EXPERIMENTAL STUDY

Co-localization of neuroendocrine hormones in the human fetal pancreas

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Abstract

Objective and Design: Co-localization of the four major pancreatic hormones, and also of islet amyloid polypeptide (IAPP), peptide tyrosine tyrosine (PYY), secretin and neurotensin, has been studied in the endocrine pancreas of human fetuses at 16, 18 and 22 weeks of gestation.

Methods: Double and triple immunofluorescence stainings have been used.

Results: All three fetal pancreata contained cells that showed insulin, glucagon, somatostatin, pancreatic polypeptide (PP), IAPP, secretin and PYY immunoreactivity. Neurotensin cells were found in the youngest fetus and gastric inhibitory polypeptide (GIP) in the two older fetuses. Co-localization of two hormones occurred in most of the endocrine cell types in the three fetuses examined, but three hormones occurred in only a few cells and especially in the youngest fetus. Somatostatin cells were the only cell type which was largely monohormonal. Our findings showed that there are two different co-localization patterns: insulin was co-localized mainly with IAPP and glucagon, while secretin and PYY occurred together with glucagon and PP.

Conclusions: These data are the first to describe secretin and neurotensin in the fetal pancreas. Two different co-localization patterns could be distinguished: insulin, IAPP and glucagon, and glucagon, secretin, PP and PYY.

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Introduction

The adult endocrine pancreas synthesizes and releases four major hormones: insulin, glucagon, somatostatin and pancreatic polypeptide (PP). Islet amyloid polypeptide (IAPP), a recently discovered polypeptide with hormonal function, has been demonstrated in the endocrine pancreas, co-localized mainly with insulin (1). A small islet cell population has also been reported to show gastric inhibitory polypeptide (GIP) immunoreactivity (2).

The pancreas develops from two buds of the primitive foregut, one dorsal and one ventral, at the level of the bile duct (3). Earliest in the evolution among endocrine cells are the glucagon-immunoreactive (IR) cells, known from the incipient formation of the pancreatic buds, followed by insulin-IR (4, 5), somatostatin-IR and PP-IR cells, all four cell types being present by embryonic week (ew) 8 to 9 (6).

Studies on fetal porcine, murine and guinea-pig pancreas have shown that the endocrine progenitor cells can co-express more than one hormone (7–11).

Corresponding studies on human pancreas are sparse, however, and have been performed with immuno-electronmicroscopic or immunofluorescence techniques. In the human fetal pancreas, cells co-expressing two (8, 12) or three of the four major pancreatic hormones have been described; a reduced frequency of co-localization was found with increasing fetal age (13, 14).

The aim of the present study was to demonstrate the co-localization not only of the four major islet hormones and IAPP but also special interest has been paid to other polypeptides not demonstrated in adult pancreas, such as peptide tyrosine tyrosine (PYY), secretin and neurotensin in the pancreas of fetuses in the second trimester, using single, double and triple immunofluorescence staining techniques.

Materials and methods

Pancreata from three human female fetuses obtained for routine histopathological examination in connection with autopsy examination were included in the study.

The pregnancies had been terminated in ew 16, 18 and 22. Gestational age was determined by means of ultrasonic fetometry, direct fetometry and analysis of maternal menstrual regularity. The youngest fetus was from a prostaglandin-induced abortion necessitated by ultrasound findings of hygroma colli and hydrothorax, while the two older fetuses were from spontaneous abortions. The fetuses measured (crown–rump length) 12.0, 19.5 and 25.5 cm respectively, and their weights were 56, 180 and 337 g. Autopsy findings in the ew 16 fetus confirmed hygroma colli and hydrothorax; a malformed right ear and horseshoe kidney were also found; the other two fetuses displayed asphyxial haemorrhages in the lung due to placental ablation. None of the mothers had a history of diabetes mellitus. This study was approved by the Medical Ethics Committee at Huddinge Hospital, Stockholm.

The pancreatic specimens obtained were routinely fixed in 10% buffered neutral formalin for 18 to 20 h at room temperature (RT) and embedded in paraffin. Sections, 5 µm thick, were cut and attached to poly-L-lysine coated glass slides and stained with haematoxylin–eosin, or immunostained to demonstrate various hormones. The streptavidin–biotin complex (ABC) technique (15), with diaminobenzidine as chromogen, was applied as a single immunostain, mainly to reveal the distribution of endocrine cell types in the pancreas, but also for the control stainings mentioned below.

