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Expression of chromogranin A and B and secretoneurin immunoreactivity in neoplastic and nonneoplastic pancreatic alpha cells

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Abstract In the endocrine pancreas, chromogranins A and B as well as secretoneurin (a biologically active peptide processed endoproteolytically from secretogranin II) are most intensely expressed in alpha (glucagon) cells. We examined whether the functional status of neoplastic and nonneoplastic human alpha cells is reflected in the expression patterns of chromogranins/secretogranins. Neoplastic alpha cells were analysed immunocytochemically in six functioning glucagonomas and 37 nonfunctioning neuroendocrine tumours (29 with alpha cells) for their immunoreactivity to chromogranin A and B, as well as secretoneurin. There was no difference in the staining intensity for either peptide between glucagonomas and nonfunctioning, alpha cell containing tumours. Nonneoplastic alpha cells from patients with a functioning glucagonoma showed a decreased glucagon immunoreactivity, whereas the expression of chromogranin A (but not chromogranin B and secretoneurin) was as intense as in alpha cells not associated with glucagonoma syndrome. These results suggest that the expression of chromogranins/secretogranins in neoplastic alpha cells of the pancreas may be independently regulated from the cells' functional status. In nonneoplastic alpha cells there seems to be an association between glucagon production and chromogranin B and secretoneurin expression.

Key words Pancreatic neuroendocrine tumours
Glucagonomas · Chromogranin · Secretoneurin
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Introduction

Chromogranins A and B and secretogranin (chromogranin C; for nomenclature see [8]) are acidic proteins widely distributed in a variety of neuroendocrine and neuronal tissues of vertebrate species including man. The function of these proteins is not yet known but they are thought to be involved in stabilization of the neuroendocrine granule structure and packaging of hormones. In addition, they may serve as prophormones of more active proteolytic cleavage products [10, 15, 16, 34].

In the normal human pancreas chromogranins A and B and secretogranin II have been demonstrated in the glucagon producing alpha cells [3, 13, 20, 22, 23, 26, 27, 31, 33]. On the light microscopical level the insulin producing beta cells showed also immunoreactivity for chromogranin A [13, 20, 22, 27, 33], but Varndell et al. were unable to confirm these findings at the ultrastructural level. Conflicting results were also reported for somatostatin producing delta cells and pancreatic polypeptide (PP) cells [13, 22, 23, 27, 31, 33].

Chromogranin A has been found in the majority of hormonally functioning as well as hormonally nonfunctioning neuroendocrine tumours of the pancreas [3, 4, 5, 7, 14, 18, 20, 21, 26, 32, 33]. Chromogranin B and secretogranin II have been immunolocalized in insulinomas [33]. In view of the strong expression of chromogranin A and, to a lesser extent also chromogranin B and secretogranin II, in alpha cells, the present study investigates the status of these three proteins in both functioning glucagonomas (glucagonomas associated with a glucagonoma syndrome [14, 19, 24] and nonfunctioning neuroendocrine pancreatic tumours with glucagon cells. Concomitantly, the immunoreactivity of chromogranins/secretogranins was also assessed in the alpha cells of islets outside the functioning glucagonomas and compared to that of alpha cells of islets adjacent to nonfunctioning tumours with glucagon production.

Materials and methods

Formalin-fixed and paraffin embedded tissues from six glucagonomas of the pancreas associated with the glucagonoma syndrome and 29 nonfunctioning pancreatic neuroendocrine tumours with immunocytochemically detectable alpha cells from 20 patients [including 13 pancreatic tumours from five patients with a multiple endocrine neoplasia (MEN)-I syndrome] and eight nonfunctioning tumours from eight patients without any glucagon cells were retrieved from the normal and consultation files of the Department of Pathology, University of Münster, Germany, the Department of Pathology, Free University of Brussels, Belgium, and the Department of Pathology, University of Zurich, Switzerland. In each case at least one tumour block and one block with normal pancreatic tissue were available for immunocytochemical studies. Serial sections were cut from all blocks at a thickness of 4 μm and mounted on protein coated glass slides.

The monoclonal antibody against chromogranin A was purchased from BioGenex (San Ramon, Calif., USA). The generation of the chromogranin B antibody against a synthetic peptide present in the amino acid sequence of human chromogranin B (amino acids 306–326; designated as DK-21) has been described previously [27]. As to the secretoneurin antibody, a peptide corresponding to rat secretogranin II amino acids 154–186 [12] was synthesized by standard solid phase to t-BOC chemistry and purified by reversed phase HPLC. The synthetic peptide (designated as secretoneurin [17] was coupled via an additional N-terminal cysteine to maleimide activated keyhole limpet hemocyanin. Subsequently rabbits were immunized following a standard protocol [9]. The antiserum was characterized in detail previously [17]. This anti-rat secretoneurin antiserum cross-reacts with human secretoneurin as there is only one amino acid substitution between these species.

