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Expression of Ghrelin and of the GH Secretagogue Receptor by Pancreatic Islet Cells and Related Endocrine Tumors

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Ghrelin is a novel gastrointestinal hormone produced by rat and human gastric X-like neuroendocrine cells, which strongly stimulates GH secretion and influences energy balance, gastric motility, and acid secretion. Ghrelin is expressed in pituitary and gastrointestinal endocrine tumors. It binds to the GH secretagogue receptor (GHS-R), which is present in a wide variety of central and peripheral human tissues. The aim of the present study was 2-fold: 1) to determine, by immunohistochemistry and mRNA analysis, whether pancreatic islet cells produce ghrelin and express GHS-R; and 2) to investigate ghrelin and GHS-R expression in pancreatic endocrine tumors. Seven cases of nonneoplastic pancreatic tissue and 28 endocrine tumors were studied. In pancreatic islets, ghrelin immunoreactivity was present in all cases and confined to β-cells. Eleven of the 28 (39%) endocrine tumors were immunoreactive for ghrelin. *In situ* hybridization and RT-PCR confirmed the immunohistochemical data for both tumors and islets but also revealed ghrelin mRNA in 8 and 11 additional tumors, respectively. GHS-R 1a and 1b mRNAs were present in 7 of 28 and 14 of 28 tumors, respectively, studied by RT-PCR. These findings demonstrate that ghrelin production is not restricted to the stomach but is also present in pancreatic β-cells and endocrine tumors (regardless of the type of pancreatic hormone produced, if any). Expression of GHS-R in some of the endocrine tumors studied indicates that autocrine/paracrine circuits may be active in neoplastic conditions. (*J Clin Endocrinol Metab* 87: 1300–1308, 2002)

GHRELIN IS A 28-amino acid acylated peptide that has recently been discovered in human and rat stomach (1–3). It is produced by approximately 20% of the neuroendocrine cell population of rat and human oxyntic glands, by cells other than ECL, EC, or D cells. Although partially contradictory data have been published, the candidate ghrelin producing cell type is the X/A-like cell, also referred to as the X-like cell (2, 4). Ghrelin has no relevant homology with the majority of biologically active peptides (1), with the notable exceptions of motilin and another novel gastric peptide known as the motilin-related peptide (5–7). Ghrelin strongly stimulates the release of GH (8–11) and is a natural ligand of the GH secretagogue receptor (GHS-R), which is specific for a family of synthetic, peptidyl, and nonpeptidyl GH secretagogues (1, 9, 12–16). Tissue distribution analysis reveals GHS-R expression in rat anterior pituitary and brain in both hypothalamic and nonhypothalamic regions, such as the dentate gyrus of hippocampal formation, the pars compacta of the substantia nigra, the ventral tegmental area, the dorsal and medial raphe nuclei, and the Edinger-Westphal nucleus (13). In parallel, ghrelin and synthetic GHS also recognize binding sites in human pituitary, hypothalamus, hippocampus, cerebral cortex, pons-medulla, and choroid plexuses, as well as in various peripheral, endocrine, and nonendocrine tissues, both in normal and neoplastic conditions (17–21).

Actually, the functions of this new hormone are not specifically related to GH secretion. Besides potent GH-releasing activity (1, 10, 11, 22), ghrelin also has stimulatory effects on lactotroph and corticotroph secretion, stimulates food intake and modulates energy expenditure, stimulates gastric motility and acid secretion, exerts cardiovascular activity, and even displays antiproliferative effects in neoplastic cells (8, 11, 21, 23–36). In particular, in addition to its cholinergic-mediated stimulatory effect on gastric contractility and acid secretion in rats (37, 38), ghrelin has a role in managing not only the GH but also the metabolic response to starvation by modulating insulin secretion and glucose metabolism (26, 27, 39, 40). This evidence supports the hypothesis that ghrelin is a newly discovered gastric hormone with a major role in endocrine regulation.

Although ghrelin has been isolated and purified from the rat and human oxyntic mucosa of the stomach, it is also produced in other tissues, including the small intestine (but not the colon or rectum) (2), the arcuate nucleus of the hypothalamus (1), the kidney (41), and the placenta (42). In addition, ghrelin expression has recently been described in pituitary adenomas and other neuroendocrine tumors, namely one thymic and five pancreatic endocrine tumors (43, 44). Gastric and, to a lesser extent, intestinal carcinoids also express ghrelin (45). While studying the ghrelin expression in these latter tumors, we observed a strong ghrelin immunoreactivity in pancreatic islets adjacent to samples of duodenal carcinoids. This prompted extensive investigation of normal pancreatic islets for the expression of ghrelin and of

Abbreviations: GHS-R, GH secretagogue receptor; IHC, immunohistochemistry; ISH, *in situ* hybridization; PP, pancreatic polypeptide; RNase, ribonuclease; rt, room temperature; SBA, Southern blot analysis; VIP, vasoactive intestinal peptide.

its receptor, the GHS-R, using immunohistochemistry (IHC), RT-PCR, and *in situ* hybridization (ISH). In addition, the second part of our study included the analysis of ghrelin and GHS-R expression in pancreatic endocrine tumors. Herein, we show that ghrelin production in the pancreatic islets is apparently restricted to insulin-producing β-cells. Conversely, some endocrine tumors express ghrelin and its receptor, the GHS-R, regardless of the type of pancreatic hormone produced.

Materials and Methods

Normal pancreas and tumors

Nontumoral pancreatic tissue was collected from the surgical pathology file of the University of Turin. Three samples had been diagnosed as chronic pancreatitis, and 4 other samples were from unaffected pancreatic parenchyma resected in the course of abdominal surgery for biliary, duodenal, or splenic diseases. Twenty-eight pancreatic endocrine tumors, for which both fresh frozen tissue and paraffin blocks were available, were collected from the University of Turin pathology files from 1990 to 2000. Storage time affected neither RNA preservation nor immunoreactivity. All cases were reviewed, histologically confirmed by positive chromogranin A and pancreatic hormone(s) immunostaining, and classified according to the World Health Organization classification of endocrine tumors (46). According to the hormonal profile, 19 functioning tumors (15 insulinomas, and 1 case each of glucagonoma, gastrinoma, vasoactive intestinal peptide (VIP)oma, and pancreatic polypeptide (PP)-producing tumor) and 9 nonfunctioning endocrine tumors were included. In addition, 4 cases of pancreatic adenocarcinoma served as controls. The study was approved by the Ethical Committee of the University of Turin.

