions of the stomach in both sexes. The number of ghrelin cells per unit area seemed to be higher in females than in males. In conclusion, trout ghrelin cells exist in the stomach and are classified into two types of cells, closed- and opened-type cells.

Key words: ghrelin, rainbow trout, stomach, immunohistochemistry, *in situ* hybridization

INTRODUCTION

Ghrelin, a brain-gut peptide, was recently isolated from the rat and human stomach as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R), and it has been shown to have a unique molecular structure, *n*-octanoyl acylation at the third serine residue (Kojima *et al*., 1999). It has been shown that this peptide exists in two forms: *n*-octanoyl ghrelin, a physiological active form, and des-n-octanoyl ghrelin, an inactive form (Hosoda *et al*., 2000). Ghrelin stimulates GH secretion from the anterior pituitary gland (Kojima *et al*., 1999; Seoane *et al*., 2000; Wren *et al*., 2000; Yamazaki *et al*., 2002), food intake (Asakawa *et al*., 2001b; Nakazato *et al*., 2001), gastric acid secretion (Masuda *et al*., 2000; Date *et al*., 2001), gastric motor activity (Masuda *et al*., 2000) and insulin secretion (Adeghate *et al*., 2002; Lee *et al*., 2002) in the rat. Moreover, in mammals, ghrelin and GHS-R are known to exist in central and peripheral organs, including the hypothalamus, pituitary gland, heart, stomach, intestine, kidney, testis and ovary (Gnanapavan *et al*., 2002), and it is thought that ghrelin regulates energy homeostasis, reproduction, gastrointestinal and cardiovascular functions.

Ghrelin has recently been identified in many species other than mammals, including birds (Kaiya *et al*., 2002) and amphibians (Kaiya *et al*., 2001). Ghrelin in chicken consists of 26 amino acids, in which the third serine residue is also *n*-octanoylated or *n*-decanoylated as it is in mammals. On the other hand, in the bullfrog, its third serine residue is replaced by threonine and is modified by *n*-octanoic acid. Ghrelin in the stomachs of goldfish (Unniappan *et al*., 2002b), tilapia (Kaiya *et al*., 2003c) and Japanese eel (Kaiya *et al*., 2003b) has also been shown to be modified by *n*-octanoic and decanoic acid at the third serine with an amidated C-terminal region. Recently, Kaiya *et al*.(2003a) purified ghrelin from the stomach of the rainbow trout (*Oncorhynchus mykiss*) and showed that four isoforms of ghrelin are produced through the amidation of alternatively spliced ghrelin gene products and that its third serine residue is also modified by octanoic acid or decanoic acid. The N-terminal

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region of rainbow trout ghrelin is highly conserved with those in other species, including mammalian species, and it has been demonstrated that ghrelin are involved in GH release in the rainbow trout.

The distribution of ghrelin cells in the gastrointestinal tract has been determined in many animals by immunohistochemical analysis (Date *et al*, 2000; Hayashida *et al*., 2001; Sakata *et al*., 2002a; Wada *et al*., 2003). Ghrelin cells are most abundant in the stomach, and there are a few cells in the small and large intestines. In this study, we studied the distribution of ghrelin-producing cells in the gastrointestinal tract of rainbow trout by immunohistochemistry (IHC) using an antibody that recognizes rat N-terminal regions of ghrelin and by *in situ* hybridization (ISH) using the trout-specific cRNA probe.

MATERIALS AND METHODS

Animals and Tissue Preparations

Adult male and female rainbow trout (*Oncorhyncus mykiss*) weighing 1467–3715 g were used in this study. The fish were allowed food once per day until they left the pellets and were kept in water at 15°C. The three parts of the stomach (cardiac portion, bliand sac, pyloric portion), intestine and pyloric cecum (Fig. 1.) were quickly removed and were fixed in 4% paraformaldehyde in 50 mM phosphate buffer (PB) overnight. The tissue blocks were dehydrated with an ascending ethanol series and immersed in xylene and then embedded in PARAPLAST PLUS (TYCO HEALTHCARE, Mansfield, MA, USA). Serial sections (7 μm thick) were made and mounted on silane (ShinEtsu Chemicals, Tokyo, Japan)-coated slides. All procedures used in this study were performed in accordance with institutional guidelines for animal care at Saitama University.