Additionally the tissues were immunostained with antibodies against GLP-1 and GLP-2 (generous gift of Professor Julia M. Polak, London, UK [14], glucagon, insulin, somatostatin, and PP. The latter four antibodies were purchased from Dakopatts (Copenhagen, Denmark).

For immunohistochemical staining following dewaxing of the sections in xylene and rehydration in a series of alcohols, the primary antibodies (Ab) were applied overnight at 4° C in a humidified chamber (dilutions in bovine serum albumin: chromogranin A Ab 1:800, chromogranin B and secretoneurin Ab 1:2000; GLP-1 and GLP-2 Ab 1:1000, glucagon Ab 1:2500, insulin Ab 1:5000, somatostatin Ab 1:5000, PP Ab 1:10000), followed by a goat-anti-mouse or goat-anti-rabbit bridging Ab (1:30 in phosphate buffered saline (PBS); 30 min at room temperature; Dako, Copenhagen, Denmark) and a polyclonal mouse- or rabbit-alkaline phosphatase antialkaline phosphatase (APAAP)-complex (1:100 in PBS; 60 min at room temperature; Dianova, Hamburg, Germany). The bridging Ab and the APAAP complex were applied on a semi-automatic immunostaining device ("Omnibus"; Quartett, Berlin, Germany). Subsequently the enzyme reaction was developed for 25 min at room temperature in a freshly prepared newfuchsin solution containing naphthol-biphosphate. Finally the sections were counterstained with haematoxylin and mounted in Kayser's glycerine gelatine. Omission of primary Ab was used as negative, normal adrenal medulla as positive controls for chromogranins and secretoneurin.

Results

All six glucagonomas stained for glucagon and GLP-2, whereas only two of six glucagonomas were positive for GLP-1. Four of the glucagonomas contained also PP immunoreactive cells. Among the 37 nonfunctioning neuroendocrine tumours, 29 showed glucagon and with two exceptions GLP-2 cells. GLP-1 cells were only seen in 6 of these tumours. Twenty-one of the 29 tumours which came from ten patients (five with the MEN-1 syndrome) were rich in glucagon cells (>50% of tumour cells), while in the remaining eight tumours less than 30% of the tumour cells stained for glucagon. Thirty of the 37 nonfunctioning tumours stained for PP. The tumours from the five MEN-1 patients and four other patients also contained some somatostatin and insulin cells.

Chromogranin A and B was present in all tumours, while secretoneurin was lacking in three. Serial sections revealed that chromogranin A labelled most glucagon positive cells of functioning glucagonomas as well as nonfunctioning tumours (Fig. 1), while chromogranin B stained glucagon positive as well as most glucagon negative tumour cells. Secretoneurin showed an inconsistent pattern with regard to glucagon positive cells. Functioning glucagonomas and nonfunctioning tumours did not differ in chromogranin/secretoneurin immunoreactivity patterns. No correlation was found between GLP-I/GLP-

