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Cystatin C in the human pancreas and gut: an immunohistochemical study of normal and neoplastic tissues

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Summary. The occurrence of immunoreactive cystatin C (CC) in normal and neoplastic cells of the human pancreas and gut was investigated using an indirect streptavidin-biotin method on formaldehyde-fixed and paraffin-embedded tissues. Virtually all pancreatic islet cells and many neuroendocrine cells throughout the gastrointestinal tract showed strong CC immunoreactivity and a granular cytoplasmic staining pattern. All 14 endocrine pancreatic tumours (insulinomas, glucagonomas, gastrinomas and non-producing tumours), as well as 16 of 17 gut carcinoid tumours, were also strongly CC immunoreactive. In addition, non-endocrine epithelial cells of pancreatic ducts and the gastrointestinal mucosa and 20 of the 24 adenocarcinomas from these sites showed weak CC immunoreactivity. Thus, CC cannot be used as a reliable immunohistochemical marker for endocrine gastro-entero-pancreatic tumours despite the fact that the protein is strongly expressed in a majority of such tumours.

Key words: Cystatin – Immunohistochemistry – Pancreas – Gut – Endocrine tumours – Adenocarcinomas

Introduction

Cystatin C (CC), previously called gamma-trace, is a protein composed of 120 amino acid residues (molecular weight 13359) and is free from carbohydrates (Grubb and Löfberg 1982; Brzin et al. 1984). CC is an important inhibitor of papain-like cysteine proteinases such as human cathepsin B, H and L (Turk and Bode 1991). The cDNA sequence is known, and the 7.3-kb CC gene has been localized to chromosome 20 (Abrahamson et al. 1989, 1990). A mutated form of CC plays a key role in the development of brain haemorrhage in patients suffering from hereditary CC amyloid angiopathy, in which the protein is deposited as amyloid in cerebral arteries (Abrahamson et al. 1990).

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The protein is known to be secreted by the choroid plexus and by alveolar macrophages in the lung (Cole et al. 1989; Warfel et al. 1987). Northern blot analysis has shown the presence of CC mRNA in many other organs, including the pancreas, stomach and gut, indicating that CC is synthesized also at these sites (Abrahamson et al. 1990). Immunohistochemical investigations have demonstrated presence of the protein in various types of neuroendocrine cells, i.e. A cells of the pancreatic islets (Löfberg et al. 1981 b), C cells of the thyroid gland (Löfberg et al. 1983), medullary cells of the adrenal glands (Löfberg et al. 1982), cortical neurons (Löfberg et al. 1981 a) and endocrine cells of the anterior lobe of the pituitary gland (Grubb and Löfberg 1982; Möller et al. 1985; Olafsson et al. 1988). A glucagonoma of the pancreas (Löfberg et al. 1981 b), a calcitonin-producing medullary thyroid carcinoma (Löfberg et al. 1983), a pheochromocytoma (Löfberg et al. 1982) and pituitary adenomas (Löfberg et al. 1984; Olafsson et al. 1988) have also been reported to contain immunoreactive CC.

We have recently shown synthesis of transthyretin (TTR) in the endocrine pancreas and in endocrine pancreatic tumours and gut carcinoids (Jacobsson 1989; Jacobsson et al. 1989a, 1990). The presence of immunoreactive CC in glucagon A cells of the pancreatic islets and in a glucagonoma (Löfberg et al. 1981 b) may suggest that CC, like TTR, is synthesized in the endocrine pancreas and that the protein may be used as an immunohistochemical marker for endocrine cell differentiation in the pancreas and gut. We thus performed a detailed survey on the cellular distribution of immunoreactive CC in normal and neoplastic human tissues from these sites and compared our data on CC with those for TTR and other known endocrine cell markers.

Materials and methods

Endocrine pancreatic tumours from 14 patients and gut carcinoid tumours from 17 patients were studied. Malignancy of the pancre-

atic tumours was determined on the basis of the presence of metastasis or of local spread beyond the pancreas at the time of surgery or autopsy, or on clinical follow-up. Four primary adenocarcinomas of the pancreas, three of the stomach, 11 of the colon and five of the rectum were also investigated. In addition, surgical specimens from the pancreas, antrum, duodenum, ileum and colon/rectum showing normal morphology were included in the study. All specimens had been fixed in 4% buffered formaldehyde overnight before routine processing to paraffin. From each tumour, one to three blocks were selected for subsequent immunostainings.