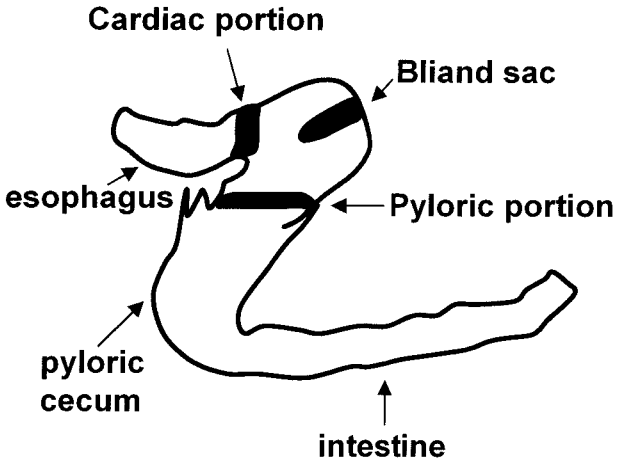

Fig. 1. Scheme of sampling portions in the trout gastrointestinal tract. Three parts of the stomach, cardiac portion, bliand sac and pyloric portion, were used for IHC and ISH.

Immunohistochemistry

Immunohistochemical detection of ghrelin cells using rabbit anti-ghrelin serum (# 603) was carried out by the ABC method. The production and specificity of the anti-ghrelin serum used in this study were previously reported (Hosoda *et al*., 2000). This antiserum recognizes the N-terminal region of ghrelin and reacts with the octanoylated form of ghrelin, physiological active ghrelin. Immunohistochemical staining was performed basically according to the previously reported procedure (Sakata *et al*., 2002a). Briefly, the sections were deparaffinized with xylene and rehydrated through descending concentrations of ethanol. Next, the sections were treated with 0.5% sodium metaperiodate to block endogenous peroxidase for 15 min at room temperature and then incubated with 1% normal horse serum and 0.4% TritonX-100 in PBS for 1 h. After washing with PBS for 15 min, the sections were incubated overnight with anti-ghrelin serum diluted 1:50000 in PBS. After washing with PBS, a second incubation was carried out for 2 h with biotin-conjugated anti-rabbit IgG serum (Vectastain ABC kit; Vector, Burlingame, CA, USA) diluted 1:300 with PBS. After washing with PBS, the sections were incubated for 30 min with an avidin-biodin-peroxidase complex (Vector) prepared according to the manufacturer's instructions and were washed with PBS for 15 min. The sections were reacted in 0.02% 3, 3-diaminobenzidine-tetrachloride (DAB) mixed with 0.006% hydrogen peroxide (H_2O_2) in 0.05 M Tris-HCl, pH 7.6, for 1–3 min to detect immunostaining. After washing with distilled water, the sections were dehydrated with a graded ethanol series, cleared in xylene, mounted with Entellan (Merck, Darmstadt, Germany), and viewed under a light microscope (BX60, OLYMPUS, Tokyo, Japan). All of the incubations were carried out in a humidity chamber at room temperature.

In situ hybridization

The sections were deparaffinized with xylene, and rehydrated through descending concentrations of ethanol, and washed twice with PBS for 15 sec. The sections were then washed with PBS, treated with 16 μg/ml proteinase K for 30 min at 37°C, and fixed with 4% paraformaldehyde in 0.067 M PB, pH 7.4. After washing with PBS for 1 min, the sections were incubated with 0.2 M HCl in water and then washed with PBS for 1 min. The sections were treated with 0.1 M triethanolamine-HCl, pH 8.0, for 1 min and 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, washed with PBS for 1 min, immersed in a graded ethanol series (70%, 80% and 90%) for 15 sec each and then immersed twice in 100% ethanol for 15 sec, and dried for 20 min. Digoxigenin (DIG)-labeled anti-sense and sense trout ghrelin cRNA probes (GenBank accession No. AB096919, nucleotides 1-490) were synthesized using a labeling kit (Roche Diagnostics GmbH, Mannheim, Germany) with SP6 or T7 RNA polymerases. The probes were diluted to 2 ng/μl with hybridization buffer (50% formamide, 3×SSC, 0.12 M DEPC-treated PB, pH 7.4, 1×Denhardt solution, 125 μg/ml tRNA, 0.1 mg/ml sonicated salmon sperm DNA, and 10% dextran sulfate) and dropped on the tissue sections. A sense RNA probe was used as a negative control. The sections were covered with PARAFILM (American National Can, CHICAGO, IL, USA) and incubated for 16 h at 42°C in a humid chamber. The covers were removed by soaking the slides in 5× SSC and immersing 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 (5 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, each twice, at 55°C. 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 (Roche Diagnostics GmbH, Mannheim, 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 GmbH, Mannheim, Germany) diluted 1:2,000 in buffer-1. The sections were then 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 4-nitroblue tetrazolium chloride (NBT) and 175 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in buffer-2) was added, and the sections were incubated until a visible signal was detected. The reaction was stopped by adding a reaction stop solution (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, pH