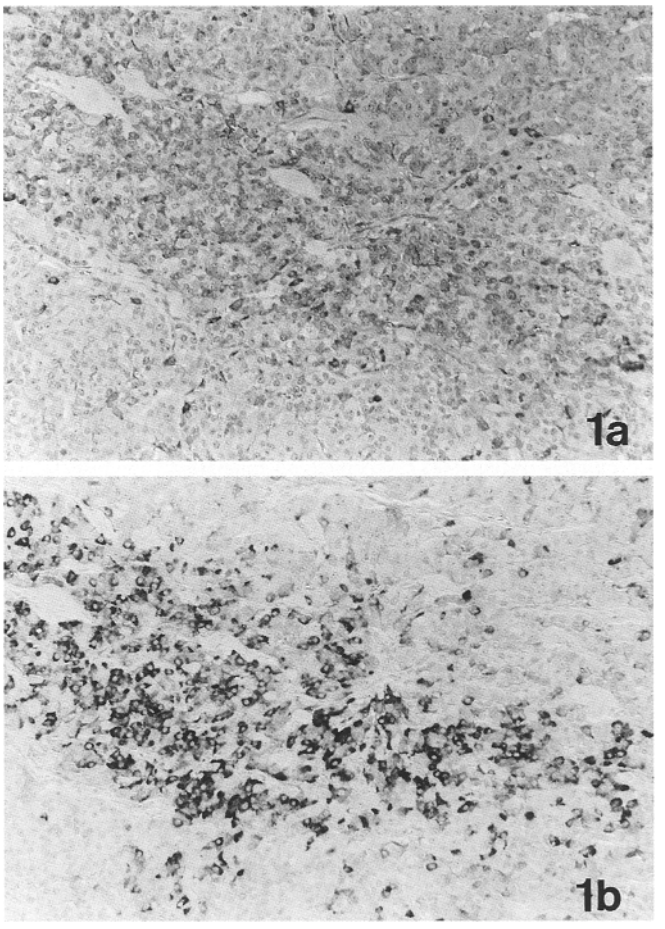


Fig. 1a, b Functional glucagonoma with (**a**) focal glucagon and (**b**) chromogranin A immunoreactivity on serial sections. ×100

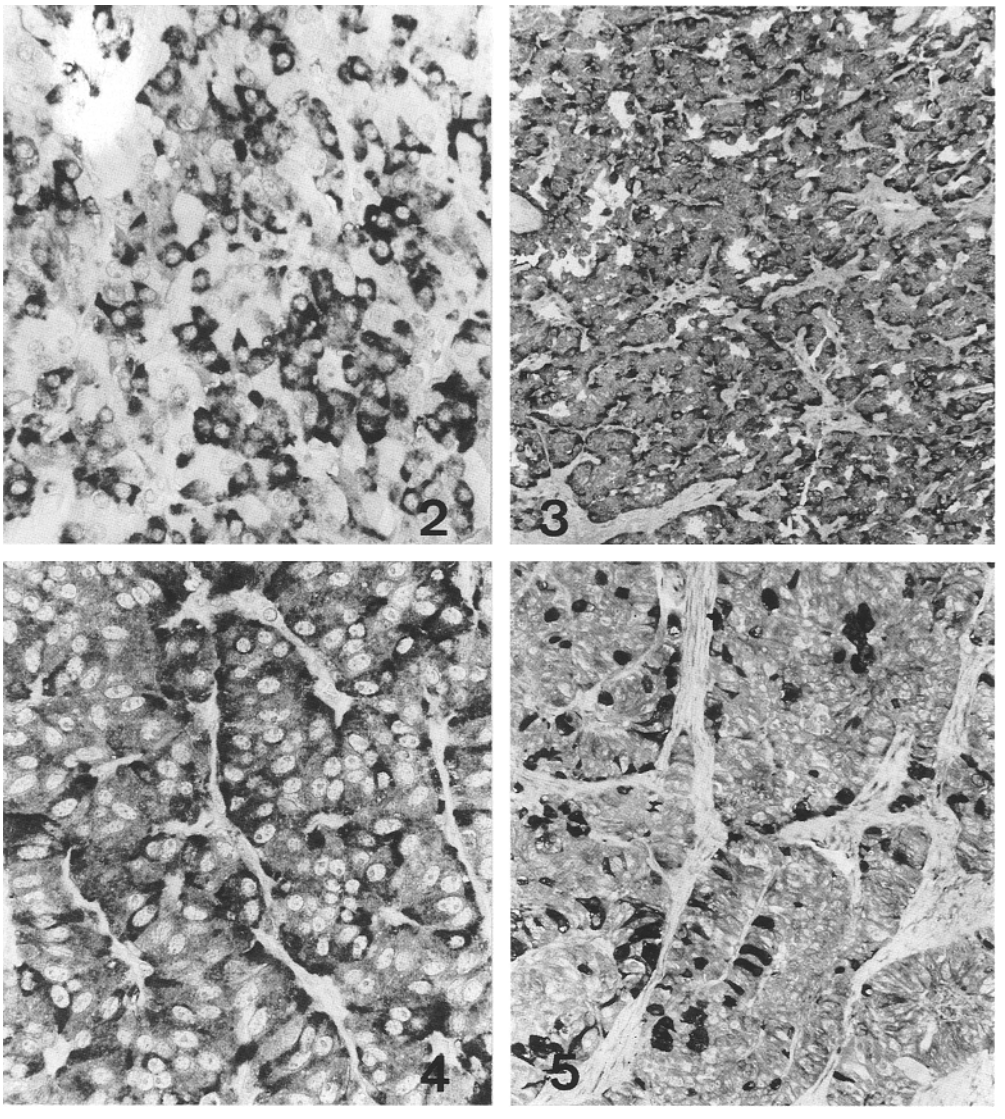
Fig. 6a–c Serial sections from a functioning glucagonoma showing (**a**) uneven chromogranin A immunoreactivity, (**b**) uniform chromogranin B immunoreactivity and (**c**) faint secretoneurin immunoreactivity. ×180

