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Characterisation of gastric ghrelin cells in man and other mammals: studies in adult and fetal tissues

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Abstract Ghrelin is a new gastric peptide involved in food intake control and growth hormone release. We aimed to assess its cell localisation in man during adult and fetal life and to clarify present interspecies inconsistencies of gastric endocrine cell types. A specific serum generated against amino acids 13–28 of ghrelin was tested on fetal and adult gastric mucosa and compared with ghrelin in situ hybridisation. Immunogold electron microscopy was performed on normal human, rat and dog adult stomach. Ghrelin cells were detected in developing gut, pancreas and lung from gestational week 10 and in adult human, rat and dog gastric mucosa. By immunogold electron microscopy, gastric ghrelin cells showed distinctive morphology and hormone reactivity in respect to histamine enterochromaffin-like, somatostatin D, glucagon A or serotonin enterochromaffin cells. Ghrelin cells were characterised by round, compact, electron-dense secretory granules of P/D_1 type in man (mean diameter 147±30 nm), A-like type in the rat (183±37 nm) and X type in the dog (273±49 nm). It is concluded that, ghrelin is produced by well-defined cell types, which in the past had been labelled differently in various mammals mostly because of the different size of their secretory granule. In man ghrelin cells develop during early fetal life.

Keywords Ghrelin · P/D_1 cells · Immunohistochemistry · Electron microscopy · In situ hybridisation

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Introduction

Ghrelin, a novel motilin-related, growth hormone-releasing and orexigenic peptide, has been recently isolated from murine stomach (Asakawa et al. 2001; Kojima et al. 1999; Tomasetto et al. 2000). Ghrelin expression was localised to X or A-like cells of rat and mouse gastric mucosa (Date et al. 2000; Tomasetto et al. 2000) as identified by previous ultrastructural analysis (Capella et al. 1971; Forssmann et al. 1969; Rubin and Schwartz 1979; Solcia et al. 1975). Light microscopy investigations in rat and human gastric mucosa showed that ghrelin cells represent a fairly abundant separate cell type, independent from other gastric endocrine cells like somatostatin D, serotonin enterochromaffin (EC) or histamine enterochromaffin-like (ECL) cells (Date et al. 2000). However, similar studies in the mouse stomach showed extensive colocalisation of the motilin-related ghrelin peptide with serotonin and somatostatin (Tomasetto et al. 2000), a finding at variance with rat and human observations for ghrelin cells and questioning their individuality and exact identification.

Up to now, ultrastructural studies of human stomach (Capella et al. 1978; D'Adda et al. 1989; Solcia et al. 1979) detected only very few cells with fairly large, round, compact granules resembling those of X cells as originally characterised in canine stomach (Solcia et al.

1975). In addition, glucagon immunoreactive cells showing round, haloed granules resembling pancreatic A cells, though moderately represented in fetal human stomach (Solcia et al. 1979, 1985), have not been observed in the oxyntic glands of adult human stomach (Capella et al. 1978; D'Adda et al. 1989; Solcia et al. 1979). Therefore the exact ultrastructural equivalent of the human ghrelin cell remains to be ascertained through direct immunocytochemical identification at the ultrastructural level.

In this paper human, rat and dog gastric mucosa were investigated at light and electron microscopy levels with immunocytochemical and hybridisation techniques to identify human ghrelin cells and to compare them with ghrelin cells of other mammals or with other types of functionally characterised endocrine cells. In addition, human fetal samples were investigated to assess the distribution of ghrelin cells at various stages of development.

Materials and methods

Generation and validation of the anti-ghrelin serum

We generated an antiserum against the carboxyl-terminal part of ghrelin. In brief, the peptide QRKESKKPPAKLQPR corresponding to the 13–28 fragment of the human ghrelin (Kojima et al. 1999) was obtained by solid phase synthesis, coupled to keyhole limpet hemocyanin and administered to two New Zealand male rabbits with incomplete Freund's adjuvant. The antiserum was tested by ELISA techniques (Lattuada et al. 1996) and western blotting as described (Tomasetto et al. 2000) to assess titration and specificity for the immunogenic peptide and synthetic human ghrelin (Europeptides, France). For ELISA control, preabsorption tests with excess antigen (10 µmol) were performed either with human ghrelin or human GHRH 1–29 (Europeptides). In addition, RT-PCR of relevant tissues and in situ hybridisation (ISH) were performed to support the results obtained by immunological methods.

Total RNA isolation and RT-PCR assay

After light anaesthesia with Avertin adult Wistar rat were killed, and gastric samples quickly isolated and snap-frozen in liquid nitrogen. Rat fetal stomachs were obtained by microdissection from rat fetuses from pregnant Wistar rats at embryonic day 21 as assessed by plug identification after mating (day 1). Human samples of gastric mucosa were obtained from two different gastrectomy specimens performed for gastric adenocarcinoma. The mucosa used for extraction was confirmed as substantially normal at histology. Total RNA was extracted from human and rat stomach according to the single-step, acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987). The integrity of extracted RNA was examined by agarose gel electrophoresis. Two hundred nanograms total RNA of each sample were subjected to reverse transcription with MMuLV followed by amplification using primers 1 (5′-TTGAGCCCAGAGCACCAGAAA-3′) and 2 (5′-AGTTGCAGAGGAG-GCAGAAGCT-3′) based on the published sequence of rat ghrelin and primers 3 (5′-GAAGAAGCCACCAGCCAAGC-3′) and 4 (5′-AAGCGAAAAGCCAGATGAGC-3′) for human ghrelin (Kojima et al. 1999). To normalise results for differences in RNA sampling, an aliquot of the same RT reaction was used to amplify a glyceraldehyde-6-phosphate (GADPH) 598 bp fragment. To ensure that PCR was performed in the linear amplification range, samples were analysed after 15, 17, 20, 25, 27, 30, 35 and 40 cycles; since the reaction proved linear over 15–27 cycles, we chose to use 22 cycles for subsequent amplifications.

