

The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas

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Received 13 February 2002; received in revised form 10 April 2002; accepted 11 April 2002

Abstract

Objectives: Ghrelin, an endogenous ligand of the growth hormone secretagogue receptor (GHS-R), was recently identified in the stomach. Ghrelin is produced in a population of endocrine cells in the gastric mucosa, but expression in intestine, hypothalamus and testis has also been reported. Recent data indicate that ghrelin affects insulin secretion and plays a direct role in metabolic regulation and energy balance. On the basis of these findings, we decided to examine whether ghrelin is expressed in human pancreas. Specimens from fetal to adult human pancreas and stomach were studied by immunocytochemistry, for ghrelin and islet hormones, and in situ hybridisation, for ghrelin mRNA. **Results:** We identified ghrelin expression in a separate population of islet cells in human fetal, neonatal, and adult pancreas. Pancreatic ghrelin cells were numerous from midgestation to early postnatally (10% of all endocrine cells). The cells were few, but regularly seen in adults as single cells at the islet periphery, in exocrine tissue, in ducts, and in pancreatic ganglia. Ghrelin cells did not express any of the known islet hormones. In fetuses, at midgestation, ghrelin cells in the pancreas clearly outnumbered those in the stomach. **Conclusions:** Ghrelin is expressed in a quite prominent endocrine cell population in human fetal pancreas, and ghrelin expression in the pancreas precedes by far that in the stomach. Pancreatic ghrelin cells remain in adult islets at lower numbers. Ghrelin is not co-expressed with any known islet hormone, and the ghrelin cells may therefore constitute a new islet cell type. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Ghrelin; Islets; Human; Development; Histochemistry; Pancreas

1. Introduction

Ghrelin, a novel peptide of 28 amino acids, was recently isolated from rat stomach as ligand of the growth hormone secretagogue receptor (GHS-R) [1]. Independently, Tomasetto et al. [2] discovered mRNA in the mouse stomach that encoded a novel protein of 117 amino acid residues with distant chemical relationship to the motilin precursor; it was named motilin-related peptide (MTRLRP) and turned out to be the mouse homologue of preproghrelin. Ghrelin/MTRLRP, further on called ghrelin, was found to be produced by a population of endocrine cells, which are most abundant in the oxyntic mucosa in the stomach, less frequent in the gastric antrum, and still fewer in the small intestine [3,4]. Ghrelin has also been detected in hypothalamus [1], kidney [5], testis [6], and placenta [7]. It has been suggested that

ghrelin, secreted primarily from the stomach, acts as a hormone to release GH from the pituitary [8]. Recent studies have shown additional actions of ghrelin. Ghrelin stimulates food intake when given either i.p. or intracerebroventricularly (i.c.v.) in rats [9,10]. Ghrelin administered i.c.v. and s.c. in rats and mice causes body weight gain with increased adiposity through changes in energy balance, including a reduction in fat utilisation [10,11]. Ghrelin stimulates gastric motility and acid secretion in rats [12]. Ghrelin mRNA is upregulated in the stomach, and plasma ghrelin levels are elevated upon fasting in rats [13]. Further, plasma ghrelin levels are elevated preprandially and reduced postprandially in humans [14,15]. Interestingly, reduced levels of plasma ghrelin have been demonstrated in human obesity [16]. Thus, many recent data indicate that ghrelin is involved in metabolic regulation and energy balance, and it has become evident that certain ghrelin actions are independent of GH [10,17]. Many of the peptides involved in metabolic regulation, e.g. neuropeptide Y (NPY), peptide YY (PYY), somatostatin, and galanin, are expressed in the pancreatic islets [18,19]; some of them, e.g. NPY and PYY, are

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upregulated during islet development [20]. We therefore decided to examine the possibility of ghrelin expression in the developing and mature human pancreas, using immunocytochemistry and in situ hybridisation.

2. Materials and methods

2.1. Tissue collection and preparation

Human specimens were collected during many years (starting 1975), according to then used ethical guidelines. Specimens from fetal (gestational age 15–26 weeks) ($n=7$), newborn (sudden infant death) ($n=1$), and adult (age range 35–75; $n=8$) pancreas, usually separate specimens from duodenal (head) and splenic (tail) portions, and from the stomach, were from legal abortions, surgical resections, and autopsies. The specimens were frozen, freeze-dried and fixed in formaldehyde (vapour or liquid) or diethyl pyrocarbonate (DEPC) vapour, and embedded in paraffin according to previously published protocols [21]. Alternatively, specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (6 μm thickness) were mounted on coated slides, deparaffinised, and hydrated before further handling.