IHC

Ghrelin immunostaining was performed on formalin-fixed and paraffin-embedded tissues, using poly-L-lysin-coated slides. The primary antibody was a polyclonal serum antihuman ghrelin (amino acids 13–28) (Phoenix Pharmaceuticals, Inc., Belmont, CA), diluted 1:15,000, and incubated for 1 h at room temperature (rt). A standard manual immunoperoxidase procedure was used, with no antigen retrieval procedure. The reaction product was revealed using a tyramide-based amplification system. Briefly, the primary antibody and the secondary biotinylated antibody steps, and incubation with the streptavidin-peroxidase kit (DAKO Corp., Glostrup, Denmark), were followed by further amplification with biotinylated-tyramide (GenPoint, DAKO Corp., diluted 1:5). The subsequent washing step was performed in hot PBS (at 95 C) to reduce the background and increase sensitivity, as described by Volante *et al.* (47). Diaminobenzidine was used as chromogen, followed by a weak nuclear counterstain with hematoxylin. Control experiments included the immunoperoxidase reaction in serial sections by either omitting the primary antibody or using the primary antibody preabsorbed with 100-fold excess of full-length (1–28) ghrelin (Europeptides). Nontumoral oxyntic mucosa of the stomach served as the positive control. Only scattered endocrine cells showed strong cytoplasmic staining using either the overdiluted antibody (1:15,000) followed by tyramide amplification or using more concentrated antibody (1:200) followed by standard streptavidin incubation. In both experimental conditions, no cross-reactivities were observed in the gastric mucosa control tissues. The antibody was raised using a C-terminal sequence of human ghrelin (amino acids 13–28), which does not encompass the Serine residue in position 3. It thus recognizes both the acylated and the desacylated form of ghrelin.

All cases were stained in parallel sections (and, in some cases, in the same section) with the following pancreatic hormones: glucagon (BioGenex Laboratories, Inc. San Ramon, CA, diluted 1:5), insulin (DAKO Corp., diluted 1:400), gastrin (BioGenex Laboratories, Inc., diluted 1:50), and somatostatin (Serotec, Oxford, UK, diluted 1:3000). Double stains were performed in sequence, first revealing ghrelin with biotinylated secondary antibody and peroxidase-conjugated streptavidin. Diaminobenzidine was used as the final reaction product, and the latter was further stained black with nickel salt precipitation. Subsequently, single pancreatic hormones were detected by the immunoalkaline phosphatase method and revealed in red. Selected cases were also stained with a double immunofluorescence procedure using the same polyclonal antibody to ghrelin and a monoclonal antibody to insulin (BioGenex Laboratories, Inc. clone HB125, diluted 1:50). This procedure relied on rhodamine-labeled secondary antirabbit antibody to reveal ghrelin immune complexes and fluorescein-labeled antimouse secondary antibody to reveal insulin-producing cells.

ISH

All cases (pancreatic islets and endocrine tumors) were studied in parallel sections using a nonradioactive ISH procedure. Two 45-nucleotide antisense probes (1), corresponding to positions 90–134 and 421–465 of pre-pro-ghrelin sequence, were synthesized and digoxigenin-labeled with the Boehringer Ingelheim GmbH (Mannheim, Germany) labeling kit, following the manufacturer's instructions. The equimolar mixture of the two probes was applied overnight at a working dilution of 33 nM for each probe per slide. Prehybridization treatments included a microwave passage (5 min at 800 W in citrate buffer, pH 6.0) and proteinase K digestion (1 μg/ml) for 4 min. The hybrids were revealed with the GenPoint kit (DAKO Corp.), as described elsewhere (48), with minor modifications, including 1:5 dilution of the tyramide kit solution and washings at a high temperature after the tyramide incubation step (47). Nontumoral oxyntic mucosa of the stomach served as the positive control. Negative controls included ISH reactions of serial sections with an unrelated probe or without any probe, as well as ribonuclease (RNase) digestion before hybridization.

RT-PCR for ghrelin and GHS-receptor

In five nontumoral pancreases and in all pancreatic endocrine tumors, RT-PCR was performed to reveal mRNA of both ghrelin and its receptor. Total RNA extraction and complementary DNA transcription were performed as described elsewhere (48). PCR for ghrelin and for GHS-R was performed following the procedure described by Gualillo *et al.* (42) for ghrelin mRNA amplification. The primers for ghrelin were synthesized according to the sequence reported by Gualillo *et al.* (42), and the sequences were 5′-TGAGCCCTGAACACCAGAGAG-3′ for the forward and 5′-AAAGCCAGATGAGCGCTTCTA-3′ for the reverse primer, respectively. Those for GHS-receptor Ia and Ib were synthesized according to Korbonits *et al.* (43) and employed for RT-PCR using the same conditions described by these authors. The following sequences were used: 5′-TCGTGGGTGCCTCGCT-3′ as the forward primer for both GHS-R 1a and GHS-R 1b, 5′-CACCACTACAGCCAGCATTTTC-3′ for the GHS-R 1a reverse primer, and 5′-GCTGAGACCCACCCAGCA-3′ for the GHS-R 1b reverse primer. The expected size of the amplicons was 327 bp, 65 bp, and 66 bp for ghrelin, GHS-R 1a, and GHS-R 1b, respectively; β2-microglobulin amplification served as a control of the RNA quality (see details in Ref. 48).

To confirm the data obtained by means of IHC and ISH on the expression of ghrelin in the endocrine and exocrine component of the normal pancreas, laser-based microdissection was employed. Islet cells and acinar cells from two cases of frozen nontumoral pancreatic specimens were isolated by means of a laser microdissection device (Olympus Corp./Cell Robotics, Albuquerque, NM) and then underwent RT-PCR analysis, following the protocols previously described by Kuecker *et al.* (49).

To further test the RT-PCR product specificity, Southern blot analysis (SBA) was performed using the probe sequences previously published by Korbonits *et al.* (43). Membranes were hybridized overnight at 42 C with 25 pmol digoxigenin-labeled ghrelin and GHS-R oligonucleotide probes. The membranes were then washed with 2× SSC-0.1% SDS for 10 min at rt and 0.5× SSC-0.1% SDS at 42 C for 30 min. Digoxigenin-labeled specific hybridization was visualized using an immunological detection system (Roche Molecular Biochemicals, Mannheim, Germany) employing antidigoxigenin antibodies conjugated with alkaline phosphatase. Detection was performed using the chemiluminescent substrate disodium 3-(4-methxyspiro; 1,2-dioxetane-3,2-(5-chloro) tricyclo(3.3.1.^{3,7}) decan;-4-yl) phenylphosphate (Boehringer Mannheim GmbH), according to the manufacturer's instructions. All blots were exposed to x-ray films with intensifying screens at rt for 3 h.

Nonradioactive in situ ghrelin binding assay

To verify whether GHS-R mRNA expression in endocrine tumors was paralleled by active ghrelin binding, a nonradioactive *in situ* binding assay was designed. Full-length ghrelin was labeled with biotin following a standard technique (50), with minor modifications. In particular, to minimize the steric hindrance between avidin (streptavidin) and the biotinylated protein, an aminocaproyl spacer arm (a modified activated biotin, caproylamidobiotin-N-hydroxysuccinimide ester) was introduced between the biotin and the activated carboxyl group of the protein (B002–6, Società Prodotti Antibiotici SpA, Milan, Italy). Ghrelin (0.5 mg/ml) was dialyzed against 0.1 M sodium-bicarbonate buffer (pH 8.5) and subsequently incubated, for 4 h at rt under continuous slow rotation, with B002–6 dissolved in DMSO (1 mg/ml). The solution was dialyzed overnight at 4 C against PBS.

Frozen serial sections of 14 endocrine pancreatic tumors were collected onto poly-L-lysine-coated slides and stored for 1 wk at −80 C. One section was stained with hematoxylin and eosin to ascertain the presence and extent of tumor tissue. Then, the sections were thawed at 37 C for 4 min and quenched in PBS. Endogenous peroxidase activity was blocked in 6% H_2O_2 for 8 min, and then the sections were incubated with biotinylated ghrelin at a concentration of 10^{-9} M for 2 h at rt. Sections were then fixed in −20 C methanol for 5 min and in acetone for 5 sec. Bound biotinylated-ghrelin was visualized by following the streptavidin/tyramide procedure described for ghrelin IHC. Control experiments included preincubation of parallel sections with unlabeled full-length ghrelin at a concentration of 10^{-7} M for 20 min at rt, before incubation with 10^{-9} labeled ghrelin, as described.