Immunofluorescence methods were used for single, double or triple staining, the latter two being applied to the same section to determine co-localization of different granular products. For triple immunofluorescence staining, the procedure was as follows: the sections were incubated with a cocktail of antibodies (one monoclonal+two polyclonal (anti-rabbit and anti-guinea-pig)), overnight incubation at RT → biotinylated goat anti-guinea-pig immunoglobulin G (IgG), 30 min at RT → a mixture of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG+Texas Red (TXRD)-conjugated goat anti-mouse IgG+aminomethyl coumarin acetic acid (AMCA)-conjugated streptavidin, 30 min at RT. Double immunostaining was performed in the same way but with exclusion of one of the primary (and corresponding secondary) antibodies. The sections were rinsed thoroughly after each step in the staining procedure. Before applying the respective primary antibodies, the sections were incubated with non-immune sera from the animal species producing the secondary antibodies.

The secondary antibodies were pre-incubated overnight with 10 µl/ml normal serum both from the species recognized by the other secondary antibodies and from the species donating the other secondary antibodies.

When two primary polyclonal antibodies raised in the same animal species (rabbit) were to be used, the staining procedure was as follows: initially the staining was performed with the first polyclonal antibody overnight at RT, followed by biotinylated swine anti-rabbit

IgG, 30 min at RT and TXRD-conjugated streptavidin, 30 min at RT. Thereafter the sections were exposed to paraformaldehyde vapour for 4 h at 80 °C (16), then incubated with the second primary antibody overnight, followed by incubation with FITC- or AMCA-conjugated goat anti-rabbit IgG for 30 min at RT.

Chemicals used

The primary antibodies are characterized in Table 1. The labelled secondary antisera were: biotinylated goat anti-guinea-pig IgG, AMCA-labelled streptavidin (Vector Laboratories, Burlingame, CA, USA), TXRD-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Inc., Birmingham, AL, USA), FITC-conjugated goat anti-rabbit IgG (Sigma, St Louis, MO, USA). All antigens were purchased from Sigma, except porcine glucagon from Novo Nordisk A/S (Bagsvaerd, Denmark) and bovine glucagon from Lilly (Indianapolis, IN, USA).

The control stainings entailed (i) omission of the primary antibody or antibodies, (ii) replacement of the first layer of antibody by non-immune serum diluted 1:10 and by the diluent alone, and (iii) preincubation (24 h) of primary antibody with the corresponding or structurally related synthetic antigens (10 and 20 nmol per ml diluted antibody solution respectively) before application to the sections. These control tests were performed with ABC technique (single staining) and immunofluorescence (co-localization studies).

Particular attention was paid to the specificity tests on the secretin, glucagon, PP and PYY antibodies, to reveal any cross-reactivity. The secretin antibodies were preincubated with porcine secretin, porcine and bovine glucagon, human PP and porcine PYY antigens. Sections from human adult pancreatic islets were used as a positive control for glucagon antibodies and as a negative control for secretin antibodies. The neutralization tests with secretin antibodies and glucagon antigens were even performed in the pancreas of the ew 22 fetus. The PP antibodies were preincubated with PP, PYY, glucagon and secretin antigens, and the PYY antibodies with the PP, PYY and glucagon antigens. All control tests turned out as expected, i.e. no cross-reaction was found between any of the antibodies. In double or triple immunofluorescence stainings, omission of one or two of the primary antibodies gave a staining result corresponding to the remaining primary antibody or antibodies.

The sections were examined in an Olympus Vanox AHBS3 fluorescence microscope (Olympus, Tokyo, Japan) equipped with filters (Olympus) giving excitation in wavelength bands of 475–555 nm for TXRD (filter no. 32821, dichroic mirror BH2-DMG), 453–488 nm for FITC (no. 32822, BH2-DMIB) and 340–375 nm for AMCA (no. 32817, BH2-DMU) respectively, while a double-band filter set (no. 39538, BH2-DFC5) for simultaneous visualization of TXRD- and FITC-labelled

Table 1 Antisera used in this study.