2.2. Immunocytochemistry

Antibodies were diluted in phosphate-buffered saline (PBS) (pH 7.2) containing 0.25% bovine serum albumin and 0.25% Triton X-100. Sections were incubated with primary antibodies (Table 1) overnight at 4 °C, followed by rinsing in PBS with Triton X-100 for 2×10 min. Thereafter, secondary antibodies with specificity for rabbit-, guinea pig-, sheep- or mouse-IgG, and coupled to either fluorescein isothiocyanate (FITC) (DAKO, Copenhagen, Denmark; Jackson, West Grove, PA, USA; Sigma, St. Louis, MO, USA), Texas-Red (Jackson), or 7-amino-4-methyl coumarin-3-acetic acid (AMCA) (Jackson) were applied on the sections. Incubation was for 1 h at room temperature. Sections were again rinsed in Triton X-100 enriched PBS for 2×10 min and then mounted in PBS/glycerol (1:1). The specificity of immunostaining was tested by using primary antisera preadsorbed with homologous

antigen (100 μg of peptide per ml antiserum in working dilution), or by omission of primary antibodies. Double or triple immunofluorescence was also used. In these studies, tests for inappropriate binding of the secondary antibodies were performed.

2.3. Probe

A synthetic oligodeoxyribonucleotide probe, complementary to the sequence 153–182 of human preproghrelin mRNA (Accession number NM 016362) [1], encoding a C-terminal portion of ghrelin, was designed. A BLAST was run, using the GenBank database, demonstrating lack of significant sequence similarity with any other mammalian mRNA. The probe was synthesised by DNA-technology, Aarhus, Denmark, and 3' -end-tailed with [³⁵S]dATP (NEN, Stockholm, Sweden).

2.4. In situ hybridisation

The in situ hybridisation protocol has been described previously [22]. In brief, deparaffinised and hydrated sections were treated with proteinase K (10 $\mu\text{g}/\text{ml}$, Sigma) for 30 min at 37 °C, fixed in 4% paraformaldehyde for 15 min, washed 5 min in PBS, and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Thereafter, sections were dehydrated in graded ethanols, and air dried. Hybridisation was carried out in sealed moisturising chambers at 37 °C overnight, using a probe concentration of approximately 1 pmol/ml, followed by stringent post-hybridisation washing ($1 \times \text{SSC}$; 0.15 M NaCl, 0.015 M sodium citrate). The slides were dipped in Ilford K.5 emulsion and stored in light sealed boxes at 4 °C for 10–18 days. They were then developed in Kodak D-19, fixed in Kodak polymax and mounted in Kaiser's glycerol gelatine. Controls included use of sense probe, or hybridisation in the presence of 100-fold excess of unlabelled probe.

2.5. Imaging

Immunofluorescence was examined in epifluorescence microscope (Olympus, BX60). By changing filters, the location of the different secondary antibodies in double and triple staining was determined. In situ hybridisation

Table 1
Details of the antibodies used for immunocytochemistry

Antigen	Code	Raised against	Raised in	Dilution	Source
Ghrelin	H-031-31	Rat ghrelin	Rabbit	1:800	Phoenix, Belmont, CA, USA
Proinsulin	9003	Human proinsulin	Guinea pig	1:2560	Eurodiagnostica, Malmö, Sweden
Glucagon	8708	Porcine glucagon	Guinea pig	1:5120	Eurodiagnostica
Somatostatin	V 1169	Human somatostatin	Mouse monoclonal	1:400	Biomedica, Foster City, CA, USA
Pancreatic polypeptide	AHP 515	Human pancreatic polypeptide	Sheep	1:640	Serotec, Oxford, UK
Chromogranin A	LK2H10	Human chromogranin	Mouse	1:160	Boehringer, Mannheim, Germany
HuC/D	A-21271	Human neuronal protein	Mouse	1:800	Molecular Probes, Leiden, NL

radiolabelling was examined in bright field. Images were captured with a digital camera (LSR UltraPlus2000).