Sections were stained with haematoxylin-eosin, according to the Grimelius modified silver nitrate method and to the Masson technique as modified by Singh (Grimelius and Wilander 1980). A streptavidin-biotin method was used to demonstrate the presence of CC, TTR, chromogranin A (CGA), neuron-specific enolase (NSE) and different regulatory peptides (Jacobsson et al. 1989b). Briefly, the sections were pretreated with 3% hydrogen peroxide for 5 min, followed by 0.1% pronase for 10 min. After blocking in 1% bovine albumin, the sections were incubated with the primary antibody for 2 h (CC, TTR, CGA and NSE) or overnight (regulatory peptides). The biotinylated secondary reagents (DACO, Santa Barbara, Calif.; dilution 1:500) were applied for 30 min followed by a peroxidase-streptavidin conjugate (DACO; dilution 1:500) for 30 min and diaminobenzidine (DAB) as the chromogen. Tris-HCl buffer 50 mM/litre, pH 7.6, with 0.15 M/litre NaCl (TBS) was used for diluting the reagents and for all washing steps.

Immunostainings performed on consecutive sections were used to establish the coexistence of CC with endocrine tumour markers and regulatory peptides. A restaining technique (Jacobsson et al. 1989a) was used to compare the distribution of argyrophilic cells with that of CC immunoreactive cells. After the initial silver staining, representative areas were photographed, and the sections were decolorized by oxidation with 3% hydrogen peroxide for 10 min and restained according to the streptavidin-biotin method.

The rabbit antiserum to CC (code no. A 451, dilution 1:400) was obtained from DACO. A mouse monoclonal antibody against TTR (clone 25) (Collins et al. 1986) was received from Professor V. Peter Collins, Institution for Pathology, Sahlgrenska Hospital, Gothenburg, Sweden, and the polyclonal rabbit antiserum to TTR (code no. A 002, dilution 1:500) was obtained from DACO.

The rabbit antiserum to CGA (dilution 1:250) was a gift from Professor D.T. O'Connor, VAMC, San Diego, CA. A rabbit antiserum to NSE (code no. 073, 1:500) and a guinea pig antiserum to insulin (code no. 083, 1:1000) were from DACO. Rabbit antisera to pancreatic glucagon (code no. 781003, 1:1000), glucagon and glicentin (code no. 781101, 1:1000), pancreatic polypeptide (PP; code no. 782308, 1:600) and somatostatin (code no. 1801, 1:1000) were from MILAB, Malmö, Sweden.

The primary antibodies were tested on tissues known to contain cells positive for the protein or the regulatory peptide in question. Sections from such normal tissues were also included in each run of immunohistochemical staining to provide an internal standard in the semiquantitative grading of the results from different experiments. Two control procedures were used: the substitution of buffer or non-immune serum for the primary antibodies, and the absorption of primary antibodies with 100–150 µg of the corresponding chemical compound per millilitre of diluted antibody solution at 4° C for 24 h before immunostaining.

A recombinant human CC protein was provided by Professor Anders Grubb, Institute for Clinical Chemistry, Lund Hospital, Lund, Sweden (Dalböge et al. 1989), and the TTR was prepared from human serum at our department as previously described (Jacobsson et al. 1989b). CGA was a gift from Professor Gudmar Lundquist, Institute for Clinical Chemistry, Akademiska Hospital, Uppsala, Sweden (Eriksson et al. 1990). Glucagon was received from Professor Victor Mutt, Karolinska Hospital, Sweden, and glicentin and insulin from Novo Industria, Denmark. Pre-absorbed antisera to PP and somatostatin were obtained from MILAB.

Results

In accordance with a previous report (Löfberg et al. 1981b), strong CC immunoreactivity was found in the argyrophilic glucagon cells of normal pancreatic islets. Immunoreactive CC was, however, also observed in the majority of non-argyrophilic islet cells (mainly insulin-producing cells), although the staining intensity was slightly less than that in the glucagon-producing cells (Fig. 1A). In addition, strongly CC-immunoreactive endocrine cells of the pancreatic ducts were demonstrated (Fig. 1B). The staining pattern for CC in the individual endocrine cells was granular. The cellular distribution and the cytoplasmic staining pattern of immunoreactive CC were similar to those of TTR and CGA. In contrast, NSE showed diffuse cytoplasmic staining in these cells.