Correlations

The data on ghrelin and GHS-R expression were correlated with the clinicopathological parameters of each tumor, including age, sex, tumor size and grade, hormonal production, and presence of metastases. Statistical evaluation was performed by means of ANOVA and chi-square (Yates-corrected) tests.

Results

Ghrelin expression in pancreatic islets

In the pancreatic endocrine islets, numerous cells expressed ghrelin in the cytoplasm, as indicated by a moderate to intense staining (Fig. 1a). The immunostaining was completely abolished by absorbing the antighrelin antibody with a 100-fold excess of full-length ghrelin. The immunohistochemical expression of ghrelin was also confirmed by ghrelin mRNA analysis. A nonradioactive ISH procedure, in fact, revealed the specific ghrelin mRNA in the same pancreatic endocrine cells (Fig. 1b). Control experiments using an unrelated probe (Fig. 1c) or omitting the probe, or using RNase pretreatment, revealed no ISH signal. The presence of the specific ghrelin mRNA was also confirmed by RT-PCR analysis of RNA extracted from a pool of islet cells obtained by a laser-based microdissection procedure, using cryostatic sections of nontumoral pancreas. Not all endocrine cells seemed to contain ghrelin. This prompted further investigation of the cell type expressing ghrelin, by means of double immunohistochemical stains. Ghrelin was found to colocalize with insulin in β-cells (Fig. 1, d–e), but not with other hormones (namely glucagon, gastrin, somatostatin, VIP, and PP). Colocalization with ghrelin was observed in the vast majority of insulin-producing cells, as shown by the virtually complete overlapping of the two fluorochromes revealing either ghrelin (*red*) or insulin (*green*) (Fig. 1f). No apparent colocalization was seen in α-cells, which have a preferential peripheral distribution within the endocrine islet, or in the less represented δ-cells.

Ghrelin expression in pancreatic endocrine tumors

As summarized in Table 1, 11 of 28 (39%) of the pancreatic endocrine tumors were immunoreactive for ghrelin in a variable percentage of tumor cells. Immunostaining never exceeded 30% of the tumor cells (Fig. 2a) and, in some cases, was restricted to isolated cells or small clusters (Fig. 2d). The cellular pattern of ghrelin reactivity was a finely granular cytoplasmic staining, generally weaker than that of the control oxyntic mucosa of the stomach and also than that of the normal islet cells. The latter two normal tissues had a com-

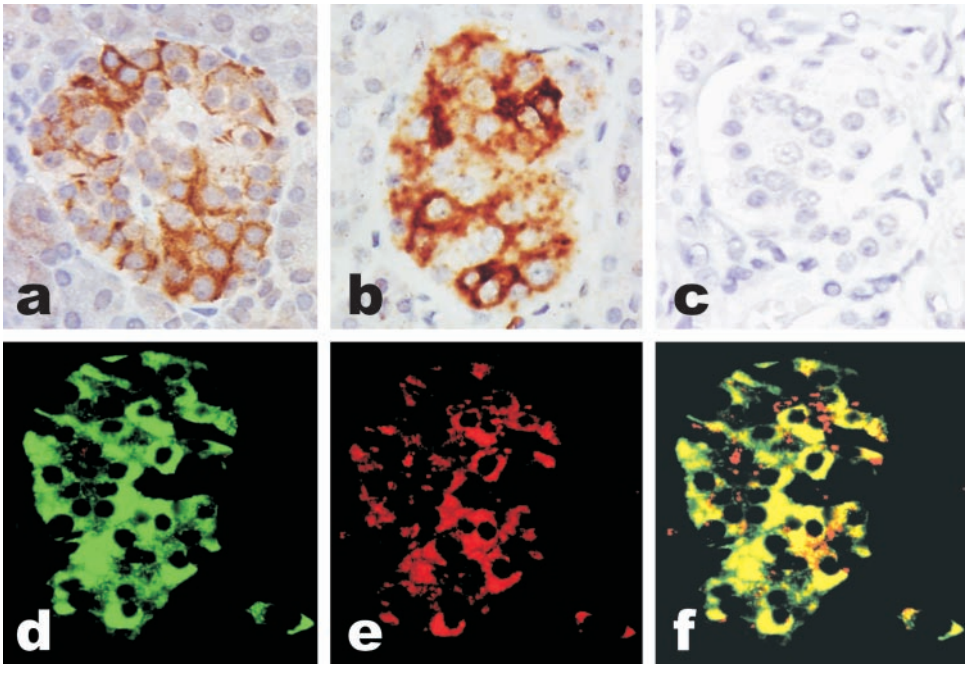

FIG. 1. Normal pancreatic islet showing a strong immunoreactivity for ghrelin in many endocrine cells (a), which is paralleled by a strong signal when ISH is performed with a specific nucleotide probe cocktail (b). A serial section stained omitting the ghrelin oligonucleotides probes is negative (c). A double immunofluorescence for insulin (*green*, d) and ghrelin (*red*, e) shows that insulin and ghrelin are colocalized in the same (β) cells, as also confirmed by the *yellow color* obtained by overlapping the two fluorescence reactions (f) (a–f, ×400).

TABLE 1. Ghrelin and GHS-R 1a and 1b expression in endocrine tumors of the pancreas

No.	Sex/age	Location/size (cm)	Diagnosis	Hormonal status	Mts	Follow-up (years)	Ghrelin			GHS-R1a	GHS-R1b
							IHC	ISH	PCR	PCR	PCR
1	M/44	Body/1	WDET	Insulin		NED 2	−	+	+	−	+
2	F/56	Head/1.4	WDET	Insulin		NED 6.5	+	+	+	−	+
3	M/58	Body/2	WDET	Insulin		NED 3.5	−	+	+	+	+
4	F/72	Tail/4	WDET	Insulin		NED 4.5	−	+	+	+	+
5	F/39	Body/1.8	WDET	Insulin		NED 4	−	+	+	+	+
6	F/55	Head/0.9	WDET	Insulin		NED 4	−	−	+	+	+
7	M/62	Tail/5	WDEC	Insulin	Liver	DOD 1	−	+	+	−	−
8	M/50	Tail/4	WDET	PP		NED 6.5	−	−	+	−	−
9	M/54	Tail/11	WDET	Glucagon		NED 4	−	+	+	−	+
10	F/63	Tail/7	WDET	VIP	LN	DOD 2	+	+	+	−	−
11	F/69	Tail/6	WDEC	Insulin		NED11	+	+	+	−	+
12	M/44	Head/5	WDET	Non-funct		DOD 0.1	+	+	+	−	−
13	F/59	Body-tail/4	WDEC	Non-funct	LN	NED 4	+	+	+	−	−
14	M/49	Head/6	WDET	Non-funct	Liver	DOC 4.5	−	−	−	−	−
15	F/54	Head/3.5	WDET	Non-funct	LN	Lost	+	+	+	−	−
16	F/44	Body/3	WDEC	Non-funct	LN	DOD 2	−	−	+	−	+
17	F/70	Head/3.5	WDET	Non-funct		NED 1	−	+	+	−	+
18	F/64	Head/2.3	WDEC	Non-funct	LN	DOD 1.2	−	−	+	−	−
19	M/55	Head/2	WDEC	Non-funct	Liver	NED	−	+	+	−	−
20	M/56	Body-tail/3	WDET	Insulin		Lost	+	+	−	−	−
21	F/59	Head/1.5	WDET	Insulin		NED 3	+	−	+	+	+
22	M/52	Body/1	WDET	Insulin		NED 11	−	−	−	−	+
23	F/26	Tail/2	WDET	Insulin		NED 5.5	+	+	+	−	−
24	M/23	Head/1	WDET	Insulin		Lost	−	+	+	+	+
25	F/29	Tail/0.5	WDET	Insulin		NED 0.6	−	−	−	−	−
26	F/59	Body/2.5	WDET	Insulin		Lost	+	+	+	+	+
27	M/28	Body-tail/7.5	WDEC	Gastrin	Liver	AWD 0.5	+	+	−	−	−
28	M/76	Head/11	PDEC	Non-funct	LN	DOD 0.5	−	−	−	−	−