Antibody raised against	Code no.	Directed against	Working dilution*	Source
Synthetic porcine GIP ^b	B35-1	—	1:80	Eurodiagnostica, Malmö, Sweden
Synthetic human glucagon ^a	Glu-001	Midportion (5–15)	1:20	Novo Nordisk S/A, Bagsvaerd, Denmark
Synthetic porcine glucagon ^b	7360061	—	1:60	Dako Corp., Santa Barbara, CA, USA
Purified porcine ¹ glucagon ^c	A565 lot 081	—	1:20	Eurodiagnostica
Synthetic human insulin ^a	B-GP310-1	A chain	1:80	Biogenex Laboratories, San Ramon, CA, USA
Synthetic human insulin ^c	MUO 29UC	A chain	1:80	Dr Per Westermark, Dept Pathology, Linköping, Sweden
Synthetic human IAPP ^b	Ma 47	A chain	1:80	Peninsula Laboratories, Belmont, CA, USA
Synthetic neurotensin ^b	RAS 7321 lot 21399-2	—	1:200	Dr E Theodorsson, Dept Clinical Chemistry, Linköping, Sweden
Synthetic human PP ^b	6-8208	C terminal (8–13)	1:50	Dako Corp.
Synthetic porcine PYY ^c	A619 lot 105	—	1:50	Eurodiagnostica
Pure porcine secretin ^b	B-GP520-1	—	1:800	Eurodiagnostica
Synthetic human somatostatin ^b	B-33-1	—	1:10	Dako Corp.
Synthetic human somatostatin ^a	A 566 lot 72	1–14	1:100	Novo Nordisk S/A
	Som-018	1–14	1:80	

^a Antisera raised in mouse (monoclonal); ^b antisera raised in rabbit; ^c antisera raised in guinea-pig. ¹ From Novo Nordisk S/A. * Dilutions used in immunofluorescence staining; for the ABC staining the dilutions were 10 to 20 times higher.

cells was also used (excitation at 550–570 nm and 480–495 nm respectively).

For triple immunofluorescence staining, all immunoreactive cells for the respective hormones were counted in three fields of vision at a magnification of $\times 400$, by changing the three different filters. The co-localization of hormones was expressed as a percentage of the total number of cells counted in each staining combination. Photomicrographs were taken using Fujicolor 800 film; the triple stainings were photographed by double-exposing the film, first through the double-band and then with the AMCA filter sets.

Results

The endocrine pancreas of the ew 16 fetus consisted of mainly small, often irregular cell clumps connected with the ducts. In the ew 18 fetus the endocrine cells formed more distinct islets, but small cell clumps were still visible, as well as single cells among the exocrine parenchyma. In the oldest fetus (ew 22) most of the endocrine cells were found in the islets which were more distinctly delimited and more numerous.

Immunoreactive cells

All three fetal pancreata contained cells displaying insulin, glucagon, somatostatin, PP, IAPP, secretin and PYY immunoreactivity. A few weakly immunoreactive GIP cells were identified, present only in the two older fetuses. A few neurotensin-IR cells were observed in the youngest fetus. In the two older fetuses, glucagon-IR

cells were apparently more numerous than PP-IR cells, while the youngest fetus contained a more or less equal number of these cell types.

In the youngest fetus, insulin-IR cells tended to be localized predominantly in small clumps, while in the two older fetuses, where islets had developed, these cells had a more central localization. Glucagon-IR cells had a greater tendency to a peripheral distribution in the cell clusters and in the islets. Somatostatin-IR cells were seen mainly in the periphery of the islets, except in the youngest fetus where they were mainly solitary and spread throughout the exocrine parenchyma. In the two younger fetuses the PP-IR cells were localized mostly in the small cell clumps and islets, with a varying distribution pattern; a few solitary cells were seen among the exocrine parenchymal cells. In the oldest fetus, PP cells were localized in the periphery of the islets. IAPP-IR cells had a distribution similar to that of the insulin-IR cells, while that of secretin-IR cells was more similar to the glucagon- and PP-IR cells. The few PYY-IR cells observed were solitary throughout the pancreas in all three fetuses.