2.6. Cell counting

The different populations of islet endocrine cells were counted in the fluorescence microscope, by two independent

observers, on double- or triple-immunostained sections from fetuses at midgestation (18–22 weeks) ($n=5$) and newborn ($n=1$), one specimen each (tail portion). All immunoreactive cells within three framed areas in one section from each specimen were counted. In addition, serial sections covering entire islets ($n=5-7$ per specimen) in adults ($n=3$) were analysed for ghrelin immunofluorescence.

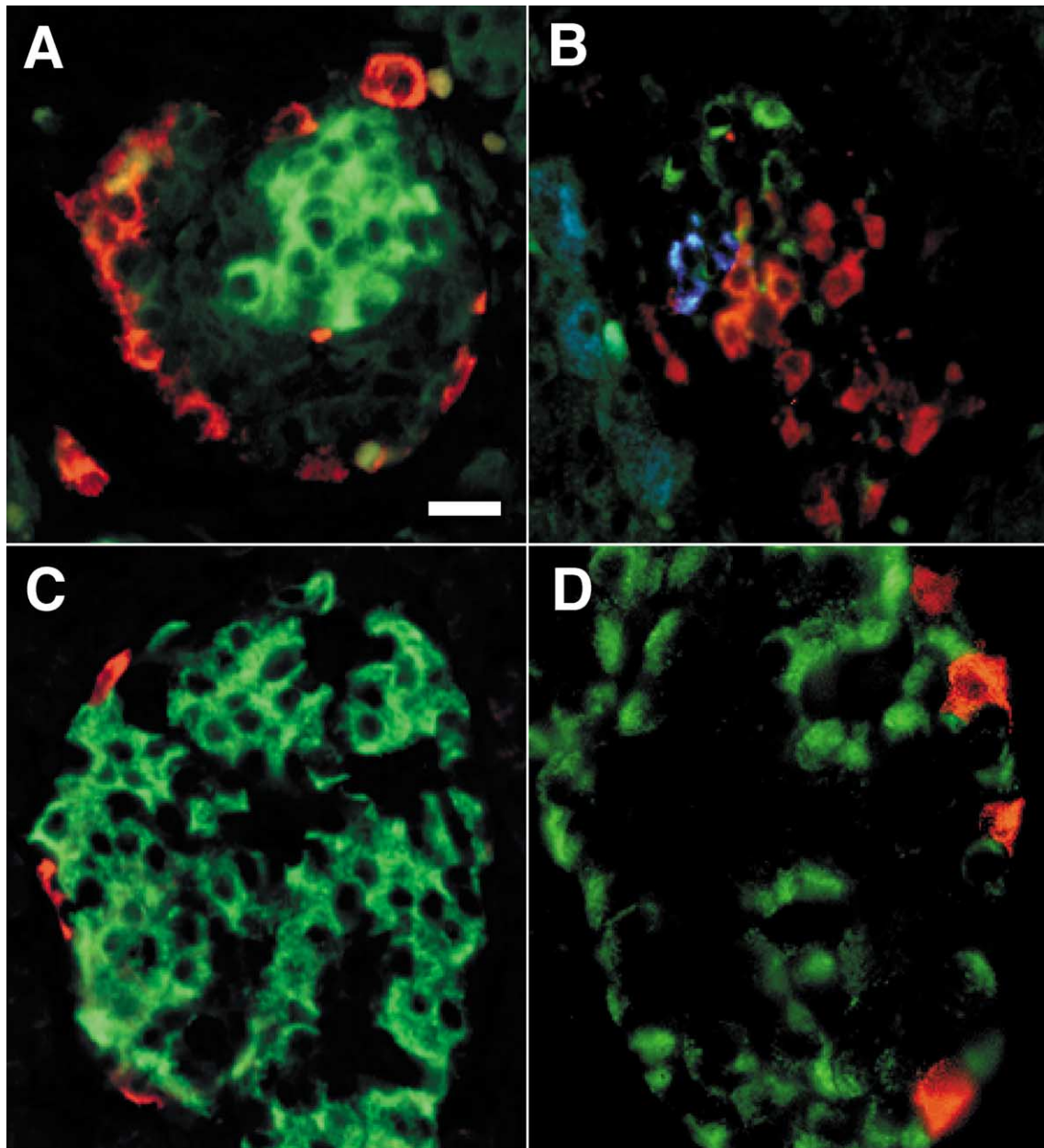


Fig. 1. Immunofluorescence photomicrographs of human islets illustrating that ghrelin-IR cells are numerous during development, but few in adults, and that they constitute a separate cell population. (A) Fetus (22 weeks), double staining for ghrelin (red) and insulin (green). Ghrelin-IR cells form an almost continuous layer at the islet peripheral rim. (B) Newborn (section through islet periphery), triple staining for ghrelin (red), insulin (blue), and somatostatin (green). Ghrelin-IR cells are still numerous at birth. (C) Adult, double staining for ghrelin (red) and insulin (green). Single scattered ghrelin-IR cells at the islet peripheral rim, some close to insulin cells. (D) Adult, double staining for ghrelin (red) and glucagon (green). Ghrelin and glucagon-IR cells are separate, sometimes juxta-posed cells. Scale bar = 20 μm .

3. Results

3.1. Immunocytochemistry

3.1.1. Fetuses and neonatal

Ghrelin-immunoreactive (IR) cells were quite numerous in the fetal and neonatal pancreas (Fig. 1A,B). At mid-gestation (18–22 weeks) and at birth, ghrelin-IR cells constituted 9% and 11% of all demonstrable islet cells, respectively (Table 2). The ghrelin-IR cells sometimes formed an almost continuous layer at the peripheral rim of the islets (Fig. 1A). Single ghrelin-IR cells were also seen in the epithelium of ducts. In the stomach, only very few ghrelin-IR cells were detected at midgestation (Fig. 2D). Comparison of ghrelin-IR cell frequency between pancreas and stomach at the same gestational stage revealed a clear predominance of ghrelin expression in the pancreas (compare Figs. 1A and 2D).

3.1.2. Adults