Mts, Metastases; M, male; F, female; WDET, well differentiated endocrine tumor; WDEC, well differentiated endocrine carcinoma; PDEC, poorly differentiated endocrine carcinoma; Non-funct, non-functioning; LN, lymph node; NED, no evidence of disease; DOD, died of disease; DOC, died of other causes; AWD, alive with disease; PCR, RT-PCR.

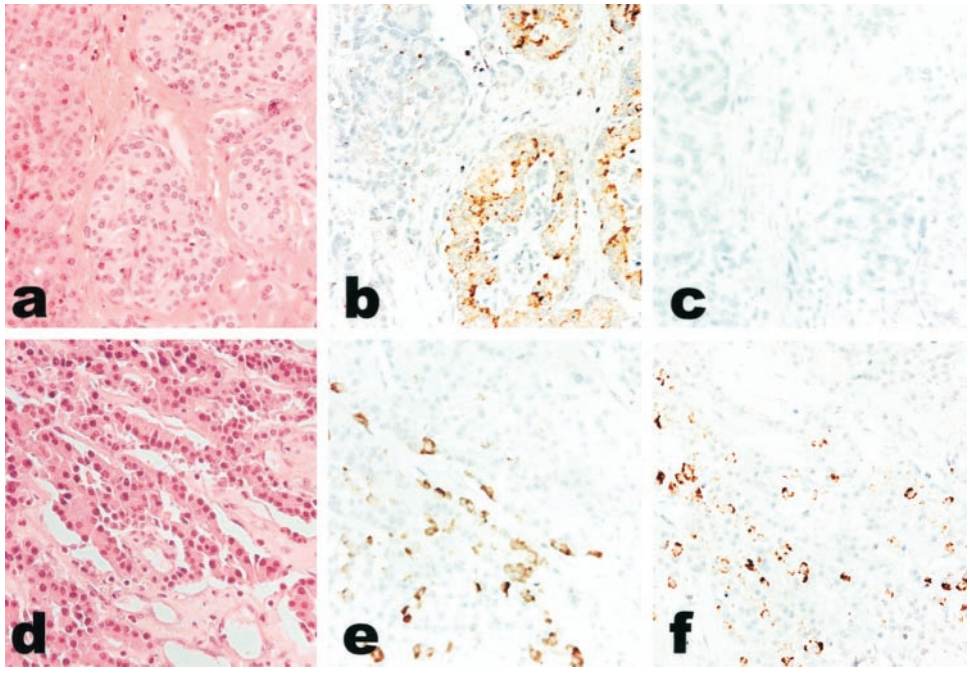

FIG. 2. A well-differentiated insulin-producing endocrine tumor (a, case 26) is strongly reactive for ghrelin immunostaining (b, *right*), as opposed to normal acinar structures (b, *left*). The reactivity is completely abolished by preabsorbing ghrelin antiserum with an excess of full-length ghrelin peptide (c). A well-differentiated nonfunctioning endocrine tumor (d, case 12) shows a focal ghrelin expression at both protein (e) and mRNA (f) levels in the same cell population, as revealed by IHC and ISH, respectively (a–f, ×200).

parable intensity of ghrelin immunostaining. No immunoreactivity was found in parallel sections stained by omitting the primary antibody or by using an antiserum preabsorbed with full-length ghrelin (Fig. 2c).

ISH revealed ghrelin mRNA in 19 of 28 (68%) tumors (Fig. 2f), including all but one case that also expressed ghrelin immunoreactivity and nine additional tumors that proved negative according to immunohistochemical analysis. In some cases, an overlapping percentage of ghrelin-positive tumor cells was observed using IHC and ISH in parallel

sections; whereas, in others, the cells containing ghrelin mRNA outnumbered the ghrelin immunoreactive ones. ISH omitting the specific probe, using an unrelated probe or RNase pretreatment, was constantly negative.

By RT-PCR, ghrelin mRNA was detected in 22 of 28 (79%) tumors. The PCR product had the expected size of 327 bp, and its specificity was also confirmed by SBA (Fig. 3).

GHS-receptor expression in pancreatic islets and in endocrine tumors

RT-PCR, to reveal GHS-receptor type 1a and 1b mRNAs, was performed in the same cases tested for ghrelin and in normal pancreas. No specific mRNA was found in 5 cases of nontumoral pancreas analyzed for either GHS-R 1a or 1b, or in microdissected acinar or islet cells. Half of the endocrine tumors expressed 1 or both types of GHS-R mRNA. The amplification products had the expected length of 65 and 66 bp for GHS-R1a and 1b, respectively. Their specificity was confirmed by SBA. In particular, GHS-R 1b mRNA was found in 14 of 28 cases (50%), 7 of which also contained GHS-R 1a mRNA (25%). All the latter cases were insulinomas, whereas the other 7 receptor-positive tumors included 4 insulinomas, as well as 1 glucagonoma and 2 nonfunctioning endocrine tumors. PCR analysis of the housekeeping gene β2-microglobulin showed a specific band in all cases.

Control tissues

The oxyntic mucosa of human stomach served as the positive control: scattered ghrelin-positive endocrine cells were observed in the mucosal glands, as expected. In the pancreas, ghrelin immunostaining was confined to pancreatic islet cells (see above), with the acinar and ductal cells proving negative overall. Interestingly, a colocalization of ghrelin and insulin was also observed in single insulin-positive cells, scattered throughout the pancreatic duct system (Fig. 4. Nerve fibers in the peripancreatic tissue were also focally immunoreactive, although more weakly than the endocrine cells. All four nonneuroendocrine pancreatic adenocarcinomas completely lacked ghrelin immunoreactivity.

Microdissected acinar cells (carefully obtained using a laser-based device in such a way as to avoid islets, ducts, and peripancreatic tissue) did not yield any ghrelin mRNA signal by RT-PCR.