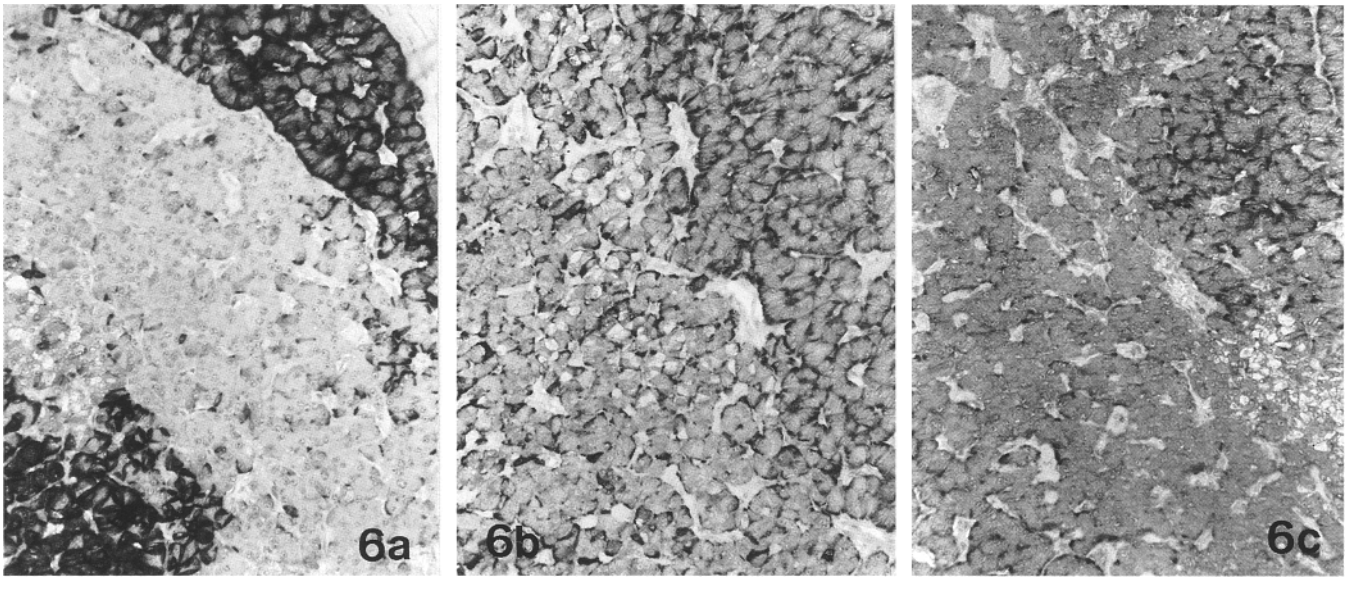
Fig. 2 Functioning glucagonoma showing a solid pattern and scattered chromogranin A immunoreactive cells. ×250

Fig. 3 Chromogranin B immunoreactivity in a functioning glucagonoma with trabecular pattern. ×100

Fig. 4 Chromogranin B immunoreactivity in a nonfunctioning endocrine pancreatic tumour. Most tumour cells are stained. ×250

Fig. 5 Functioning glucagonoma with trabecular pattern; scattered secretoneurin immunoreactive cells. ×125