In situ hybridisation studies

Adult Wistar rat stomachs were fixed by immersion in 4% paraformaldehyde and snap frozen. Consecutive 20- and 6-µm-thick sections were processed for ISH and immunocytochemistry, respectively (see following paragraph). For ISH two oligodeoxynucleotide sequences (ghrel1: 5′-TCTCTGCTGG GCTTTCTGGT GCTCTGGGCT CAAGAAGCTG GAACC-3′; ghrel2: 5′-GGAGAGTGCT GGGAGTTGCA GAGGAGGCAG AAGCTGGATG TGAGT-3′) were chosen in unique regions of the rat ghrelin mRNA (rat ghrelin cDNA Gene Bank accession number AB029433; ghrel1 and ghrel2 are complementary to nucleotides 100–144 and 427–471, respectively) following analysis for mRNA secondary structure using Genetic Computer Group Sequence Analysis Software 7.1 (GCG, USA). The oligonucleotide probes were labelled at the 3′ end using ^{35}S-dATP (Amersham, UK) and terminal deoxynucleotidyl transferase (Boehringer, Germany) following the specifications of the manufacturer at a specific activity of 100–300 kBq/pmol. The labelled probes were separated from unincorporated ^{35}S-dATP using NucTrap push columns (Stratagene, USA), precipitated in ethanol and resuspended in distilled water containing 50 mM dithiothreitol. The ISH procedure was carried out according to Zoli et al. (1995). Probes were applied at a concentration of 2,000–3,000 Bq/30µl per section (corresponding to around 15 fmol/section). The slides were exposed for 7 days to BioMax MR film (Kodak) and then for 1 month to a photographic emulsion (K5; Ilford). Specificity controls included the demonstration that: (1) both probes showed an identical labelling pattern, (2) probes with the same base composition but different sequence did not show the specific labelling pattern and (3) a 100-fold excess of cold probe added to the incubation mixture abolished the labelling.

Conventional light microscopy studies

Samples from the stomach of adult Wistar rats and Beagle dogs, or from non-pathologic human gut, pancreas and lung (from the files of the Departments of Pathology of Pavia and Brescia) were fixed in formalin or Bouin's fluid and embedded in paraffin.

For developmental studies, human fetal tissues were obtained after either elective termination of the pregnancy or natural abortion. Gastrointestinal, pancreatic and lung samples were microdissected from 24 fetuses between 9 and 37 weeks of gestation and fixed in formalin.

Serial paraffin sections (2–4 µm) were brought to water and incubated with anti-ghrelin serum 1:5,000, anti-human human vesicular monoamine transporter 2 (VMAT2) 1:20,000 (Chemicon International, USA), anti-recombinant rat histidine decarboxylase (HDC) 1:6,000 (Euro Diagnostica, Sweden) using the avidin-biotin-peroxidase (ABC) technique (Hsu et al. 1981) with (ghrelin and VMAT2) or without (HDC) microwave antigen retrieval (Cattoretti et al. 1993). Tests for chromogranin A (CgA), glucagon (with pancreatic glucagon-specific, C-terminal antibodies; Biogenesis, UK), somatostatin and serotonin were performed as detailed elsewhere (Rindi et al. 1993; Solcia et al. 1985). For double localisation studies, serial 2-µm paraffin sections were used. Specificity tests for ghrelin immunostain consisted of absorption of the serum with the specific antigen (5–10 nmol/ml diluted antiserum) or with the non-homologous antigen VMAT2 (10 nmol/ml diluted antiserum). Further controls consisted of omission of the first layer and use of tissues known to harbour (stomach) or to lack (liver, spleen) ghrelin (Gualillo et al. 2001; Kojima et al. 1999).

Electron microscopy studies

Small samples of normal human, rat and dog gastric mucosa were fixed in 2.5% glutaraldehyde or in a mixture of 2.5% glutaralde-

hyde and 2% paraformaldehyde solution in 0.1 M cacodylate buffer, pH 7.4. Part of the samples was postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, and embedded in Epon-Araldite. Samples fixed in aldehyde solutions only were embedded in the hydrophilic London White Resin (LWR; London Resin, UK) according to standard procedures. Areas of interest were trimmed, sectioned with a Sorvall Porter-Bloom MT2 ultramicrotome (60–100 nm), collected on uncoated nickel 200–300 mesh grids, desiccated, counterstained with uranyl acetate and Reynold's lead citrate and observed in a Zeiss 10CR transmission electron microscope (Zeiss, Germany). Single and double immunogold labelling studies were performed according to the gold-labelled immunoglobulin (Bio Cell Research Laboratories, UK) methods (Tapia et al. 1982). Specificity tests consisted of omission of the first layer and use of unrelated primary antibodies.

Computer-assisted quantitative analysis of immunogold-labelled electron-dense granules was performed on digitised pictures of ghrelin cells taken at ×10,000 or ×20,000 (human samples) and at ×12,000 (rat and dog). The diameter of each granule was measured using the image analysis software Scion 1.6 (Scion, USA) and the data were expressed as mean ± standard deviation (SD).

Results

Adult gastric mucosa

Validation of the anti-ghrelin serum and light microscopy studies

In ELISA tests the anti-ghrelin serum here generated proved effective in detecting as low as 15 ng human ghrelin at 1:2,500 dilution, remaining effective at 1:100,000 dilution when the wells were coated with 1 µg human ghrelin. Preabsorption with excess (10 µmol) human ghrelin or the immunogenic peptide, but not with human GHRH 1–29, completely prevented any positive signal at 1:2,500 dilution. Tested by standard western blotting techniques (Fig. 1), the antibody recognised synthetic ghrelin as a band of ca 5 kDa and an additional band of ca 10 kDa possibly corresponding to ghrelin dimers (Fig. 1 *hG*). A comparable band of ca 5 kDa was detected in protein extracts of rat gastric mucosa together with an additional band of ca 13 kDa (Fig. 1 *rS*) corresponding to the expected weight-size of rat preproghrelin (Kojima et al. 1999).