Binding experiments

Nine of 14 endocrine tumors tested with biotinylated ghrelin revealed ghrelin binding sites. Although the tissue preservation quality of the frozen sections was suboptimal in most cases (in the lack of any fixation of the tissue), the ghrelin binding was specifically identified at the cell membrane or in a peripheral cytoplasmic location. Preincubation with an excess of unlabeled full-length ghrelin abolished the reactivity (Fig. 5). The 5 cases lacking ghrelin binding activity also lacked GHS-R1a mRNA by RT-PCR, and only 2 of them showed GHS-R 1b expression. However, GHS-R 1a and 1b were also absent in a fraction of tumors with ghrelin binding sites, possibly indicating the existence of another receptor subtype or even of a different family of receptors.

Correlation

In endocrine tumors, ghrelin expression was observed in 39% of the cases by IHC and in 68% and 79% of the cases by ISH and RT-PCR, respectively. The number of cases proven positive by RT-PCR was slightly higher than that obtained by ISH, but double that of ghrelin immunoreactive tumors. The two procedures (protein *vs.* mRNA analysis) had concordant results in nearly half of all cases. The lack of complete agreement was probably attributable to the different sensitivity of the various methods and to the fact that the ghrelin gene may be transcribed but the protein not synthesized (or stored) in all cases. In two cases (nos. 20 and 27), ghrelin expression was detected by IHC and ISH but not by RT-PCR. This could be related to tumor heterogeneity, because the fragment of tumor tissue used for RNA extraction was adjacent, but not identical, to that used for immunostaining. As an alternative, the quality or amount of ghrelin mRNA may have been suboptimal, despite a sufficient β2-microglobulin housekeeping gene signal.

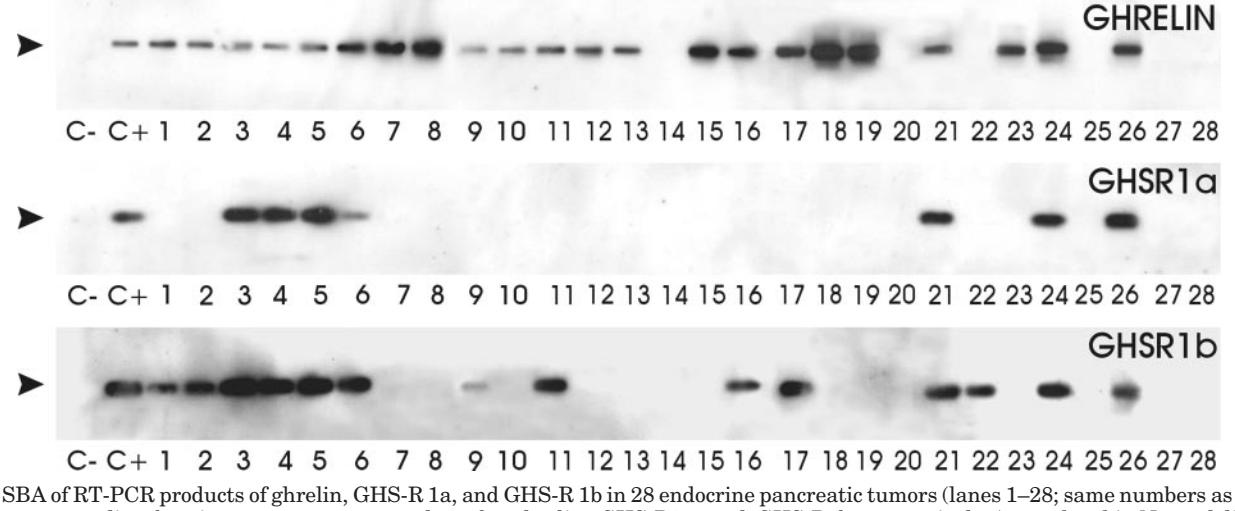

FIG. 3. SBA of RT-PCR products of ghrelin, GHS-R 1a, and GHS-R 1b in 28 endocrine pancreatic tumors (lanes 1–28; same numbers as in Table 1). The corresponding bp sizes were 327, 65, and 66 for ghrelin, GHS-R1a, and GHS-R1b, respectively (*arrowheads*). Normal liver and hypothalamus served as negative (C−) and positive (C+) controls, respectively, for both ghrelin and GHS-R mRNA amplification.

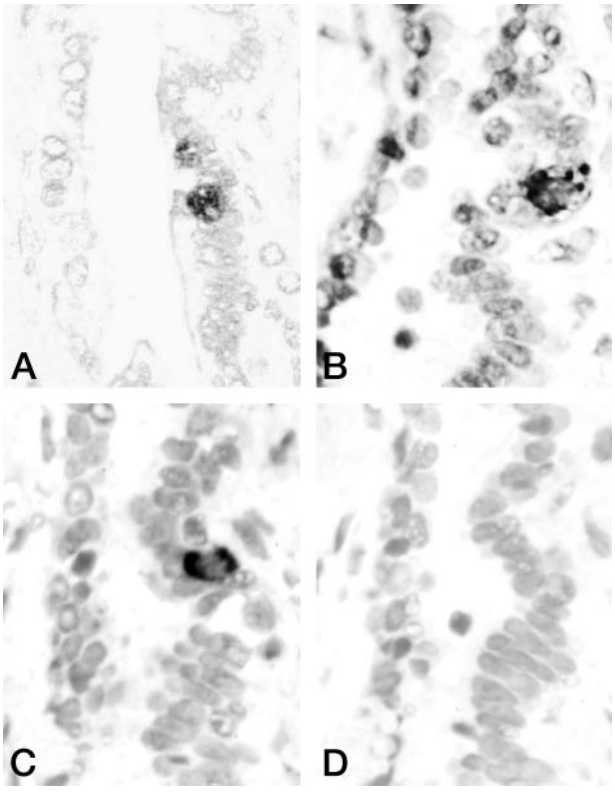

FIG. 4. Normal pancreas. A pancreatic exocrine duct shows a single isolated endocrine cell, containing insulin, as detected by insulin immunostaining (a). The same cell also expresses ghrelin, as shown by IHC (b) and ISH (c) performed on serial sections. No staining is present in a parallel section stained by ISH omitting the specific probe (d) (a–d, ×400).

GHS-R 1a and 1b mRNAs were found in 14 cases, 13 of which also expressed ghrelin mRNA by RT-PCR, although only 4 exhibited ghrelin immunoreactivity.

In endocrine tumors, no correlation was observed between ghrelin expression and age, sex, tumor size or grade or stage, or with the hormonal status of the cases investigated. No GH-release related symptoms (*i.e.* acromegalic features and symptoms) were identified in the patients for whom this information was obtained; the blood level of ghrelin was, however, unknown in this retrospective case series. As for the cell type expressing ghrelin, a colocalization with insulin-producing β-cells was seen in normal pancreatic tissue; whereas, in the endocrine tumors (if functioning), no colocalization with individual pancreatic hormones was observed.