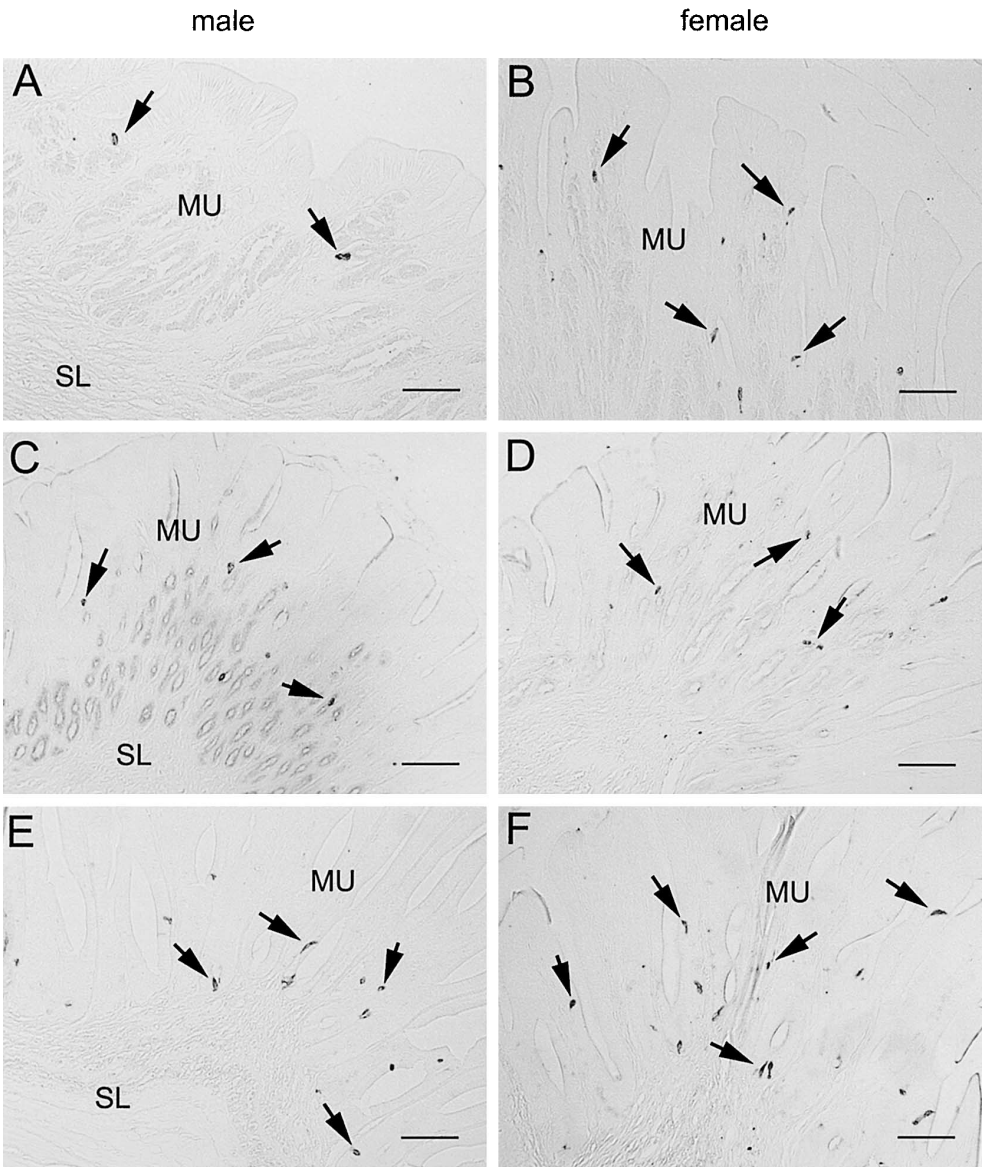

Fig. 2. Microphotographs of ghrelin-ip cells in the trout stomach by the immunohistochemistry. Ghrelin-ip cells were only found in the mucosal layer and were observed throughout the stomach (A, B; cardiac portions, C, D; bliand sac, E, F; pyloric portions) in the male trout (A, C, E) and female trout (B, D, F). The number of ghrelin-ip cells in the stomach increased gradually in the direction to the pyloric portions of the stomach in both sexes. MU, mucosa; SL, smooth muscle layer; (A–F) bar: 100 μm

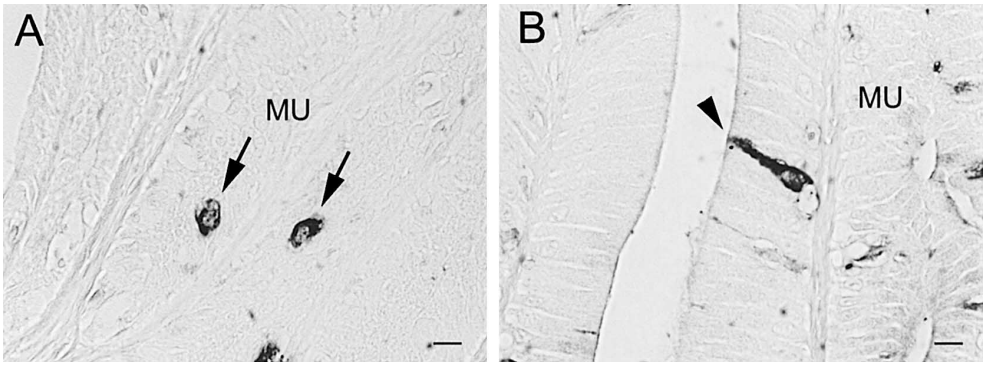

Fig. 3. High magnification of ghrelin-ip cells in the male trout stomach by the immunohistochemistry. Ghrelin-ip cells can be classified into two types: closed-type endocrine cells (arrows, A) and opened-type endocrine cells (arrowheads, B), processes of which extended to the gut lumen. MU, mucosa, (A, B) bar: 10 μm

8.0). The sections were washed with PBS, mounted with 90% glycerol in PBS, and then viewed and photographed 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.

Morphometric analysis

The densities of ghrelin-immunopositive (ghrelin-ip) cells in the three regions of the rainbow trout stomach were estimated. After taking digital photographs under a light microscope with a digital camera (DP70, OLYMPUS, Tokyo, Japan), the number of ghrelin-ip cells in each section was counted and the area of the mucosal layer in each section was measured using a computerized image analysis program, Scion Image (Scion Corporation, Frederick, MD, USA). The ghrelin cell density was calculated as the number of immunopositive mucosal cells per unit area (cells/mm^2). All of the data are expressed as means±S. E. Three fishes were used in this study, and five independent areas were measured in each portion. The differences between cell densities were evaluated using Fisher's least significant difference with StatView statistics software (SAS Institute, Cary, NC, USA). $p<0.05$ was considered statistically significant.

RESULTS

Localization of ghrelin cells in the stomach

Ghrelin-ip cells were found in the mucosal layer of both male and female trout stomachs but not in the myenteric plexus (Fig. 2A–F), and no ghrelin-ip cells were observed in