In islets of adults, ghrelin-IR cells were regularly, but less frequently seen (Fig. 1C,D); analysis of serial sections revealed an average of 3–5 ghrelin-IR cells per islet, with some islets lacking demonstrable ghrelin-IR cells. Counting revealed that approximately 1% of all islet cells were ghrelin-IR cells in the adult. The ghrelin-IR cells were evenly distributed in the pancreas in that there was no overt difference in number of ghrelin-IR cells between head and tail portions. Single ghrelin-IR cells were also seen in the epithelium of ducts (Fig. 2B), and in the exocrine tissue, sometimes closely applied to another endocrine cell (Fig. 2C). Interestingly, double staining for ghrelin and a marker for nerve cell bodies (human neuronal protein HuC/D) revealed that ghrelin-IR cells were occasionally located within pancreatic ganglia, closely applied to nerve cell bodies (Fig. 2A). In the stomach of adults, ghrelin-IR cells were numerous (not shown), as previously reported [3], outnumbering those in the pancreas.

3.1.3. Cell features

The ghrelin-IR cells were usually round or ovoid in shape, but sometimes also displayed one or two cytoplasmic extensions (Fig. 1A–D). The ghrelin-IR cells were often

located at the periphery of the islets, as single cells or small clusters of cells. To verify that the ghrelin-IR cells were endocrine cells, we used chromogranin A (CgA) as an endocrine cell marker. Double staining for ghrelin and CgA showed that the ghrelin-IR cells were weakly to moderately CgA-positive (not shown). To investigate if ghrelin-IR cells co-expressed any of the “classical” pancreatic hormones, triple staining for ghrelin/somatostatin/insulin, double staining for ghrelin/pancreatic polypeptide (PP), and double staining for ghrelin/glucagon were performed. No co-localisation could be detected at any stage (Fig. 1A–D). However, the ghrelin-IR cells were often located in the immediate vicinity of glucagon-IR cells (Fig. 1D), or somatostatin-IR cells (Fig. 1B), and occasionally situated close to insulin-IR cells (Figs. 1C and 2C) or PP-IR cells. In fetuses, co-localisation of two “classical” islet hormones could be detected in a few islet cells, in agreement with previous observations [23]. DEPC vapour fixation was superior to any of the other fixations tested to demonstrate ghrelin-IR cells.