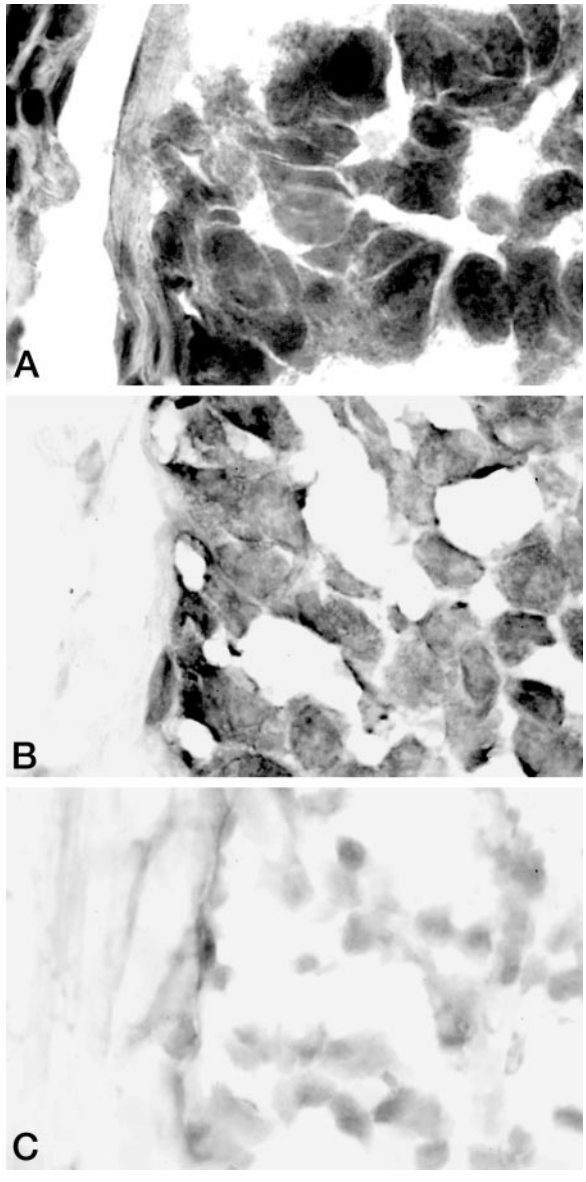

FIG. 5. Ghrelin binding sites in a pancreatic gastrinoma (a, case 27). In an unfixed, frozen section, the tumor cells were intensely stained with a nonradioactive *in situ* binding assay using biotinylated ghrelin, with a preferential cell membrane or peripheral cytoplasmic distribution (b). A peritumoral blood vessel was negative (b, *left*), as were the tumor cells in a parallel section incubated with biotinylated ghrelin after displacing treatments with 100-fold excess of unlabeled ghrelin (c) (a–c, ×1000).

Discussion

In this study, we have demonstrated that normal pancreatic islet cells (namely, β-cells) and the majority of pancreatic endocrine tumors produce ghrelin, a novel gastrointestinal hormone recently isolated from the rat and human stomach (1–3). In addition, we showed that the extent of ghrelin detection is largely dependent on the detection method used, because the amount of ghrelin (mRNA or protein) stored in the cells may be lower than the threshold of detectability of *in situ* localization methods (*e.g.* IHC or ISH). In most cases, however, highly sensitive techniques, such as RT-PCR, are able to reveal the specific mRNA.

Normal pancreas

Ghrelin was originally isolated from the rat stomach and then also found to be expressed by the arcuate hypothalamic nucleus, the small intestine, the kidney, the placenta, and the pituitary (including neoplastic conditions) (1, 41–44). In the literature, no reports that normal pancreas is a primary ghrelin production site have appeared to date. In this study, immunoreactive ghrelin was confined to pancreatic islets.

Not all cells were stained, and double immunocytochemical staining revealed that ghrelin expression was restricted to insulin-producing β-cells. This was also the case for the rare insulin-producing cells scattered throughout the exocrine duct system of the pancreas. The significance of the colocalization of insulin and ghrelin in β-cells is unknown. Interestingly, we have recently shown that ghrelin has hyperglycemic effects coupled with an inhibitory effect on insulin secretion (40). These effects of the natural ligand of GHS-R, *i.e.* ghrelin, are not shared by peptidyl synthetic GH secretagogues, such as hexarelin, although the molecules possess similar GH-releasing activity (40). Evidence that peptidyl synthetic GHSs do not affect insulin and glucose levels suggests that ghrelin's modulatory effect on them is not mediated by the classical GHS-R type 1a, which, by definition, is activated by both natural and synthetic GHS.

Further insights into these interactions may derive from the analysis of GHS-R distribution. Using a radioreceptor assay, we found a limited number of ghrelin binding sites in the membranes obtained from whole pancreas, 1000-fold less than the number of sites in GHS-R rich tissues, such as the pituitary gland or the heart (19). These findings are congruent with the negative RT-PCR data obtained when seeking GHS-R 1a and 1b mRNAs in normal pancreatic tissue. In contrast, a small (but detectable) amount of GHS-R-specific mRNA has been detected by some authors in human normal pancreatic tissue by means of RNase protection assay (51). These results by no means exclude the presence of binding sites. In fact, RT-PCR was designed to reveal GHS-R types 1a and 1b, the only currently known ghrelin receptors; yet other receptors may exist, as implied by previous data (19, 21, 52). Analysis of microdissected specimens may help more clearly to determine the receptor status of normal pancreatic cells. Laser-based microdissection makes single cell preparations possible and is optimal for pure islet cell component separation. RT-PCR analysis of GHS-R, using dissected specimens, corroborated the data obtained on whole pancreatic tissue, indicating that the specific mRNA is absent in both the endocrine and the acinar cell components.

Endocrine tumors

In a fraction of the pancreatic endocrine tumors, either the ligand, ghrelin, or its receptor, GHS-R, were expressed. The exact percentage of ghrelin-producing endocrine tumors of the pancreas is hard to assess, because it is largely influenced by the method used to reveal it. Immunoreactive ghrelin is focally present in approximately one third of the cases, although the specific mRNA is present in up to 79% of the cases when ISH or the highly sensitive RT-PCR techniques are employed. The fact that some cases or some tumor areas showed ghrelin mRNA, but no detectable peptide, may be related to a translational deficit or to limited storage of the protein within the cell. The latter is a relatively common event in endocrine tumors, especially in poorly differentiated ones (*e.g.* chromogranin A content in small cell lung carcinoma) (53). The significance of ghrelin production or even of mRNA transcription is not known; and, in the absence of any correlation with clinicopathological parameters, no definite conclusion can be drawn from these data.

The 11 ghrelin-immunoreactive cases (*i.e.* those with a fair amount of hormone stored in the cytoplasm) included 6 insulinomas, 3 nonfunctioning tumors, 1 gastrinoma, and one vipoma. In all these cases, the corresponding mRNA was detected either by ISH or RT-PCR or by both methods. In the tumors studied, there was therefore no preferential association of ghrelin expression with any given hormone type. Moreover, no statistical correlation (see *Materials and Methods*) with age, sex, tumor size, tumor grade, presence of metastases, or clinical outcome was observed. Four of 10 cases having lymph node or liver metastases at diagnosis were ghrelin-positive. The same pattern of ghrelin immunoreactivity was also found in the corresponding metastatic tissues (when available for immunostaining) of these cases.

The clinical impact of chronic ghrelin hypersecretion is still unknown. Precise clinical and biochemical monitoring of hormonal parameters in patients bearing ghrelin-positive pancreatic tumors will be necessary to demonstrate the potential existence of functioning, ghrelin-producing, pancreatic endocrine tumors. In this context, it must be emphasized that the antibody and probes employed in this study recognize both octanoylated and desoctanoylated forms of ghrelin (54) and are unable to distinguish between tissues producing acylated and nonacylated forms. This is another critical point, because desoctanoyl ghrelin, though inactive in terms of GH secretion, exerts antiproliferative effects similar to those of acylated ghrelin (21).