Co-localization of hormones

The co-localization patterns are shown in Table 2. Co-localization of two hormones occurred in most of the endocrine cell types in all three fetuses examined. Somatostatin-IR cells were the only cell type which was largely monohormonal. The frequency of cells expressing co-localization of hormones was highest in the ew 16 fetus; on the whole, co-localization decreased with age.

Table 2 Co-localization of hormones in three human fetuses, embryonic weeks 16, 18 and 22, expressed as a percentage of the total number of cells counted by double or triple staining, by using different filters. The figures within parentheses refer to the total number of cells in each staining combination, i.e. those cells immunostained solely by the first primary antibody, plus those stained solely by the second primary antibody, plus those showing double or triple staining.

Hormones	(Percentage of co-localized cells)					
	16 weeks		18 weeks		22 weeks	
	%	(n)	%	(n)	%	(n)
Insulin + glucagon	8.3	(124)	8.1	(605)	1.5	(543)
Insulin + somatostatin	1.1	(190)	2.5	(290)	2.1	(381)
Insulin + PP	6.2	(283)	1.5	(387)	0.6	(355)
Insulin + IAPP	18.8	(112)	16.4	(404)	49.5	(297)
Insulin + secretin	0		0		0	
Insulin + PYY	0		0		0	
Glucagon + somatostatin	3.8	(258)	1.6	(418)	1.1	(345)
Glucagon + PP	47.4	(233)	7.2	(290)	1.4	(179)
Glucagon + IAPP	7.2	(129)	72.5	(379)	35.6	(326)
Glucagon + secretin	67.3	(49)	100	(88)	100	(86)
Glucagon + PYY	1.6	(129)	0.8	(237)	0	
Glucagon + neurotensin	few		0		0	
Somatostatin + PP	3.6	(302)	3.4	(296)	2.1	(235)
Somatostatin + IAPP	1.1	(92)	4.3	(161)	2.6	(156)
Somatostatin + secretin	0		0		0	
Somatostatin + PYY	0		0		0	
PP + IAPP	NE		NE		NE	
PP + secretin	92.9	(56)	20.6	(34)	68.8	(26)
PP + PYY	41.9	(31)	14.7	(34)	5.3	(29)
Insulin + glucagon + somatostatin	0	(83)	0	(216)	0	(219)
Insulin + glucagon + PP	1.5	(133)	0.5	(185)	0	(194)
Insulin + somatostatin + PP	0.4	(210)	0	(270)	0	(236)
Glucagon + somatostatin + PP	1.0	(203)	0.6	(161)	0	(181)
IAPP + insulin + glucagon	1.8	(147)	0.2	(458)	0.8	(132)
IAPP + glucagon + somatostatin	0.9	(109)	0.5	(214)	0	(159)

NE, not estimated.

The various hormones in the endocrine pancreas occurred in different combinations, the most frequent being glucagon with secretin (Fig. 1, 1A and 1B), followed by PP with secretin (Fig. 1, 2A and 2B), except in the ew 18 fetus. Other hormones with a high percentage of co-localization were glucagon with IAPP (Fig. 1, 3A and 3D), insulin with IAPP (Fig. 1, 3B, 3D, 4A and 4B), and PP with PYY. The other co-localization combinations occurred only in small cell populations (Fig. 1, 5A-5D).

Triple co-localization occurred in just a few cell clumps (Fig. 1, 3A-D). Neurotensin was demonstrated in the ew 16 fetus and was found to be co-localized with glucagon (Fig. 1, 6A-6C) but not with insulin.

Discussion

Few ontogenic studies of endocrine fetal pancreas are performed on humans due to problems in obtaining