To assess the efficacy and specificity of our anti-ghrelin serum at histological level, tests were performed on rat, dog and human gastric mucosa. The serum proved effective in staining discrete, round to ovoid, endocrine cells of the oxyntic mucosa (Fig. 2A). The staining was abolished after absorption with ghrelin 13–28 (Fig. 2B), but not after absorption with human VMAT2 (not shown). With double immunostaining ghrelin cells were found to correspond to a fraction of CgA and VMAT2-positive cells, though negative for HDC antibodies (Fig. 2C, D). In addition, VMAT2 antibodies stained a larger population of ghrelin-negative, HDC-positive, elongated to angular cells corresponding to ECL cells.

In situ hybridisation for rat ghrelin and immunohistochemistry with our ghrelin antiserum were applied to consecutive 20- or 6-µm sections, respectively, of rat oxyntic mucosa. With both methods a similar mucosal cell population was demonstrated (Fig. 2E), thus proving that the serum here generated was effective in identifying true ghrelin-producing cells. Finally, RT-PCR analysis of total RNA from human and rat stomach yielded a DNA fragment of the expected length (292 and 348 bp for human and rat ghrelin, respectively) in all samples tested, including the rat fetal stomach at 21 days of gestation (Fig. 2F).

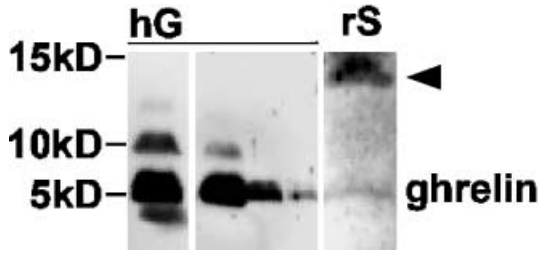


Fig. 1 Western blot for ghrelin. A band of ca 5 kDa is observed in the lanes of both human synthetic ghrelin (*hG*) and protein crude extract of rat gastric mucosa (*rS*). The additional band observed at ca 10 kDa in hG possibly corresponds to ghrelin dimers, while the band below the 15-kDa size marker (between 10 and 14 kDa standards in other experiments) in rS (*first two lanes from left*) corresponds to the preproghrelin estimated size (ca 13 kDa, *arrowhead*). *hG* Four lanes loaded *left to right* with 5, 0.1, 0.05 and 0.01 µg synthetic peptide; *rS* 50 µg extracted total proteins

Altogether the above findings demonstrate that, at both immunochemical and immunohistological levels, the serum generated is specific for ghrelin, does not crossreact either with human GHRH 1–29 or with the unrelated protein VMAT2 and colocalises ghrelin-expressing cells as detected by ISH.

Electron microscopy studies

Ultrastructurally, human ghrelin cells (Fig. 3A–D) were characterised by round, compact to thin-haloed secretory granules, small elongated mitochondria and fairly abundant microfilaments and lysosomal dense (lipofuscin) bodies (Fig. 3B). Dense particles resembling secretory granules and related coalescence or fragmentation products were frequently found inside such bodies, to which cytoplasmic granules were also seen to adhere, thus suggesting active crynophagia and granulolysis. Accurate size analysis of 815 granules from 19 human ghrelin immunoreactive cells in aldehyde-fixed/LWR-embedded tissue (Fig. 3A, C, D) showed a mean diameter of 130±32 nm (mean ± SD). In aldehyde-osmium-fixed/Epon-Araldite-embedded preparations with better preserved and contrasted perigranular membranes (Fig. 3B), ghrelin immunoreactive granules (n=921 from 14 cells) showed a mean diameter of 147±30 nm, ranging in individual cells from 137 to 168 nm. The ghrelin-reactive cells displayed ultrastructural features consistent with P/D_1 cells of previous studies (Capella et al. 1978; D'Adda et al. 1990; Solcia et al. 1979).