A separate comment is deserved for the simultaneous presence of GHS-R and ghrelin mRNA in nearly half of the pancreatic endocrine tumors. The ghrelin production reported in some endocrine tumors of the pancreas may represent an *in vivo* experimental model to further understanding of pancreatic activities of ghrelin, of synthetic GHS, and of their analogs, including the antagonist D-Lys^3-GHRP6 (1). Knowledge about the target organs is mandatory, and the identification of binding sites for ghrelin and GH secretagogues was reported in several human and rat peripheral tissues (1, 2, 19, 55) and tumors (20, 21, 43, 45, 56). In the present study, we identified specific mRNA of GHS-R 1b in half of the tumors examined and of type 1a in a quarter of the tumors. All but 1 endocrine tumor expressing the receptor were also found to express ghrelin at either the protein or the gene level. Unfortunately, because no anti-GHS-R antibody is commercially available, the exact localization of the receptor molecules could not be defined at the cellular level. In addition, we detected ghrelin binding sites in tissue sections of 9 of 14 endocrine tumors by means of an *in situ* nonradioactive biotinylated-ghrelin binding assay. Curiously, only 6 of the 9 positive cases had detectable GHS-R mRNA as revealed by RT-PCR, suggesting that receptor subtypes other than GHS-R 1a and 1b may exist as effectors of ghrelin-mediated signals. It is, however, of great interest that a significant number of pancreatic endocrine tumors, as opposed to normal endocrine pancreatic tissue, coexpress ghrelin and its receptor. Autocrine/paracrine circuits may be active in endocrine pancreatic tumors and may be involved in growth control mechanisms, as demonstrated for ghrelin in other neoplastic models (20, 21).

In conclusion, we have demonstrated that: 1) ghrelin is produced by pancreatic islet cells, in particular by insulin-

producing β cells; 2) a consistent number of endocrine pancreatic tumors focally express ghrelin; and 3) most of these tumors also contain the specific GHS-R mRNA, the only currently known ghrelin receptor.

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References

1. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K** 1999 Ghrelin is a growth hormone-releasing acylated peptide from stomach. Nature 402:656–660
2. **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
3. **Gualillo O, Caminos JE, Kojima M, Kangawa K, Arvat E, Ghigo E, Casanueva FF, Dieguez C** 2001 Gender and gonadal influences on ghrelin mRNA levels in rat stomach. Eur J Endocrinol 144:687–690
4. **Dornonville de la Cour C, Bjorkqvist M, Sandvik AK, Bakke I, Zhao CM, Chen D, Hakanson R** 2001 A-like cells in the rat stomach contain ghrelin and do not operate under gastrin control. Regul Pept 99:141–150
5. **Tomasetto C, Karam SM, Ribieras S, Masson R, Lefebvre O, Staub A, Alexander G, Chenard MP, Rio MC** 2000 Identification and characterization of a novel gastric peptide hormone: the motilin-related peptide. Gastroenterology 119:395–405
6. **Folwaczny C, Chang JK, Tschop M** 2001 Ghrelin and motilin: two sides of one coin? Eur J Endocrinol 144:R1–R3
7. **Del Rincon JP, Thorner MO, Gaylinn BG** 2001 Motilin-related peptide and ghrelin: lessons from molecular techniques, peptide chemistry, and receptor biology. Gastroenterology 120:587–589
8. **Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K** 2000 Ghrelin strongly stimulates growth hormone release in humans. J Clin Endocrinol Metab 85:4908–4911
9. **Bowers CY** 2001 Unnatural growth hormone-releasing peptide begets natural ghrelin. J Clin Endocrinol Metab 86:1464–1469
10. **Arvat E, DiVito L, Broglio F, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F, Ghigo E** 2000 Preliminary evidence that ghrelin, the natural GH secretagogue (GHS)-receptor ligand strongly stimulates GH secretion in humans. J Endocrinol Invest 23:493–495
11. **Arvat E, Maccario M, DiVito L, Broglio F, Benso A, Gottero C, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F, Ghigo E** 2001 Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone. J Clin Endocrinol Metab 86:1169–1174
12. **Ghigo E, Arvat E, Muccioli G, Camanni F** 1997 Growth hormone-releasing peptides. Eur J Endocrinol 136:445–460
13. **Smith RG, van der Ploeg LHT, Howard AD, Feighner SD, Cheng K, Hickey GJ, Wyvratt Jr MJ, Fisher MH, Nargund RP, Patchett AA** 1997 Peptidomimetic regulation of growth hormone secretion. Endocr Rev 18:621–645
14. **Dieguez C, Casanueva FF** 2000 Ghrelin: a step forward in the understanding of somatotroph cell function and growth regulation. Eur J Endocrinol 142: 413–417
15. **Kojima M, Hosoda H, Matsuo H, Kangawa K** 2001 Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. Trends Endocrinol Metab 12:118–122
16. **Matsumoto M, Katajima Y, Iwanami T, Hayashi Y, Tanaka S, Minamitake Y, Hosoda H, Kojima M, Matsuo H, Kangawa K** 2001 Structural similarity of ghrelin derivatives to peptidyl growth hormone secretagogues. Biochem Biophys Res Commun 284:655–659
17. **Muccioli G, Ghè C, Ghigo MC, Papotti M, Arvat E, Boghen MF, Nilsson MH, Deghenghi R, Ong H, Ghigo E** 1998 Specific receptors for synthetic GH secretagogues in the human brain and pituitary gland. J Endocrinol 157:99–106
18. **Muccioli G, Papotti M, Locatelli V, Ghigo E, Deghenghi R** 2001 Binding of ^{125}I-labeled ghrelin to membranes from human hypothalamus and pituitary gland. J Endocrinol Invest 24:RC7–RC9
19. **Papotti M, Ghè C, Cassoni P, Catapano F, Deghenghi R, Ghigo E, Muccioli G** 2000 Growth hormone secretagogue binding sites in peripheral human tissues. J Clin Endocrinol Metab 85:3803–3807
20. **Cassoni P, Papotti M, Catapano F, Ghe C, Deghenghi R, Ghigo E, Muccioli G** 2000 Specific binding sites for synthetic growth hormone secretagogues in nontumoral and neoplastic human thyroid tissue. J Endocrinol 165:139–146
21. **Cassoni P, Papotti M, Ghè C, Catapano F, Sapino A, Graziani A, Deghenghi R, Reissmann T, Ghigo E, Muccioli G** 2001 Identification, characterization and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues in human breast carcinomas and cell lines. J Clin Endocrinol Metab 86:1738–1745
22. **Peino R, Baldelli R, Rodriguez-Garcia J, Rodriguez-Segade S, Kojima M, Kangawa K, Arvat E, Ghigo E, Dieguez C, Casanueva FF** 2000 Ghrelin-induced growth hormone secretion in humans. Eur J Endocrinol 143:R11–R14
23. **Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, Bloom SR** 2000 The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 141:4325–4328
24. **Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I** 2000 Central effect of ghrelin, an endogenous hormone secretagogue, on hypothalamic peptide gene expression. Endocrinology 141:4797–4800
25. **Seoane LM, Tovar S, Baldelli R, Arvat E, Ghigo E, Casanueva FF, Dieguez C** 2000 Ghrelin elicits a marked stimulatory effect on GH secretion in freely-moving rats. Eur J Endocrinol 143:R7–R9
26. **Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S** 2001 A role for ghrelin in central regulation of feeding. Nature 409:194–198
27. **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
28. **Tschop M, Smiley DL, Heiman ML** 2000 Ghrelin induces adiposity in rodents. Nature 407:908–913
29. **De Gennaro-Colonna V, Rossoni G, Bernareggi M, Muller EE, Berti F** 1997 Cardiac ischemia and impairment of vascular endothelium function in hearts from growth-hormone deficient rats: protection by hexarelin. Eur J Pharmacol 334:201–207
30. **De Gennaro-Colonna V, Rossoni G, Cocchi D, Rigamonti AE, Berti F, Muller EE** 2000 Endocrine, metabolic and cardioprotective effects of hexarelin in obese Zucker rats. J Endocrinol 166:529–536
31. **Locatelli V, Rossoni G, Schweiger F, Torsello A, De Gennaro-Colonna V, Bernareggi M, Deghenghi R, Muller EE, Berti F** 1999 Growth hormone-independent cardioprotective effects of hexarelin in the rat. Endocrinology 140:4024–4031
32. **Rossoni G, De Gennaro-Colonna V, Bernareggi M, Polvani GL, Muller EE, Berti F** 1998 Protectant activity of hexarelin or growth hormone against post-ischemic ventricular dysfunction in hearts from aged rats. J Cardiovasc Pharmacol 32:260–265
33. **Muccioli G, Broglio F, Valetto MR, Ghe C, Catapano F, Graziani A, Papotti M, Bisi G, Deghenghi R, Ghigo E** 2000 Growth hormone releasing peptides and the cardiovascular system. Ann Endocrinol (Paris) 61:27–31
34. **Nagaya N, Kojima M, Uematsu M, Yamagishi M, Hosoda H, Oya H, Hayashi Y, Kangawa K** 2001 Hemodynamic and hormonal effects of human ghrelin in healthy volunteers. Am J Physiol Regul Integr Comp Physiol l280:1483–1487
35. **Broglio F, Benso A, Valetto MR, Gottero C, Quaranta L, Podio V, Arvat E, Bobbio M, Bisi G, Ghigo E** 2001 Growth hormone-independent cardiotropic activities of growth hormone-releasing peptides in normal subjects, in patients with growth hormone deficiency, and in patients with idiopathic or ischemic dilated cardiomyopathy. Endocrine 14:105–108
36. **Filigheddu N, Fubini A, Baldanzi G, Cutrupi S, Ghe C, Catapano F, Broglio F, Bosia A, Papotti M, Muccioli G, Ghigo E, Deghenghi R, Graziani A** 2001 Hexarelin protects H9c2 cardiomyocytes from doxorubicin-induced cell death. Endocrine 14:113–119
37. **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
38. **Date Y, Nakazato M, Murakami N, Kojima M, Kangawa K, Matsukura S** 2001 Ghrelin acts in the central nervous system to stimulate gastric acid secretion. Biochem Biophys Res Commun 280:904–907
39. **Hewson AK, Dickson SL** 2000 Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. J Neuroendocrinol 12:1047–1049
40. **Broglio F, Arvat E, Benso A, Gottero C, Muccioli G, Papotti M, van der Lely AJ, Deghenghi R, Ghigo E** 2001 Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. J Clin Endocrinol Metab 86:5083–5086