Figure 1 (1A and 1B) Human fetal pancreas (ew 18) double-stained for (1A) glucagon (FITC), and (1B) secretin (Texas Red). All the immunostained cells show co-localization of these two hormones. (2A and 2B) Human fetal pancreas (ew 16) double-stained for (2A) PP (FITC), and (2B) secretin (Texas Red). The same cells display both immunoreactivities. Bar (1A-2B) = 48 μ m. (3A-3D) Human fetal pancreas (ew 18) triple-stained for (3A) glucagon (FITC), (3B) insulin (Texas Red), and (3D) IAPP (AMCA). Glucagon and insulin appear in different cells with one exception, seen as yellow with the double-band filter (3C); this cell also contains IAPP, i.e. shows triple hormonal co-localization. IAPP occurs in all the glucagon cells and in a few insulin cells. Bar = 18 μ m. (4A-4C) Human fetal pancreas (ew 22) double-stained for (4A) insulin (FITC), and (4B) IAPP. All the cells immunostained for insulin show co-localization with IAPP, as illustrated by the yellow colour visualized with the double-band filter set (4C), but IAPP also occurs in non-insulin cells. Bar = 24 μ m. (5A-5D) Human fetal pancreas (ew 16) triple-stained for (5A) PP (FITC), (5B) somatostatin (Texas Red), and (5D) insulin (AMCA). There is co-localization of PP and somatostatin in a few cells only, illustrated as yellow with the double-band filter (5C). Some of the insulin cells also display PP immunoreactivity (arrows). Bar = 24 μ m. (6A-6C) Human fetal pancreas (ew 16) double-stained for (6A) neurotensin (FITC), and (6B) glucagon (Texas Red). In a few cells, distinct co-localization of these two polypeptides is visualized as yellow with the double-band filter set (6C). Bar = 48 μ m.

proper tissue material. For obvious reasons the material was also limited in number in the present study. Due to the small size of the pancreata no systemic randomization of the histological sections in each staining combination was performed, but the staining technique in itself, with single, double and triple stainings in serial sections implies a form of randomization. The main purpose of this study was to investigate the co-localization of the four major islet hormones, IAPP and other polypeptides not demonstrated in adult pancreas.

It is well known that the islet cell composition varies in different parts of the pancreas, where the processus uncinatus developing from the ventral anlage contains a large proportion of PP cells and few glucagon cells, while the converse is the case in the remaining pancreas. As only archival material has been available, we were not able to separate the processus uncinatus from the remaining gland. As pancreata from the two older fetuses contained few PP cells and a relatively large proportion of glucagon cells, our sections probably represented the body-tail region. In the pancreas from the youngest fetus the PP and glucagon cells appeared to be more or less equal in number, which indicates that these glandular sections also included the processus uncinatus.

The present study demonstrated the occurrence of secretin, neurotensin, PYY, GIP and IAPP in fetal pancreas, besides the four classical hormones. Secretin and neurotensin, not present in the adult pancreas, are here reported for the first time, whereas the presence of the other hormones confirms previous findings.

In the present study numerous secretin-IR cells were seen in fetal pancreas. Moreover, the high content of secretin-IR cells in the fetus suggests that secretin may play a functional role in fetal development. Secretin and glucagon, which belong to the same peptide family and display quite considerable amino acid sequence homology, were often co-localized. Our secretin antibodies were raised against pure porcine secretin (Prof. V Mutt, Karolinska Institute, Stockholm, Sweden) and our extensive control tests on the secretin antibodies revealed no signs of cross-reactivity with glucagon. An explanation for this co-localization pattern could be that they share a common molecular precursor. Our findings corroborate the genetic relationship of secretin with glucagon suggested by studies in transgenic mice expressing the simian virus 40 oncoprotein large T-antigen (Tag), controlled by the secretin gene, which developed glucagon-producing endocrine tumours in the colon (17). Besides glucagon, secretin was also co-localized with PP. These two polypeptides do not share any amino acid sequence homology and our control tests did not show any antibody cross-reactivity. Some of these PP cells most probably also contain glucagon. The higher frequency of PP/secretin cells compared with glucagon/secretin cells indicates that there are cells that contain PP but not glucagon. These findings point to the

possibility that secretin can also arise in the absence of a glucagon-secretin precursor.

In contrast to the numerous secretin cells, the other newly detected polypeptide, neurotensin, was found in small amounts, only in glucagon cells and only in early fetal age. The importance of neurotensin in the fetal pancreas is unclear, but a growth factor function has been suggested in the pancreas and intestinal tract (18).

The co-localization of PYY and glucagon agrees with findings by Ali-Rachedi *et al.* (12) in the human fetal pancreas and by Upchurch *et al.* (11) and Jackerott *et al.* (19) in mice. Co-localization of PYY and glucagon-related peptides has also been reported in adult human intestinal mucosa. However, in contrast to Ali-Rachedi *et al.* (12) we also found PP co-localized with PYY. Although PYY-IR cells occurred in small numbers, decreasing with fetal age, our finding suggests that PYY plays a role in the early development of the pancreas.