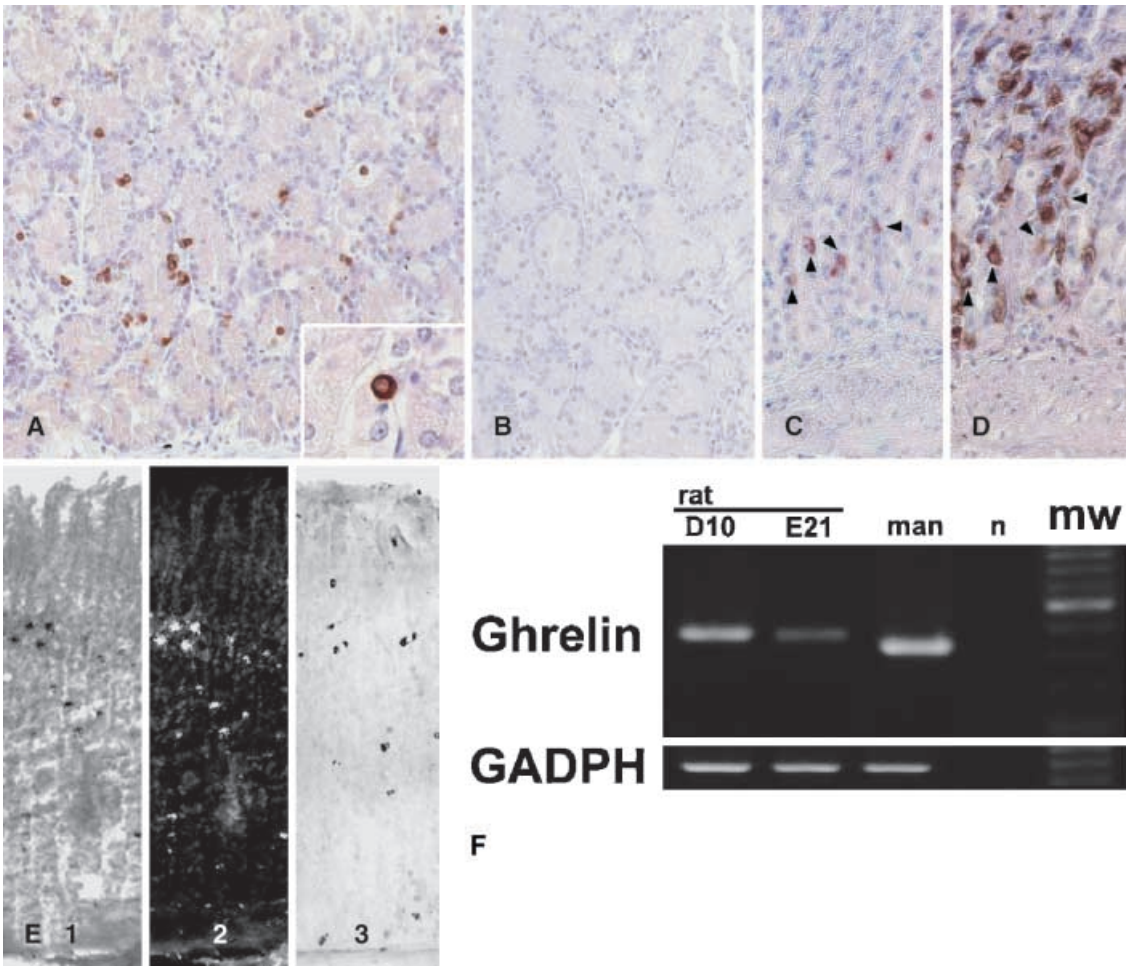
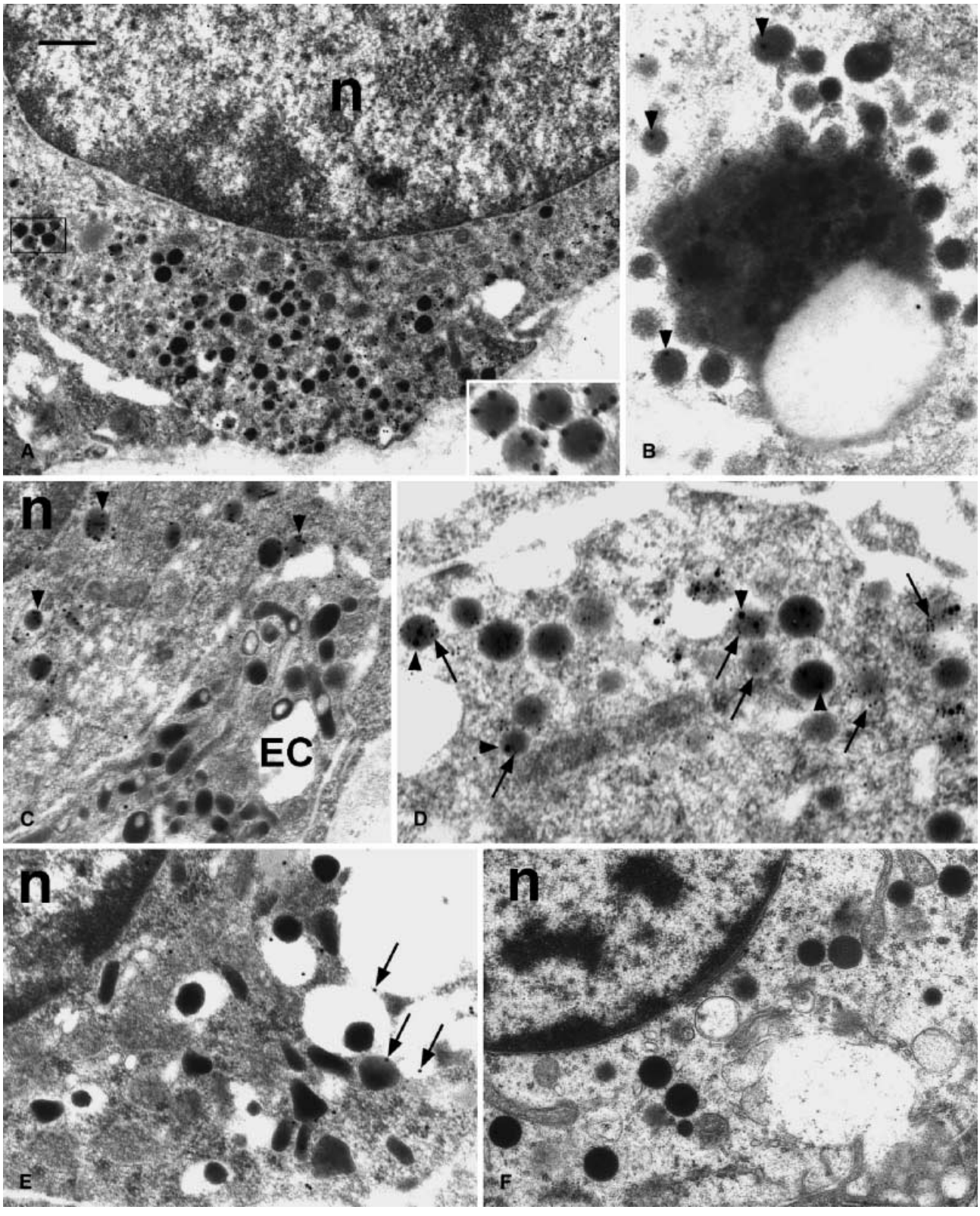


Fig. 2A–F Ghrelin expression in acidopeptic mucosa. **A**, **B** Human oxyntic mucosa; ×150. Ghrelin-immunoreactive cells dispersed in the oxyntic glands frequently displaying a round to ovoid shape (**A**, *inset*; ×470), without any contact with the gland lumen (closed type endocrine cell); the immunoreactivity is abolished after absorption with ghrelin 13–28 (**B**). **C**, **D** Rat oxyntic mucosa; ×150. Ghrelin immunoreactive cells (*arrowheads*) display vesicular monoamine transporter 2 (VMAT2) immunoreactivity in a serial section; note that ghrelin cells are a minor fraction of the VMAT2-positive cells. **E** Consecutive, 20-µm cryostat sections of rat oxyntic mucosa labelled with an antisense oligonucleotide for ghrelin mRNA (bright-field *1* and dark-field *2* of the same emulsion-coated ISH preparation; oligo ghrel1) and anti-ghrelin antiserum (*3*); for both methods the obtained signal is partly overlapping on distinct mucosal cells; ×50. **F** RT-PCR analysis of normal gastric mucosa of adult man and rat at day 10 after birth (*D10*) and at embryonic day 21 (*E21*). A band of 348 bp for rat and of 292 bp for human ghrelin mRNA is observed; the corresponding GADPH amplicons of 598 bp are shown below. *n* Negative control, *mw* molecular weight marker. **A–D** and **E***3* immunoperoxidase, ABC method, haematoxylin counterstain