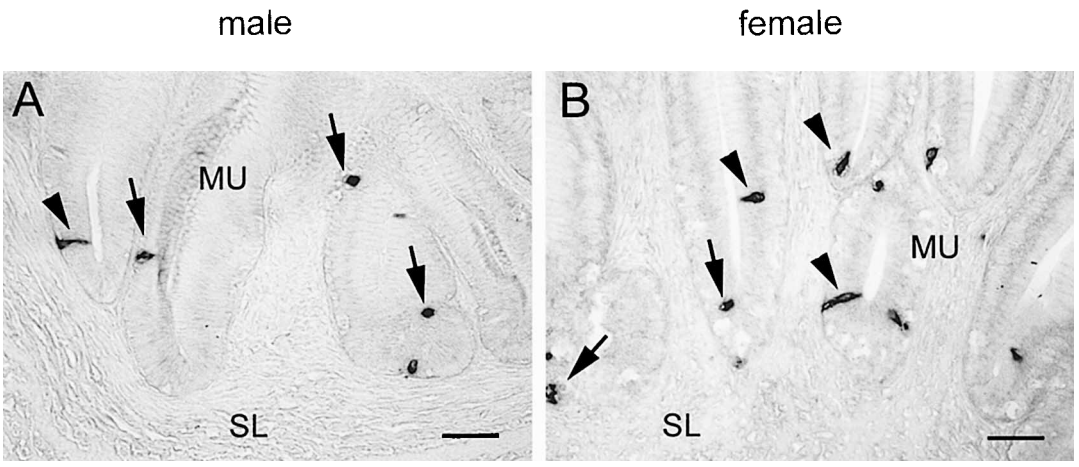

Fig. 4. Microphotographs of ghrelin-ex cells in the trout stomach. (A) Ghrelin-ex cells in the male stomach. (B) Ghrelin-ex cells in the female stomach. The numbers and distribution patterns of these cells correspond to the results of IHC shown in Fig. 1. Ghrelin-ex cells can also be classified into two types: closed-type endocrine cells (arrows) and opened-type endocrine cells (arrowheads) MU, mucosa; SL, smooth muscle layer; (A, B) bar: 100 μm

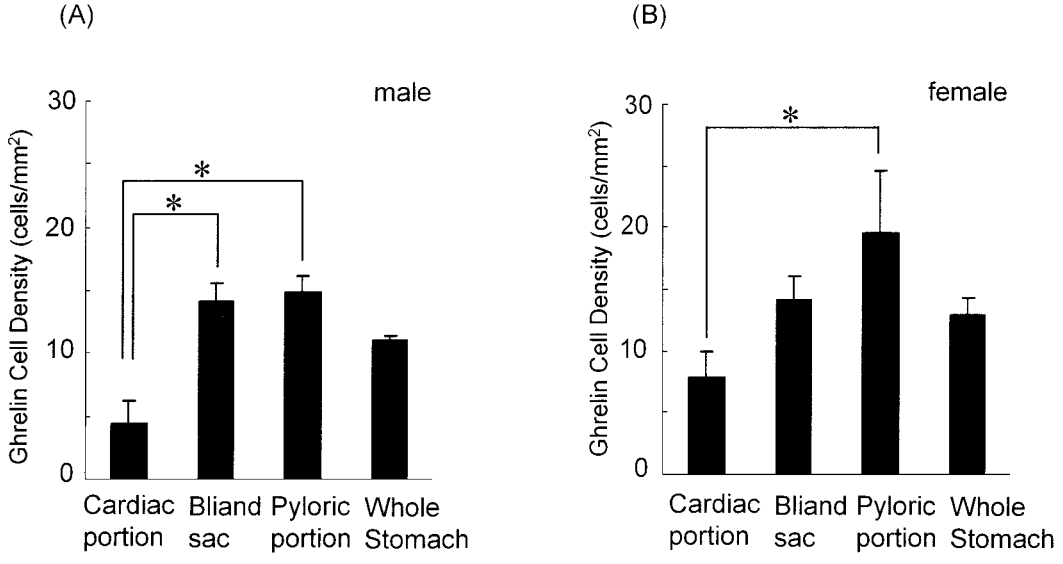

Fig. 5. Histogram showing the densities of ghrelin-ip cells (cells/mm^2) in different parts of the stomach. Ghrelin cell densities in the male (A) and female (B) are shown as number of cells per square mm. The density of ghrelin-ip cells increased gradually in the direction to the pyloric portion of the stomach in both sexes. Although there were no significant differences, the mean values of ghrelin-ip cells densities in the female were greater than that in the male in the cardiac, pyloric portions and whole stomach. Three fishes were used in this study, and five independent areas were measured in each portion. Asterisk, $P<0.05$