3.2. In situ hybridisation

Specimens fixed in formaldehyde (vapour or liquid) were found suitable for in situ hybridisation. Labelling for ghrelin mRNA was detected in numerous cells at the periphery of fetal islets, but only in few cells in islets of adult (Fig. 3A,B). In sections from the stomach of adults, scattered cells in the mucosal glands were strongly labelled (not shown). Only very weak background labelling was seen when hybridisation was performed in the presence of excess unlabelled probe, or when sense probe was used (not shown).

4. Discussion

In this study, we show that ghrelin is expressed in human islets from early gestation to adult. Importantly, the immunocytochemical results correspond closely to the data obtained by in situ hybridisation of ghrelin mRNA expression. The ghrelin cells do not express any of the known pancreatic hormones (insulin, glucagon, somatostatin, or PP) at any stage. Ghrelin cells may therefore constitute a new islet cell type. While this work was in progress, Date et al. [24] presented data indicating that ghrelin is co-localised with glucagon in adult human and rat pancreas, whereas Volante et al. [25] obtained results indicating that ghrelin is co-localised with insulin in human islets. As is obvious from our results, we could not confirm any of these data; rather our data indicate that both ghrelin peptide and mRNA expression is confined to a distinct cell type. The reasons for the discrepancy in results are not clear; they may be related to differences in methodology or antibody specificity. In the fetal pancreas at midgestation, cell counting revealed that ghrelin-IR cells constituted 9% of all endo-

Table 2

Result of cell counts, yielding number (mean \pm SEM) and percentage (mean \pm SEM) of different islet cell populations, in pancreas (tail portion) of fetuses at midgestation ($n=5$) and in newborn ($n=1$)

Immunofluorescence	Midgestation		Newborn	
	Number of cells	% of total	Number of cells	% of total
Ghrelin	24 \pm 13.3	9 \pm 2.3	36	11
Glucagon	100 \pm 31.3	36 \pm 5.3	112	34
Insulin	86 \pm 32.8	31 \pm 4.4	103	31
Somatostatin	71 \pm 50.6	23 \pm 8.5	78	23
PP	2 \pm 0.9	1 \pm 0.5	3	1