No ghrelin immunogold labelling was detected in ECL, D, or EC cells (Fig. 3C). Double immunogold labelling with ghrelin and VMAT2 sera demonstrated that ghrelin cells co-express VMAT2 in their granules (Fig. 3D), while ECL cells showed immunogold labelling for VMAT2 only (Fig. 3E) and the remaining cell types were unreactive for both markers (not shown). Only two "X cells" with round, compact secretory granules of rather large mean diameter (ca 300 nm) were observed in the present preparations and they too proved unreactive for our ghrelin antiserum (Fig. 3F). Consistent with previous investigations (Capella et al. 1978; Solcia et al. 1979) no A-like cells similar to glucagon-producing A cells of the human pancreas were observed.

Fig. 3A–F Electron microscopy of endocrine cells in the human oxyntic mucosa. **A** A ghrelin cell reactive for ghrelin antibodies over small electron-dense granules of P/D_1 type; the intense gold labelling is detailed in the *inset* (2.8 times magnification of the *square at the left of the micrograph*). Note that the granules are frequently located near the basal cytoplasm membrane, suggesting exocytosis of their content in the extracellular space. Aldehyde/London White Resin (LWR), immunogold (20 nm) preparation, uranyl acetate and lead citrate counterstain; ×11,300. *n* Nucleus. **B** Ghrelin cells frequently show lysosomal dense bodies with coalescing secretory granules. Note that in this osmicated section the immunogold labelling for ghrelin is less intense than in aldehyde-LWR preparations. Aldehyde-osmium/Epon-Araldite, immunogold (20 nm) preparation, uranyl-lead counterstain; ×45,000. **C** Ghrelin immunogold labelling (*arrowheads*) concentrated on granules of P/D_1 type. Note that the variably shaped, electron-dense granules of a nearby serotonin EC cell (*right, lower* ▶



part of the micrograph) are unlabeled. Aldehyde/LWR, immunogold (20 nm), uranyl-lead; ×22,500. **D** The granules of P/D_1 type display both ghrelin (*arrowheads*) and VMAT2 (*arrows*) immunogold labelling. Aldehyde/LWR, double immunogold (20 nm for ghrelin, 10 nm for VMAT2), uranyl-lead; ×45,000. **E** VMAT2 (*arrows*) immunoreactivity in the large, vacuolar granules of an ECL cell. Aldehyde/LWR, immunogold (20 nm), uranyl-lead; ×36,000. **F** Endocrine cell showing large (compare with **A**), electron-dense granules of "X" type unreactive for ghrelin antibodies. Aldehyde-osmium/Epon-Araldite, immunogold (20 nm), uranyl-lead; ×11,900. *Scale bar* 880 nm in **A**; 220 nm in **B**, **D**; 440 nm in **C**; 270 nm in **E**; 840 nm in **F**

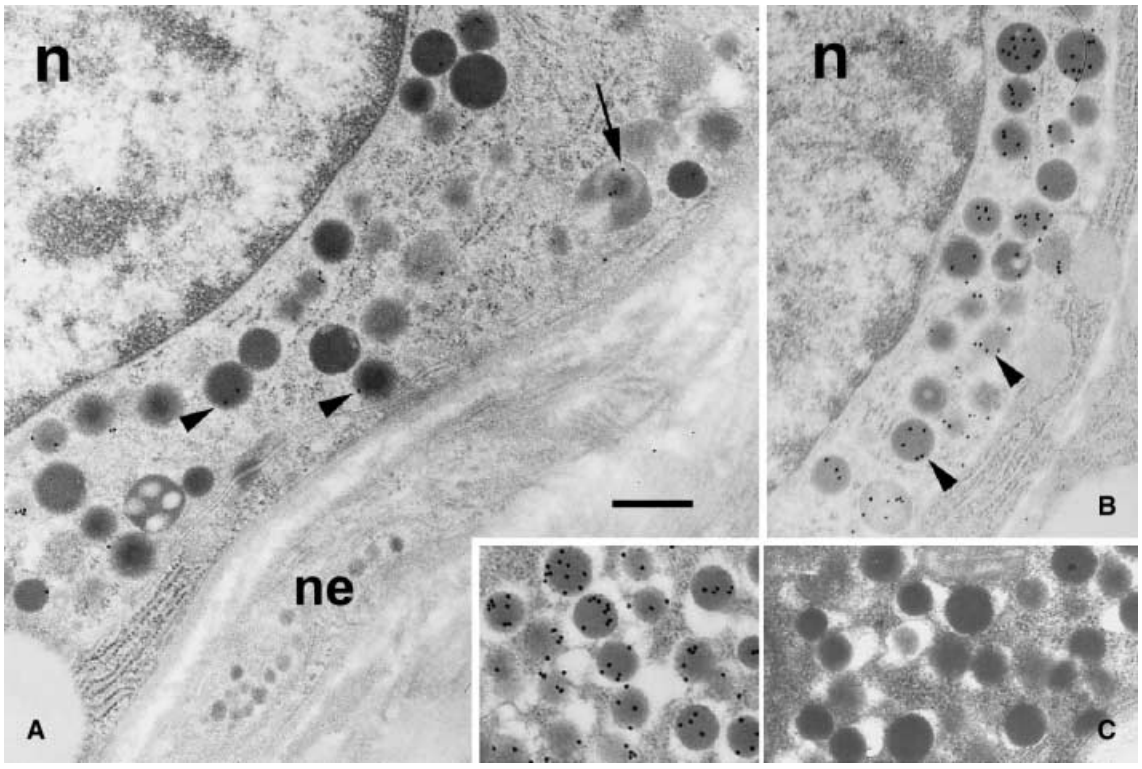


Fig. 4A–C Electron microscopy of ghrelin and glucagon cells in the same preparation of dog oxyntic mucosa; aldehyde-osmium/Epon-Araldite, immunogold (20 nm), uranyl-lead; ×24,000. **A** Ghrelin cell displaying moderate labelling for ghrelin antibodies (*arrowheads*) on large, round, solid granules of X type; gold particles in a lysosomal dense body (*arrow*) suggest active granulolysis. Note a nerve ending (*ne*) close to the ghrelin cell. *n* Nucleus. **B** Intense ghrelin immunogold labelling (*arrowheads*) over large round, compact granules of X type. **C** Detail of secretory granules in a gastric glucagon A cell. Large, haloed granules void of ghrelin immunogold particles (*right*), with glucagon immunoreactive core (*left*) in an adjacent section. *Scale bar* 416 nm in **A–C**