other parts of the gastrointestinal tract. A few ghrelin-ip cells were found in the villi of the mucosa, but most ghrelin-ip cells were observed at the bottom of the fundus. Ghrelin cells in the stomach could be classified into two types: closed-type endocrine cells which were observed in the base of the mucosal layer (Fig. 3A; arrows) and opened-type endocrine cells, whose processes extended to the gut lumen (Fig. 3B; arrowhead), and no marked differences were found between immunoreactivities of the two types of ghrelin-ip cells. Moreover, notable differences between staining patterns in male and female specimens were not found. Using the ISH method, ghrelin mRNA-expressing cells (ghrelin-ex cells) were observed throughout the stomach mucosa (Fig. 4). Ghrelin-ex cells detected by ISH showed the same localization as that of ghrelin-ex cells detected by IHC, and two cell types with the same properties as those found by IHC were also detected. Morphometric analysis revealed that ghrelin-ip cells in the stomach increased gradually in the direction toward the pyloric portion of the stomach in both sexes and that the densities of ghrelin cells in the pyloric portions were significantly higher than those in the cardiac portions (Fig. 5A, B). Although significant differences in cell density were not found, the densities of ghrelin-ip cells in female trout had a tendency to be greater than that in male trout in the cardiac and pyloric parts of the stomach. (Fig. 5A, B).

DISCUSSION

Ghrelin has been identified in several vertebrates (Hayashida *et al*., 2001; Kaiya *et al*., 2001; Kaiya *et al*., 2002; Kaiya *et al*., 2003a, b, c), and it has been found that the N-terminal regions of the ghrelins were evolutionally highly conserved. Recently, Kaiya *et al*. (2003a) successfully isolated trout ghrelin and reported that its third serine residue was modified by either octanoic or decanoic acid. Interestingly, the first seven amino acids of the N-terminal region of trout ghrelin (GSSFLSP) and the octanoylated form are identical to those in humans, rats and chickens. N-terminal region-recognizing rabbit anti-rat ghrelin serum, which is known to react with *n*-octanoylated ghrelin but not with des-acyl ghrelin (Hosoda *et al*., 2000), was used for IHC analysis in this study. Since the N-terminal region of trout ghrelin is identical to that of rat ghrelin, it is thought that this antiserum can specifically recognize trout ghrelin cells. Moreover, we compared the cell densities determined by IHC or ISH and found that no differences between ghrelin-ip and -ex cell densities, suggesting that almost all ghrelin-ex cells contain the mature type of ghrelin.

Although ghrelin cells exist only as closed-type cells in the rat stomach and chicken stomach (Sakata *et al*., 2002; Wada *et al*., 2003) we demonstrated in this study that ghrelin cells in the rainbow trout stomach include both opened- and closed-type cells. Opened-type cells are thought to be functionally regulated by receiving luminal information such as on nutrients and pH, while, closed-type cells are thought to be regulated by some hormones, neuronal stimulation or mechanical distention (Solcia *et al*., 1975; Lewin *et al*., 1986). The presence of these two distinct ghrelin cell types in the stomach of rainbow trout may indicate that ghrelin secretions from these morphologically unique cell types are regulated by different mechanisms and may have distinct physiological effects compared to mammals or birds.

As reported in the rat stomach (Sakata *et al*., 2002b), the densities of ghrelin-ip cells in the rainbow trout seemed to be higher in the female than that in the male. Ghrelin mRNA levels were also reported to be significantly higher in female than in male tilapia (Parhar *et al*., 2003). Pagotto *et al*. (2003) reported that ghrelin levels in hypogonadal patients increased after testosterone treatment, indicating that sex hormones modulate circulating ghrelin concentrations in humans. Moreover, Matsubara *et al*. (2004) recently reported that ghrelin-ip cells express estrogen receptor α and that the number of ghrelin cells and plasma ghrelin level significantly increased three days after ovariectomy, suggesting that sex steroids regulate the expression of ghrelin. Therefore, the effect of sex steroids on ghrelin expression in the rainbow trout must be studied.

It is known that the trout stomach is composed of cardiac, bliand sac and pyloric portions, and that the digestive function, tissue structure and gastric glands are different in each gastric portion (Yokote, 1982). In this study, we clearly demonstrated that the densities of ghrelin cells in these portions differed. Although the difference in cell densities in gastric portions is an interesting observation, the significance of this difference is not yet clear, and further studies to determine the significance are needed.

In summary, we have demonstrated the localization of ghrelin cells in the trout stomach and have shown that ghrelin cells exist as two types of cells, closed- and opened-type cells.

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