The endocrine pancreatic tumours, their staining characteristics and the clinical syndromes which were associated with these individual tumours are shown in Table 1. All endocrine pancreatic tumours were found to contain immunoreactive CC and the staining intensity was strong in most cases. The two benign insulinomas

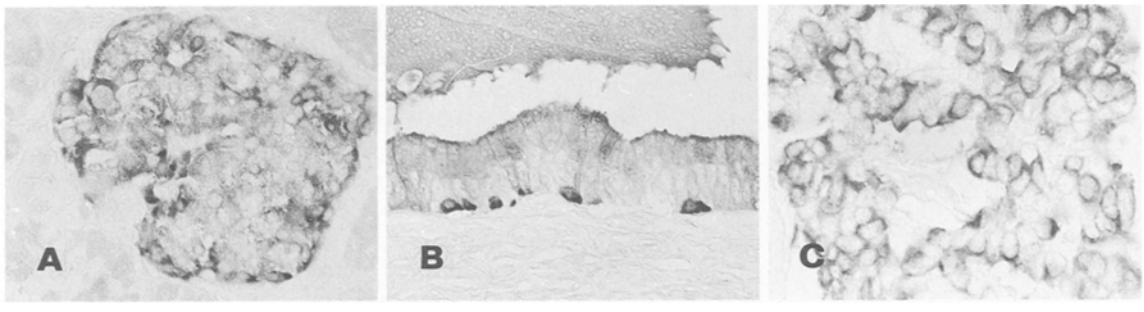

Fig. 1. A Pancreatic islet demonstrating strong cystatin C immunoreactivity (CC-IR) in virtually all islet cells. The glucagon cells in the peripheral parts of the islet show the most intense CC staining. ×260. **B** Pancreatic duct. Weak to moderately strong CC-IR is present in almost all non-endocrine epithelial cells; the CC-IR is most intense in the apical (luminal) parts of these cells. The CC staining is also present extracellularly in the adjacent secretory fluid. The endocrine cells along the base of the duct epithelium are strongly CC immunoreactive. The duct was dilated, and more pronounced reactive epithelial changes, as well as infiltration of inflammatory cells, were present in adjacent parts of the duct. ×260. **C** Gastrinoma. Strong CC-IR in a majority of the tumour cells. ×410. All sections were immunostained with antibodies to CC followed by the streptavidin-biotin method and diaminobenzidine as the chromogen

Table 1. Endocrine pancreatic tumours and their corresponding clinical syndromes

Case no.	Clinical syndrome	Tumours[a, b]					
		CC	TTR	CGA	Grim	NSE	Hormone
1	Insulinoma	4+/2#	2+/1#	3+/1#	4+/2#	3+/4#	Ins
2	Insulinoma	2+/4#	2+/4#	3+/4#	–	3+/4#	Ins
3	Insulinoma	4+/4#	4+/4#	4+/4#	4+/4#	3+/4#	Ins
4	Insulinoma	4+/4#	4+/4#	3+/4#	4+/4#	2+/4#	Ins/Som
5	Glucagonoma	4+/4#	4+/4#	4+/4#	4+/4#	3+/4#	Glu/Gli
6	Glucagonoma	3+/3#	4+/4#	3+/4#	4+/4#	2+/4#	Glu/Gli
7	Glucagonoma	3+/3#	4+/4#	3+/3#	4+/4#	2+/4#	Glu
8	MEN I	4+/4#	4+/4#	4+/3#	4+/4#	–	Glu/Gli/PP
9	MEN I	4+/4#	4+/4#	3+/4#	4+/4#	2+/4#	Glu/Gli/PP
10	Zollinger-Ellison	3+/4#	–	3+/4#	4+/4#	2+/4#	Gastr
11	Zollinger-Ellison	3+/4#	–	4+/3#	4+/3#	2+/4#	Gastr
12	Non-producing	3+/1#	4+/4#	–	3+/3#	3+/4#	–
13	Non-producing	3+/2#	3+/2#	4+/2#	4+/2#	–	–
14	Non-producing	3+/3#	4+/4#	4+/4#	4+/4#	3+/4#	Glu/Gli/PP

Results of immunostainings for cystatin C (CC), transthyretin (TTR), chromogranin (CGA), neuron-specific enolase (NSE), insulin (Ins), glucagon (Glu), glicentin (Gli), somatostatin (Som), pancreatic polypeptide (PP) and gastrin (Gastr) and of silver stainings with the Grimelius technique (Grim)
MEN I, Multiple endocrine neoplasia type I