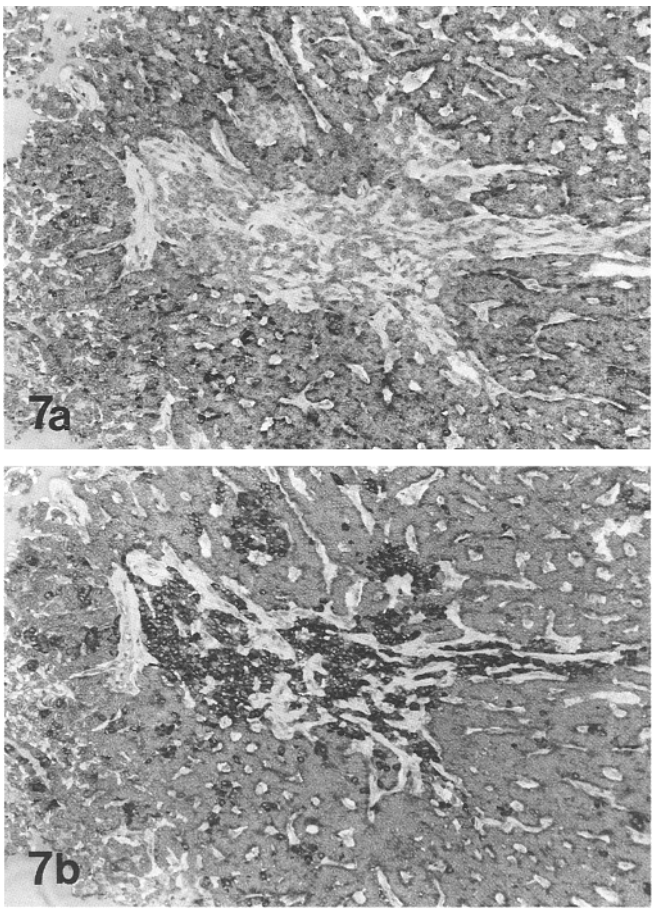


Fig. 7a, b Nonfunctioning pancreatic endocrine tumour showing mirror immunostaining for (**a**) chromogranin A and (**b**) secretoneurin. ×100

2 expression and the chromogranin/secretoneurin staining patterns.

Chromogranin A was predominantly localized in areas with a trabecular pattern and less frequent in solid tumour areas (Fig. 2). Moreover, its staining intensity varied from area to area within the same tumour (Figs. 6a, 7a). In contrast chromogranin B positivity was more evenly distributed within the tumours and found with all growth patterns (Figs. 3, 4, 6b). Secretoneurin, was usually less intense expressed that the other chromogranins (Figs. 5 and 6c). In some tumours associated with the MEN-I syndrome small tumour foci were strongly positive while the surrounding negative tissue stained for chromogranin A (Fig. 7).

In most islets of the nontumour pancreatic tissue adjacent to functioning glucagonomas the glucagon immunoreactivity was found to be distinctly decreased when compared to islets from pancreases containing a nonfunctioning tumour (Fig. 8a, c). A similar pattern was recognized for the chromogranin B and secretoneurin staining intensity (data not shown). The chromogranin A immunoreactivity, however, remained unchanged, regardless from where the alpha cells came from (Fig. 8b, d), Beta cells, in contrast to alpha cells, showed only a faint chromogranin A immunostaining.

Discussion

This study shows that the staining patterns of chromogranin A and B and secretoneurin in neoplastic alpha (glucagon) cells associated with a glucagonoma syndrome do not differ from those of neoplastic alpha cells in nonfunctioning endocrine pancreatic tumours. The functional status of neoplastic alpha cells is therefore not associated with a concomitant change in the expression of the chromogranins/secretogranins in these cells. In nonneoplastic alpha cells of islets outside functioning glucagonomas chromogranin B and secretoneurin, but not chromogranin A expression, seems to reflect the hormonal activity of these cells.

Chromogranins/secretogranins are most likely precursors of peptides the function of which is yet not fully understood. Pancreastatin, a peptide derived from chromogranin A, has been shown to inhibit the release of insulin [30]. It has been reported that the release of catecholamines from the adrenal medulla is suppressed in vitro by another peptide derived from chromogranin A, chromostatin [11]. A different distribution of pancreastatin and chromostatin has recently been demonstrated immunocytochemically in normal human pancreatic islets [6]. Pancreastatin was mainly localized in alpha cells, while chromostatin was exclusively found in beta cells. In an in vitro study secretoneurin led in rat striatal slices to a specific dopamine release [25] which is the first biological effect demonstrated for this peptide. The finding that histamine specifically induces secretogranin II mRNA in cultured bovine chromaffin cells [2] together with the expression of secretoneurin in capsaicin-sensitive sensory afferent neurons may indicate a possible role of this peptide in inflammatory conditions.

Chromogranin A was found in all functioning glucagonomas and nonfunctioning neuroendocrine tumours of our series, thus confirming the results from earlier studies [4, 7, 14, 18, 20]. Neoplastic glucagon positive cells were usually stained by chromogranin A. However, comparison of the intensity of the immunoreactivity for chromogranin A between functioning and nonfunctioning glucagon positive tumours revealed no differences. This was also true for chromogranin B and secretoneurin. Production and secretion of chromogranins/secretogranins seem therefore to be unrelated to glucagon biosynthesis and release of neoplastic alpha cells.

In this tumour series, chromogranin B was found to be the most reliable marker since it was not only identified in all tumours (regardless whether they contained alpha cells or not) but labelled also almost all tumour cells. Secretoneurin, which was not detected in all tumours, showed a particularly patchy distribution often alternating with chromogranin A positivity. This mirror image distribution has already been described in gangliocytomas and extra-adrenal paragangliomas [28, 29].