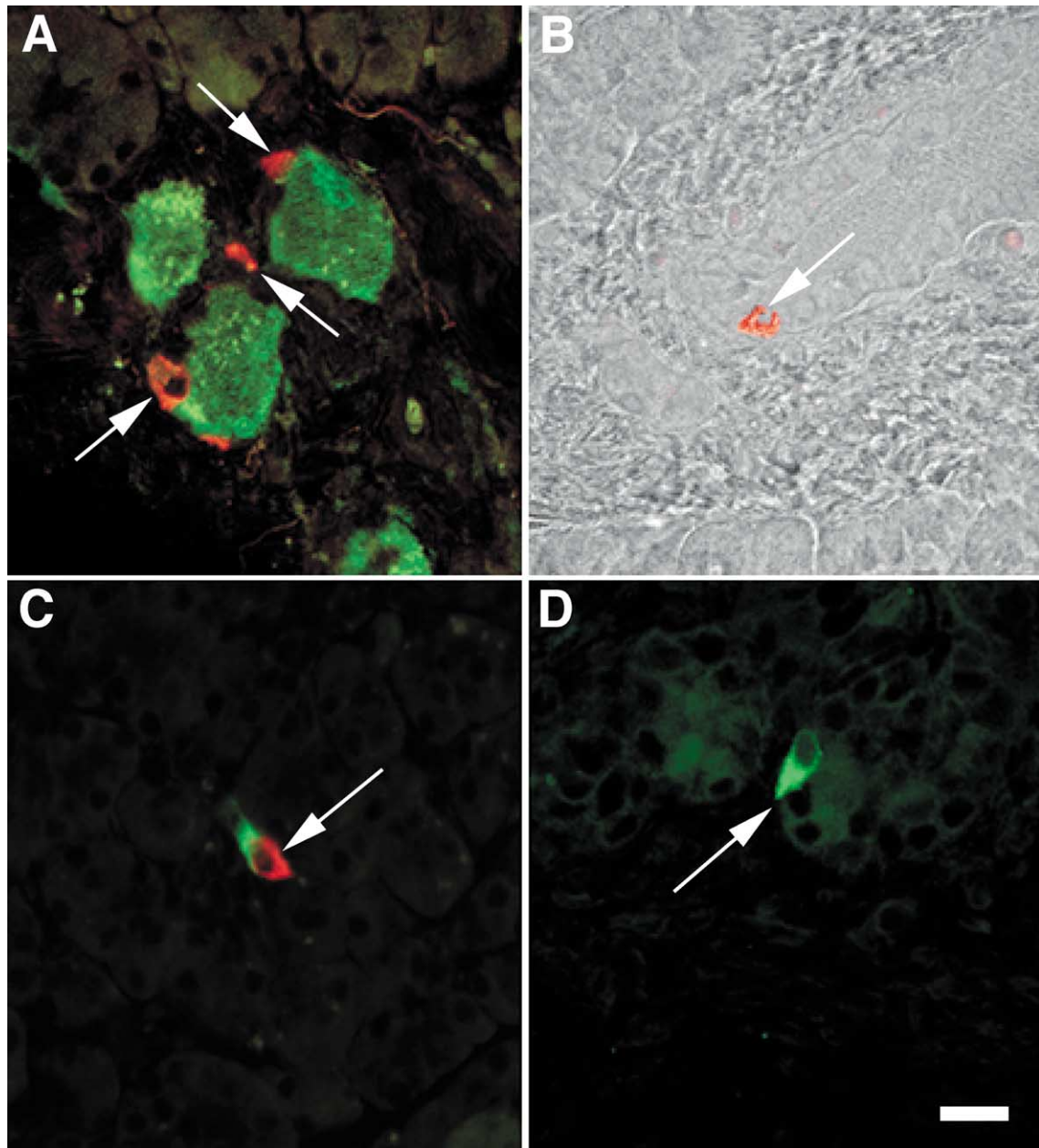


Fig. 2. Immunofluorescence photomicrographs illustrating extra islet occurrence of ghrelin-IR cells in human pancreas and in stomach. (A) Pancreatic ganglion of adult, double-stained for ghrelin (red) and human neuronal protein HuC/D (green). Ghrelin-IR cells are in contact with nerve cell bodies. (B) Combined phase contrast and fluorescence photomicrograph of pancreatic duct of adult, stained for ghrelin. Single ghrelin-IR cell in ductal epithelium. (C) Adult, double staining for ghrelin (red) and insulin (green). Single ghrelin-IR cell in exocrine tissue, in close contact with an insulin-IR cell. (D) Fetal (22 weeks) stomach mucosa, stained for ghrelin. Single, immunostained cell indicates low frequency of ghrelin cells. Arrows indicate ghrelin-IR cells. Scale bar = 20 μ m.

crine cells. However, a few non-ghrelin-IR endocrine cells co-expressed “classical” islet hormones [23], and since we did not account for this, the actual percentage of ghrelin cells at this fetal stage may be slightly higher. The expression of ghrelin in a quite prominent endocrine cell population in fetal pancreas suggests a developmental role. Interestingly, therefore, ghrelin has recently been reported to have trophic effects, as studied in the colonic epithelial cell line HT-29 [26], and in the hepatocellular carcinoma cell line HepG2 [27]. One function of ghrelin in the

developing pancreas might be to locally promote cell growth and maturation, and perhaps also, by hormonal actions, promote growth at distant sites.

Interestingly, the onset of ghrelin expression in the pancreas by far precedes that in the stomach. This is reminiscent of gastrin, another gastric hormone which, as studied in the rat, is expressed in pancreatic cells during a time period starting well before birth, but in the stomach only postnatally [28]. A role for pancreatic gastrin as a local trophic factor was suggested [28], and later substantiated