The finding of GIP-IR cells in fetal pancreas agrees with a previous report (20). Co-localization studies on GIP were not performed in the present investigation due to lack of material. However, GIP has been reported in glucagon cells in both adult and fetal human endocrine pancreas (2, 21). Although both these peptides belong to the same family, our control tests in adult pancreas suggest that there is no mutual cross-reactivity.

The finding of co-localization of insulin and IAPP agrees with previous reports (1, 22, 23). These results corroborate the attributed functional relationship between these two polypeptides in response to glucose stimulus, as reported by Rindi *et al.* (1). However, IAPP was found not only in insulin cells, but also in varying numbers of glucagon cells, which is consistent with the findings of Lukinius *et al.* (23) in their ew 14 fetus. This latter report also describes co-localization of IAPP with somatostatin, a finding which we were able to confirm. In agreement with other observations, insulin was also found together with glucagon immunoreactivity (8, 13, 14). Furthermore, these two immunoreactive polypeptides have been found in the same secretory granules (8, 13). *In vitro* studies have also suggested a functional relationship between insulin and glucagon, as glucagon seems to be a potent stimulus of insulin release (24, 25).

Only a small number of somatostatin cells displayed co-localization with either insulin or glucagon, but such co-localization is in agreement with other reports (8, 13), although we have shown a smaller number of somatostatin cells displaying insulin immunoreactivity than previously described; in a few cells, somatostatin and PP were co-localized in all three fetuses, a finding not reported earlier in humans. Our findings do suggest, however, that somatostatin cells are to a large extent monohormonal, possibly because somatostatin cells may derive from a different precursor cell lineage.

Knowledge of the physiological significance of polypeptides during fetal development is limited. Evidence that regulatory peptides in the animal fetus are released

has only been shown for gastrin, PP and GIP (26, 27). The differences in frequency of co-localization of the various polypeptides at different fetal ages suggest that they have a regulatory role also in human. It is possible that various peptides may be involved in fetal metabolism or may exert regulatory actions and/or interactions in the complex growth and differentiation of the developing pancreas (28). In this sense, somatostatin and PYY have been reported to have growth inhibitory effects (29, 30), while neurotensin seems to induce pancreatic growth (18). However, it remains to be proved if these growth effects are valid for the fetus as it has been shown that peptide actions in the animal fetus can differ from those in the adult (31).

The development of the fetal endocrine pancreas appears to undergo three phases: (i) from ew 10 to 15, when the endocrine cells start to form distinct islets, (ii) from ew 16 to 20, when the islets acquire their characteristic vascular system, and (iii) from ew 21 to 26 when the islet innervation is developed (32). Of the present cases, two are included in the mid phase and one in the last. A recent study (33) showed the highest proliferation rate of the endocrine pancreas to occur at ew 10 and during ew 14 to 16. This is coincident with the highest co-localization of hormones found in the present study.

The reason for the varying co-localization pattern of polypeptides in the fetal pancreatic endocrine cells is also still unclear. Based on co-localization studies in an insulin-Tag transgenic mouse model, Alpert *et al.* (34) proposed the hypothesis that the four major pancreatic endocrine cells must derive from a progenitor stem cell line displaying glucagon immunoreactivity. In agreement with previous ultrastructural studies, the present findings showed that glucagon was co-localized with all the other hormones examined, which supports, but does not prove, this hypothesis (8, 13, 23). Recent studies on transcriptional regulation of rat pancreatic endocrine cells suggest, however, that insulin cells do not derive from a glucagon progenitor cell (35–38).

In conclusion, our study has shown that the human fetal pancreas expresses secretin and neurotensin, as well as the previously reported polypeptides PYY, GIP and IAPP, besides the four classical hormones. A complex co-localization pattern of polypeptide hormones was found, except for somatostatin, which was mostly monohormonal. Co-localization of three hormones was rare. Two different co-localization patterns could be distinguished: (i) insulin, IAPP and glucagon, and (ii) glucagon, secretin, PP and PYY.

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