Murine and canine ghrelin-immunoreactive cells closely resembled those of the human stomach in their general ultrastructure, including structural patterns of their compact granules. In aldehyde-osmium-fixed/Epon-Araldite-embedded gastric mucosa, dog ghrelin cells showed obviously larger granules (mean diameter 273±49 nm, 774 granules of 17 cells, fitting with those previously reported for dog X cells; Solcia et al. 1975; Fig. 4A, B) than rat (mean diameter 183±37 nm, 709 granules from 11 cells) and human ghrelin cell granules (147±30 nm, see above). No ghrelin immunogold staining was detected in dog glucagon A (Fig. 4C), somatostatin D, histamine ECL and serotonin EC cells identified by the distinctive ultrastructure and hormone immunoreactivity of their secretory granules or by HDC immunoreactivity.

Human fetal tissues

Like P/D_1 cells of previous ultrastructural investigations (Capella et al. 1978), ghrelin immunoreactive cells were fairly represented in human fetal stomach, duodenum, pancreas and lung from week 10 of gestation (Fig. 5A–D). In keeping with ultrastructural studies of P/D_1 cells, ghrelin cells were found to be among the earliest cells to differentiate in fetal gastric epithelium, earlier than ECL, parietal and chief cells of the oxyntic gland. Parallel investigations of adult human tissues showed in addition to gastrin cells, rare ghrelin immunoreactive cells in the upper small intestine and lung and no reactive cells in the pancreas.

Discussion

This study provides direct characterisation of the human ghrelin cell as a distinct endocrine type, ultrastructurally and cytochemically different from other, known endocrine cells of the oxyntic mucosa, such as the histamine-producing ECL cell, the somatostatin D cell or the serotonin EC cell (D'Adda et al. 1989; Date et al. 2000; Håkanson et al. 1984; Solcia et al. 1975, 1979, 1987). Human ghrelin cells are mainly characterised by round, solid or thin-haloed secretory granules with a mean diameter of 147±30 nm in standard aldehyde-osmium preparations, abundant cytoplasm microfilaments and frequent lysosomal dense (lipofuscin) bodies. All of these features have been described in early ultrastructural studies of the human gastric mucosa as typical of D_1 cells (Solcia et al. 1975), later tentatively separated on the basis of size and inner structure of their granules into P and D_1 cells (Capella et al. 1978; D'Adda et al. 1989). Since in the present study both P and D_1 cells proved to react with ghrelin antibodies, it seems reasonable to con-

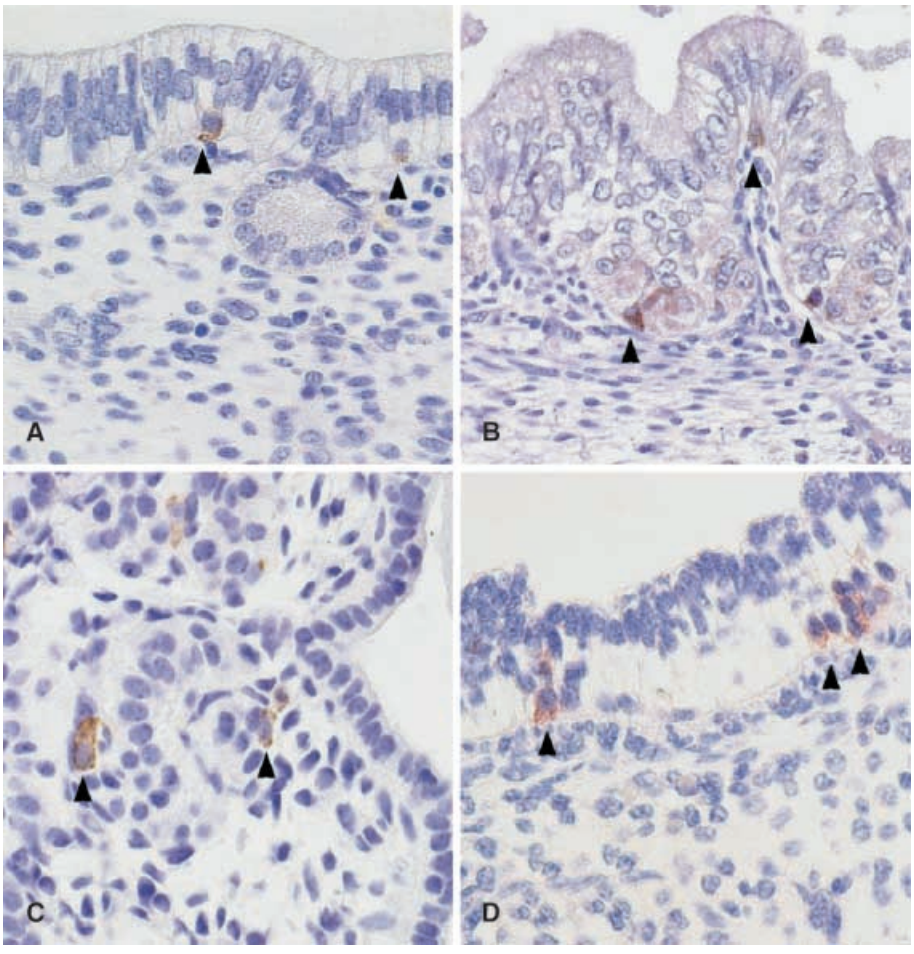


Fig. 5A–D Ghrelin expression in man during development. **A**, **B** Scattered, closed-type cells expressing ghrelin (*arrowheads*) in the gastric mucosa at 12 (**A**) and 16 (**B**) weeks of gestation; ×470. **C** Ghrelin immunoreactive cells (*arrowheads*) in a developing pancreatic islet (10 weeks of gestation); ×470. **D** Ghrelin immunoreactive cells either isolated (*arrowhead*) or in small clusters (*arrowheads at right, upper part of the micrograph*) in a developing lung (10 weeks of gestation); ×470. **A–D** immunoperoxidase, ABC method, haematoxylin counterstain

sider them as two ultrastructural variants of a single functional type, the ghrelin cell.