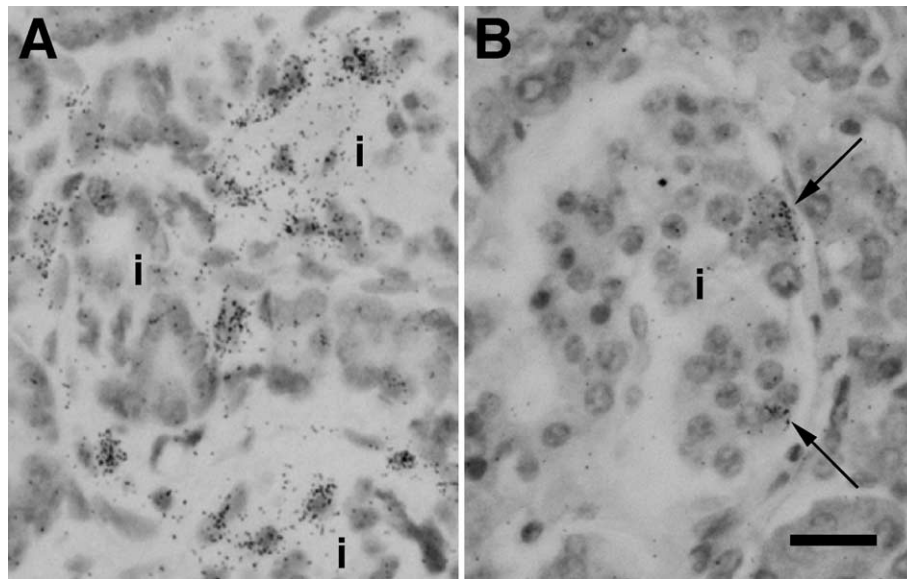


Fig. 3. In situ hybridisation autoradiographs of human islets, with labelling for ghrelin mRNA: (A) 22-week fetus; (B) adult. The labelled cells are numerous along the periphery of fetal islets, but they are few in islets of adult. Arrows in B indicate the two cells labelled for ghrelin mRNA; i indicates islet centre. Scale bar = 20 μ m.

[29]. It is of note that several additional peptides in the rodent endocrine pancreas are developmentally regulated, and there is a tendency for a specific pattern of developmental expression for each peptide. Among such peptides is NPY, which is expressed in the insulin cells for a short period during late gestation and early postnatally [20]. The structurally related peptide PYY is expressed very early during islet ontogeny, mainly in the glucagon cells. PYY remains highly expressed during the subsequent development, but is gradually down-regulated after weaning, as studied in both rat and mouse [20,30]. Both NPY [18,31] and PYY [32] are known to inhibit insulin secretion. In analogy, we have found pancreatic ghrelin expression to be developmentally regulated also in the rat islets [33], and ghrelin has recently been shown to inhibit insulin secretion, both in man [34] and rodents [35,36], although stimulating effects of ghrelin on insulin secretion have also been reported [24,37]. Thus, ghrelin is yet another potential modulator of insulin secretion that is upregulated in the islets during development.

It is of interest to note that many of the peptides that ghrelin interacts with in the hypothalamus are expressed also in the islets, and that GHS-R expression in pancreas has been reported [38]. Thus, ghrelin may influence islet peptide secretion by local actions. Ghrelin, given i.c.v., upregulates NPY in hypothalamic neurons [17,39,40], and provided similar mechanisms are operating in the islets, the inhibitory effect of ghrelin on insulin secretion could be exerted via locally released NPY. In adult humans, plasma ghrelin rises upon fasting [15]. Since ghrelin inhibits insulin secretion and causes hyperglycemia [34], one role of ghrelin in the islets postnatally may be to inhibit insulin secretion upon fasting. Interestingly, recent data have shown that in

humans, gastrectomy fails to abolish circulating ghrelin, rather 35% remains [41]. It is tempting to suggest that at least part of the remaining circulating ghrelin emanates from the pancreas.

The observed close relationship between neurones in pancreatic ganglia and single ghrelin cells is remarkable and may suggest specific neuronal actions of ghrelin. In fact, recent data indicate that ghrelin induced inhibition of exocrine pancreatic secretion is neuronally mediated [42].

In conclusion, ghrelin is expressed in human islets, and is developmentally regulated. The onset of expression in the islets precedes that in the stomach. Since ghrelin is not co-expressed with any other known islet hormone, we propose that ghrelin cells constitute a new islet cell type. The specific role of islet ghrelin remains to be established.

Acknowledgements

Grant supports from: Swedish Medical Research Council (Project No. 4499), Swedish Diabetes Association, and the Novo Nordic, Pahlsson, and Gyllensternska Krapperup Foundations. We thank Eva Hansson, Karin Jansner, Ann-Christin Lindh, and Doris Persson for expert technical assistance.

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