The observed marked decrease in the glucagon immunoreactivity of the pancreatic islets from patients with functioning glucagonomas has already been observed by Bani et al. [1] and suggests an inhibition of glucagon

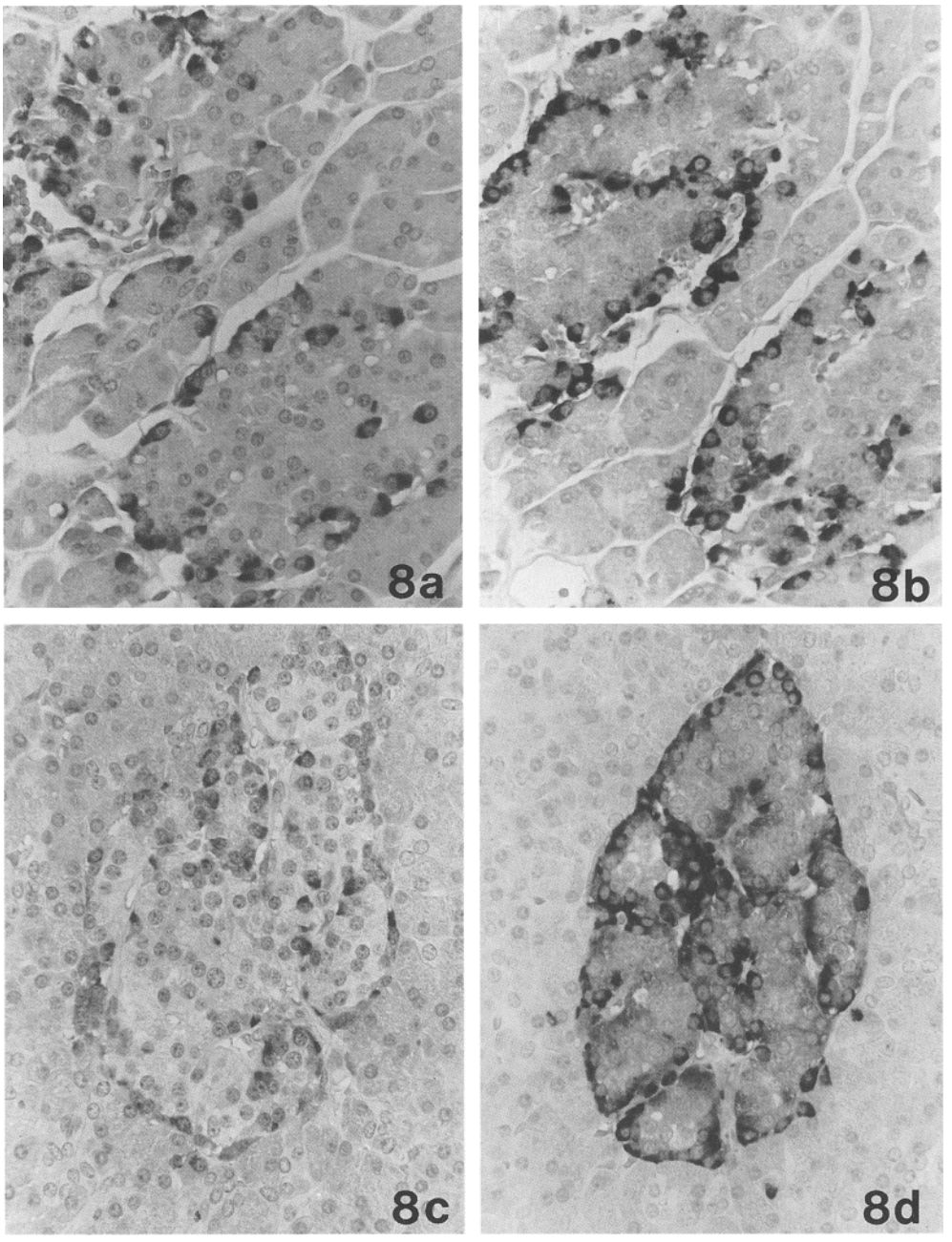


Fig. 8a–d Glucagon immunostaining in pancreatic islets (**a**, **b**) not associated and (**c**, **d**) associated with a functioning glucagonoma. Distinct immunostaining for glucagon as well as chromogranin A in **a** and **b**, but decreased glucagon immunoreactivity obtained with well preserved chromogranin A labelling in **c** and **d**. ×250

production and secretion in the nonneoplastic alpha cells by inappropriate glucagon release from the tumour cells. As both the distribution and staining intensity of chromogranin A appeared to be unchanged in the degranulated and functionally suppressed alpha cells, it is concluded that the production of glucagon and chromogranin A is independently regulated in nonneoplastic alpha cells. The same does obviously not apply to chromogranin B and secretoneurin because their expression was similarly diminished as that of glucagon in functionally inactive alpha cells.