In the human oxyntic mucosa, both ghrelin cells and ultrastructurally characterised P/D_1 cells account for ca 20–30% of endocrine cells (D'Adda et al. 1989; Date et al. 2000), thus representing a substantial proportion of the endocrine cell population. On the contrary, the cells with large, round, compact granules so far interpreted as the human counterpart of mammalian X cells (Capella et al. 1978; Solcia et al. 1979), account for less than 1% of total volume fraction of endocrine cells in quantitative ultrastructural studies (D'Adda et al. 1989), consistent with the rare "X cells" negative for ghrelin antibodies found in the present study. On the other hand here we demonstrate that dog X cells are true ghrelin cells and differ from human or rat ghrelin cells only by the larger mean size of their secretory granules.

It is concluded that ghrelin cells correspond to those cells which, in earlier ultrastructural classifications, had been labelled as "X cells" in the dog (Solcia et al. 1975), "A-like or X cells" in the rat (Capella et al. 1971; Forssmann et al. 1969) and "P/D_1" cells in man (Capella et al. 1978; D'Adda et al. 1989; Solcia et al. 1979). In addition, our findings indicate that ghrelin cells and authentic glucagon-producing A cells are separate cell types in spite of their ultrastructural similarity in murine tissues (Forssmann et al. 1969).

The functional significance of the abundant microfilaments here reported in ghrelin cells remains uncertain. A possible role in sensing mucosal distension, a likely factor in modulation of ghrelin secretion, may be suggested by analogy with filament-rich tuft cells (Luciano and Reale 1990). It seems pertinent to recall that P/D_1 cells form the main component of the neuroepithelial (sensory?) bodies in the human gastric mucosa (D'Adda and Bordi 1988) and that ghrelin stimulates gastric emptying and food intake (Asakawa et al. 2001; Masuda et al. 2000). Our ultrastructural findings suggest a relationship of dense bodies with lysosomal granulolysis (crynophagia), a process known to regulate hormonal storage in several endocrine cells including gastrin-stimulated ECL cells (Orci et al. 1968; Smith and Farquarh 1966; Zhao et al. 1998).

We confirm and extend to the dog the distribution of ghrelin cells reported in the gastric mucosa of man and rodents (Date et al. 2000; Dornonville de la Cour et al. 2001; Tomasetto et al. 2000), with high concentration in the oxyntic glands and few cells in the pyloric and intestinal mucosa. In addition, we detected ghrelin cells in human fetal stomach, intestine, pancreas and lung, where P/D_1 cells had been described by electron microscopy in-

vestigation (Capella et al. 1978). Ghrelin mRNA was shown by RT-PCR in rat stomach at day 21 of gestation and at day 10 after birth (Fig. 2F) and also confirmed by immunohistochemical tests (G. Rindi, data not presented here). The early and widespread development of ghrelin cells in embryonic tissues may indicate some important role for fetal ghrelin either in promoting body growth through stimulation of GH release, or by fulfilling some local trophic and morphogenetic role, as already suggested for placental ghrelin (Gualillo et al. 2001). The development of gastric ghrelin cells long before ECL and chief cells is of interest also because the latter cells are a major source of leptin (Bado et al. 1998), the anorexigenic peptide known to negatively regulate ghrelin secretion (Asakawa et al. 2001).

We have shown here that ghrelin cells display substantial VMAT2 immunoreactivity, a functional marker of histamine-producing ECL cells which has been reported to be specific for this cell type in man (Kölby et al. 1997; Rindi et al. 2000). Our observation is consistent with data reported in the rat mucosa where VMAT2 immunoreactivity was also observed in unidentified endocrine elements other than ECL cells (Zhao et al. 1997). Separate analysis of the two cell types requires differential staining by ghrelin and histamine (Håkanson et al. 1986) or by ghrelin and HDC (Kubota et al. 1984) antibodies, respectively.

In conclusion, in the human, canine and rodent stomach, ghrelin is produced by a well-defined cell type, distinct from other functionally characterised endocrine cells of the oxyntic mucosa, as histamine ECL, somatostatin D, serotonin EC or glucagon A cells. The identification of human ghrelin cells with previously reportedP/D_1 cells, of dog ghrelin cells with X cells and of rat ghrelin cells with A-like/X cells, allows to reclassify all these ultrastructurally heterogeneous cells under a single functional type. As a result, all main gastric endocrine cell types identified in mammalian stomach have now obtained hormonal characterisation. The early fetal development of ghrelin cells suggests the opportunity to investigate ghrelin secretion during pregnancy to assess its physiological role.