41. **Mori K, Yoshimoto A, Takaya K, Hosoda K, Ariyasu H, Yahata K, Mukoyama M, Sugawara A, Hosoda H, Kojima M, Kangawa K, Nakao K** 2000 Kidney produces a novel acylated peptide, ghrelin. FEBS Lett 486:213–216
42. **Gualillo O, Caminos JE, Blanco M, Garcia-Caballero T, Kojima M, Kangawa K, Dieguez C, Casanueva F** 2001 Ghrelin, a novel placental-derived hormone. Endocrinology 142:788–794
43. **Korbonits M, Bustin SA, Kojima M, Jordan S, Adams EF, Lowe DG, Kangawa K, Grossman AB** 2001 The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors. J Clin Endocrinol Metab 86:881–887
44. **Korbonits M, Kojima M, Kangawa K, Grossman AB** 2001 Presence of ghrelin in normal and adenomatous human pituitary. Endocrine 14:101–104
45. **Papotti M, Cassoni P, Volante M, Deghenghi R, Muccioli G, Ghigo E** 2001 Ghrelin–producing endocrine tumors of the stomach and intestine. J Clin Endocrinol Metab 86:5052–5059
46. **Solcia E, Kloppel G, Sobin L** 2000 International histological classification of tumors. In: Histological typing of endocrine tumors. 2nd ed. Geneva: WHO; 62–64
47. **Volante M, Pecchioni C, Bussolati G** 2000 Post-incubation heating significantly improves tyramide signal amplification. J Histochem Cytochem 48: 1583–1585
48. **Papotti M, Croce S, Macri L, Funaro A, Pecchioni C, Schindler M, Bussolati G** 2000 Correlative immunohistochemical and reverse transcriptase-polymerase chain reaction analysis of somatostatin receptor type 2 in neuroendocrine tumors of the lung. Diagn Mol Pathol 9:47–57
49. **Kuecker SJ, Jin L, Kulig E, Oudraogo GL, Roche PC, Lloyd RV** 1999 Analysis of PRL, PRL-R, TGFβ1, and TGFβ-RII gene expression in normal and neoplastic breast tissues after laser captured microdissection. Appl Immunohistochem Mol Morphol 7:193–200
50. **Mouton CA, Pang D, Natraj CV, Shafer JA.** 1982 A reagent for covalently attaching biotin to protein via a cleavable connect. Arch Biochem Biophys 218:101–108
51. **Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, Smith RG, Van der Ploeg LH, Howard AD** 1997 Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res Mol Brain Res 48:23–29
52. **Bodart V, Bouchard JF, McNicoll N, Escher E, Carriere P, Ghigo E, Sejlitz T, Sirois MG, Lamontagne D, Ong H** 1999 Identification and characterization of a new growth hormone-releasing peptide receptor in the heart. Circ Res 85: 796–802
53. **Hamid Q, Corrin B, Sheppard MN, Huttner WB, Polak JM** 1991 Expression of CGA mRNA in small cell carcinoma of the lung. J Pathol 163: 293–297
54. **Hosoda H, Kojima M, Matsuo H, Kangawa K** 2000 Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun 279:909–913
55. **Shuto Y, Shibasaki T, Wada K, Parhar I, Kamegai J, Sugihara H, Oikawa S, Wakabayashi I** 2001 Generation of polyclonal antiserum against the growth hormone secretagogue receptor (GHS-R): evidence that the GHS-R exists in the hypothalamus, pituitary and stomach of rats. Life Sci 68:991–996
56. **Korbonits M, Jacobs RA, Aylwin SJ, Burrin JM, Dahia PL, Monson JP, Honegger J, Fahlbush R, Trainer PJ, Chew SL, Besser GM, Grossman AB** 1998 Expression of the growth hormone secretagogue receptor in pituitary adenomas and other neuroendocrine tumors. J Clin Endocrinol Metab 83: 3624–3630