In summary, we have demonstrated that the expression patterns of chromogranins/secretogranins do not help to distinguish between functioning and nonfunctioning glucagon containing tumours of the pancreas. The functional status of nonneoplastic alpha cells may, however, be reflected by the expression of chromogranin B and secretoneurin but not chromogranin A.

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Referenes

1. Bani D, Biliotti G, Bani Sacchi T (1991) Morphological changes in the human endocrine pancreas induced by chronic excess of endogenous glucagon. Virchows Arch [B] 60: 199–206
2. Bauer JW, Kirchmair R, Egger C, Fischer-Colbrie R (1993) Histamine induces a gene-specific synthesis regulation of secretogranin II but not of chromogranin A and B in chromaffin cells in a calcium dependent manner. J Biol Chem 268:1586–1589
3. Bishop AE, Bretherton-Watt D, Hamid QA, Fahey M, Sheperd N, Valentino K, Tatemoto K, Ghatei MA, Bloom SR, Polak JM (1988) The occurrence of pancreastatin in tumours of the diffuse endocrine system. Mol Cell Probes 2:225–235
4. Bordi C, Pilato FP, D'Adda T (1988) Comparative study of seven neuroendocrine markers in pancreatic endocrine tumours. Virchows Arch [A] 413:387–398
5. Buffa R, Rindi G, Sessa F, Fini A, Capella C, Jahn R, Navone F, Camilli P de, Solcia E (1988) Synaptophysin immunoreactivity and small clear cell vesicles in neuroendocrine cells and related tumours. Mol Cell Probes 1:367–381
6. Cetin Y, Aunis D, Bader M-F, Galindo E, Jörns A, Bargsten G, Grube D (1993) Chromostatin, a chromogranin A-derived bioactive peptide, is present in human pancreatic insulin (β) cells. Proc Natl Acad Sci USA 90:2360–2364
7. Chejfec G, Falkmer S, Grimelius L, Jacobsson B, Rodensjo M, Wiedenmann B, Franke WW, Lee I, Gould VE (1987) Synaptophysin. A new marker for pancreatic endocrine tumors. Am J Surg Pathol 11:241–247
8. Eiden LE, Huttner WB, Mallet J, O'Connor DT, Winkler H, Zanini A (1987) A nomenclature proposal for the chromogranin/secretogranin proteins. Neuroscience 21:1019–1021
9. Fischer-Colbrie R, Schober M (1987) Isolation and characterization of chromogranins A, B and C from bovine chromaffin granules and a rat pheochromocytoma. J Neurochem 48:262–270
10. Fischer-Colbrie R, Hagn C, Schober M (1987) Chromogranins A, B, and C: widespread constituents of secretory vesicles. Ann N Y Acad Sci 493:120–134
11. Galindo E, Rill A, Bader M-F, Aunis D (1991) Chromostatin, a 20 amino acid peptide derived from chromogranin A, inhibits chromaffin cell secretion. Proc Natl Acad Sci USA 88:1426–1430
12. Gerdes H-H, Phillips E, Huttner WB (1988) The primary structure of rat secretogranin II deduced from a cDNA sequence. Nucleic Acids Res 16:11811
13. Grube D, Aunis D, Bader F, Cetin Y, Jörns A, Yoshie S (1986) Chromogranin A (CGA) in the gastro-entero-pancreatic (GEP) endocrine system. I. CGA in the mammalian endocrine pancreas. Histochemistry 85:441–452
14. Hamid QA, Bishop AE, Sikri KL, Varndell IM, Bloom SR, Polak JM (1986) Immunocytochemical characterization of 10 pancreatic tumours, associated with the glucagonoma syndrome, using antibodies to seperate regions of the pro-glucagon molecule and other neuroendocrine markers. Histopathology 10:119–133
15. Helle KB, Reed RK, Ehrhard M, Aunis D, Hogue-Angeletti R (1990) Chromogranin A: osmotically active fragments and their susceptibility to proteolysis during lysis of the bovine chromaffin granules. Acta Physiol Scand 138:565–574
16. Huttner WB, Gerdes H-H, Rosa P (1991) The granin (chromogranin/secretogranin) family. Trends Biochem Sci 16:37–30
17. Krichmair R, Hogue-Angeletti R, Guitarrez J, Fischer-Colbrie R, Winkler H (1993) Secretoneurin – a neuropeptide generated in brain, adrenal medulla and other endocrine tissues by proteolytic processing of secretogranin II (chromogranin C). Neuroscience 53:359–365