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References

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

Bado A, Levasseur S, Attoub S, Kermorgant S, Laigneau JP, Bortoluzzi MN, Moizo L, Lehy T, Guerre-Millo M, Le Marchand-Brustel Y, Lewin MJ (1998) The stomach is a source of leptin. Nature 394:790–793

Capella C, Vassallo G, Solcia E (1971) Light and electron microscopic identification of the histamine-storing argyrophil (ECL) cell in murine stomach and of its equivalent in other mammals. Z Zellforsch Mikrosk Anat 118:68–84

Capella C, Hage E, Solcia E, Usellini L (1978) Ultrastructural similarity of endocrine-like cells of the human lung and some related cells of the gut. Cell Tissue Res 186:25–37

Cattoretti G, Pileri S, Parravicini C, Becker MH, Poggi S, Bifulco C, Key G, D'Amato L, Sabattini E, Feudale E, et al (1993) Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. J Pathol 171:83–98

Chomczynski P, Sacchi NS (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem:156–159

D'Adda T, Bordi C (1988) Ultrastructure of a neuroendocrine complex in oxyntic mucosa of normal human stomach. Cell Tissue Res 254:465–467

D'Adda T, Bertele A, Pilato FP, Bordi C (1989) Quantitative electron microscopy of endocrine cells in oxyntic mucosa of normal human stomach. Cell Tissue Res 255:41–48

D'Adda T, Corleto V, Pilato FP, Baggi MT, Robutti F, Delle Fave G, Bordi C (1990) Quantitative ultrastructure of endocrine cells of oxyntic mucosa in Zollinger-Ellison syndrome. Correspondence with light microscopic findings. Gastroenterology 99:17–26

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

Dornonville de la Cour C, Björkvist M, Sandvik AK, Bakke I, Zhao C-M, Chen D, Håkanson R (2001) A-like cells in the rat stomach contain ghrelin and do not operate under gastrin control. Regul Pept 99:141–150

Forssmann WG, Orci L, Pictet R, Renold AE, Rouiller C (1969) The endocrine cells in the epithelium of the gastrointestinal mucosa of the rat. An electron microscope study. J Cell Biol 40:692–715

Gualillo O, Caminos JE, Blanco M, Garcia-Caballero T, Kojima M, Kangawa K, Dieguez C, Casanueva FF (2001) Ghrelin, a novel placental-derived hormone. Endocrinology 142:788–794

Håkanson R, Ekelund M, Sundler F (1984) Activation and proliferation of gastric endocrine cells. In: Håkanson R, Falkmer S, Sundler F (eds) Evolution and tumour pathology of the neuroendocrine system. Elsevier, Amsterdam, pp 371–398

Håkanson R, Bottcher G, Ekblad E, Panula P, Simonsson M, Dohlsten M, Hallberg T, Sundler F (1986) Histamine in endocrine cells in the stomach. A survey of several species using a panel of histamine antibodies. Histochemistry 86:5–17

Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase technique. J Histochem Cytochem 29:577–580

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

Kölby L, Wängberg B, Ahlman H, Jansson S, Forsell-Aronsson E, Erickson JD, Nilsson O (1997) Gastric carcinoid with histamine production, histamine transporter and expression of somatostatin receptors. Digestion 59:160–166

Kubota H, Taguchi Y, Tohyama M, Matsuura N, Shiosaka S, Ishihara T, Watanabe T, Shiotani Y, Wada H (1984) Electron microscopic identification of histidine decarboxylase-containing endocrine cells of the rat gastric mucosa. An immunohistochemical analysis. Gastroenterology 87:496–502

Lattuada D, Casnici C, Gregori S, Berrini A, Secchi C, Franco P, Marelli O (1996) Monoclonal antibodies against recombinant human growth hormone as probes to study immune function. Hybridoma 15:211–217

Luciano L, Reale E (1990) Brush cells of the mouse gallbladder/a correlative light- and electron-microscopical study. Cell Tissue Res 262:339–349

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

Orci L, Junod A, Pictet R, Renold AE, Rouiller C (1968) Granulolysis in a cells of endocrine pancreas in spontaneous and experimental diabetes in animals. J Cell Biol 38:62–66

Rindi G, Luinetti O, Cornaggia M, Capella C, Solcia E (1993) Three subtypes of gastric argyrophil carcinoid and the gastric neuroendocrine carcinoma: a clinicopathologic study. Gastroenterology 104:994–1006

Rindi G, Paolotti D, Fiocca R, Wiedenmann B, Henry J-P, Solcia E (2000) Vesicular monoamine transporter 2 as a marker of gastric ECL cell tumors. Virchows Arch 436:217–223

Rubin W, Schwartz B (1979) Gastric ECL, APUD, and enterochromaffin-like cells. Gastroenterology 77:1166

Smith RE, Farquarh MG (1966) Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland. J Cell Biol 31:319–347

Solcia E, Capella C, Vassallo G, Buffa R (1975) Endocrine cells of the gastric mucosa. Int Rev Cytol 42:223–286

Solcia E, Capella C, Buffa R, Usellini L, Frigerio B, Fontana P (1979) Endocrine cells of the gastrointestinal tract and related tumors. Pathobiol Annu 9:163–204

Solcia E, Fiocca R, Capella C, Usellini L, Rindi G, Schwartz TW, Yanaihara N (1985) Glucagon and related peptides of intestinal L cells and pancreatic A or PP cells. Possible interrelationships of peptides and cells during evolution, fetal development and tumour growth. Peptides 6:223–229

Solcia E, Capella C, Buffa R, Usellini L, Fiocca R, Sessa F (1987) Endocrine cells of the digestive system. In: Johnson LR (ed) Physiology of the intestinal tract. Raven Press, New York, pp 111–130

Tapia FJ, Varndell IM, Probert L, De Mey J, Polak JM (1982) Double immunogold staining method for the simultaneous localization of regulatory peptides. J Histochem Cytochem 30:195–208

Tomasetto C, Karam SM, Ribieras S, Masson R, Lefebvre O, Staub A, Alexander G, Chenard M-P, Rio M-C (2000) Identification and characterization of a novel gastric peptide hormone: the motilin-related peptide. Gastroenterology 119:395–405

Zhao C-M, Jacobsson G, Chen D, Håkanson R, Meister B (1997) Exocytotic proteins in enterochromaffin-like (ECL) cells of the rat stomach. Cell Tissue Res 290:539–551

Zhao C-M, Chen D, Andersson K, Håkanson R (1998) ECL cells of the rat stomach: development of lipofuscin in response to sustained gastrin stimulation. Cell Tissue Res 291:315–323

Zoli M, Le Novère N, Hill JAJ, Changeux J-P (1995) Developmental regulation of nicotinic receptor subunit mRNAs in the rat central and peripheral nervous systems. J. Neurosci 15: 1912–1939