18. Klöppel G, In't Veld P (1990) Neural and endocrine markers as diagnostic tools in pancreatic and gastrointestinal endocrine tumors. Acta Histochem Suppl (Jena) 28:93–98
19. Klöppel G, Höfler H, Heitz P (1993) Pancreatic endocrine tumours in man. In: Polak JM (ed) Diagnostic histopathology of neuroendocrine tumours. Churchill Livingstone, Edinburgh, pp 91–121
20. Lloyd RV, Mervak T, Schmidt K, Warner TF, Wilson BS (1984) Immunohistochemical detection of chromogranin and neuron-specific enolase in pancreatic endocrine neoplasms. Am J Surg Pathol 8:607–614
21. Lloyd RV, Iacangelo A, Eiden LE, Cano M, Jin L, Grimes M (1989) Chromogranin A and B messenger ribonucleic acids in pituitary and other normal and neoplastic human endocrine tissues. Lab Invest 60:548–556
22. O'Connor DT, Burton D, Deftos LJ (1983) Immunoreactive human chromogranin A in diverse polypeptide hormone producing human tumors and normal endocrine tissues. J Clin Endocrinol Metab 57:1084–1086
23. Rindi G, Buffa R, Sessa F, Tortora O, Solcia E (1986) Chromogranin A, B and C immunoreactivities of mammalian endocrine cells. Distribution, distinction from costored hormones/prohormones and relationship with the argyrophil component of secretory granules. Histochemistry 85:19–28
24. Ruttmann E, Klöppel G, Bommer G, Kiehn M, Heitz PU (1980) Pancreatic glucagonoma with and without syndrome. Immunocytochemical study of five tumour cases and review of the literature. Virchows Arch [A] 388:51–67
25. Saria A, Troger J, Kirchmair R, Fischer-Colbrie R, Hogue-Angeletti R, Winkler H (1993) Secretoneurin releases of dopamine from rat striatal slices: a biological effect of a peptide derived from secretogranin II (chromogranin C). Neuroscience 54:1–4
26. Schmid KW, Newman GR, Fischer-Colbrie R, Hagn C, Jasani B, Mikus G, Winkler H (1987) Chromogranin A und B und Secretogranin II im endocrinen Pankreas. Verg Dtsch Ges Pathol 71:311–313
27. Schmid KW, Weiler R, Xu RW, Hogue-Angeletti R, Fischer-Colbrie R, Winkler H (1989) An immunological study on chromogranin A and B in human endocrine and nervous tissue. Histochem J 21:365–373
28. Schmid KW, Dockhorn-Dworniczak B, Fahrenkamp A, Kirchmair R, Tötsch M, Fischer-Colbrie R, Böcker W, Winkler H (1993) Chromogranin A, secretogranin II and vasoactive intestinal peptide (VIP) in phaeochromocytomas and ganglioneuromas. Histopathology 22:527–533
29. Schmid KW, Schröder S, Dockhorn-Dworniczak B, Kirchmair R, Tötsch M, Böcker W, Fischer-Colbrie R (1994) Immunohistochemical demonstration of chromogranin A, chromogranin B, and secretogranin II in extra-adrenal paragangliomas. Mod Pathol 7:347–353
30. Tatemoto K, Efendic S, Mutt V, Makk G, Feistner GJ (1986) Pancreastatin, a novel pancreatic peptide that inhibits insulin secretion. Nature 324:476–478
31. Varndell IM, Lloyd RV, Wilson BS, Polak JM (1985) Ultrastructural localization of chromogranin: a potential marker for the electron microscopical recognition of endocrine cell secretory granules. Histochem J 17:981–992
32. Weiler R, Fischer-Colbrie R, Schmid KW, Feichtinger H, Bussolati G, Grimelius L, Krisch K, Kerl H, O'Connor D, Winkler H (1988) Immunological studies on the occurrence and properties of chromogranin A and B and secretogranin II in endocrine tumors. Am J Surg Pathol 12:877–884
33. Wiedenmann B, Waldherr R, Buhr H, Hille A, Rosa P, Huttner WB (1988) Identification of gastroenteropancreatic neuroendocrine cells in normal and neoplastic human tissue with antibodies against synaptophysin, chromogranin A, secretorgranin I (chromogranin B), and secretogranin II. Gastroenterology 95:1364–1374
34. Winkler H, Fischer-Colbrie R (1992) The chromogranins A and B: the first 25 years and future perspectives. Neuroscience 49:497–528