[a] Average immunoreactivity per cell: 4+, very strong; 3+, strong; 2+, moderate; 1+, weak; −, negative. Number of positive cells: 4#, more than 50%; 3#, 25–50%; 2#, 5–25%; 1#, less than 5%

[b] The tumours in cases 1, 2, 5, 8 and 9 were benign; all other tumours were malignant

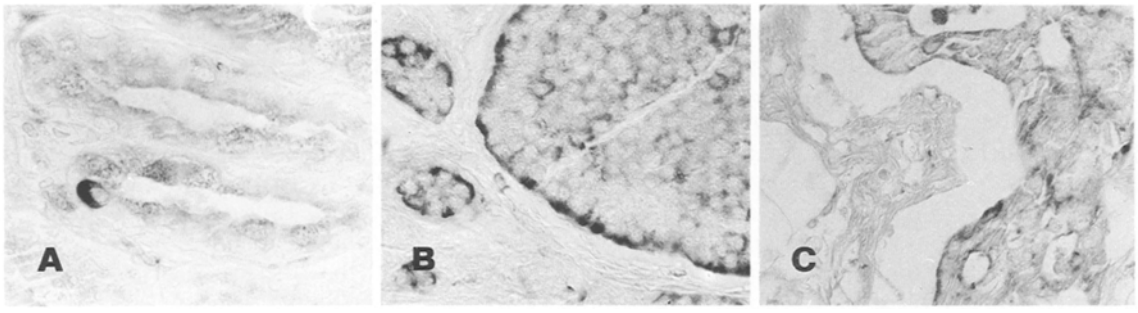

Fig. 2. A Duodenal mucosa. Weak cystatin C immunoreactivity (CC-IR) is present in the non-endocrine epithelial cells of the crypts whereas the endocrine cell to the left is strongly CC immunoreactive. ×410. **B** Ileal carcinoid tumour demonstrating strong CC-IR in a majority of the tumour cells. ×260. **C** Colon adenocarcinoma. CC-IR of weak to moderate intensity is seen in virtually all tumour cells. ×260. All sections were immunostained with antibodies to CC followed by the streptavidin-biotin method and diaminobenzidine as the chromogen

showed a weaker degree of CC immunoreactivity (case 2) or contained a lower number of CC positive cells (case 1) than their malignant counterparts (cases 3 and 4). All three glucagonomas showed immunoreactive CC. The two patients with multiple endocrine neoplasia type I (MEN I) did not suffer from the glucagonoma syndrome, although their tumours contained many glucagon-positive tumour cells in addition to PP-positive cells. The tumours from both these patients contained immunoreactive CC. The two gastrinomas as well as the three so-called non-producing tumours also contained CC (Fig. 1C).

The distribution of tumour cells showing CC, TTR and CGA immunoreactivity and the argyrophil reaction was similar in many tumours as shown by the restaining technique (not illustrated), although there were exceptions (Table 1). CC was also frequently colocalized with insulin, glucagon, PP or gastrin in the tumour cells. As in normal islet cells, the cytoplasmic staining pattern for CC appeared in a granular form.

In the antral part of the stomach and in the duodenum, many endocrine cells expressed strong CC immunoreactivity and the distribution of these cells was almost the same as that of gastrin-containing cells (Fig. 2A). Many CC-positive endocrine cells were also demonstrated in the mucosa of the ileum as well as in the colon.

All 17 gut carcinoid tumours were argyrophilic and TTR, CGA or NSE positive, confirming their endocrine nature. All nine midgut and one each of the five foregut and three hindgut tumours were also argentaffin as revealed with the Masson silver technique, indicating serotonin production (Grimelius and Wilander 1980). In agreement with our findings in normal gastrointestinal tissues, all gut carcinoid tumours except one foregut tumour were found to express CC immunoreactivity. In

Table 2. Adenocarcinomas of the pancreas and gut

Case no.	Tumour localization	CC	Case no.	Tumour localization	CC	Case no.	Tumour localization	CC
1	Pancreas	2+/4#	9	Colon	1+/4#	17	Colon	1+/3#
2	Pancreas	2+/4#	10	Colon	2+/4#	18	Colon	–
3	Pancreas	1+/2#	11	Colon	2+/4#	19	Colon	–
4	Pancreas	2+/4#	12	Colon	1+/3#	20	Rectum	1+/3#
5	Pancreas	–	13	Colon	2+/3#	21	Rectum	1+/3#
6	Stomach	1+/3#	14	Colon	1+/3#	22	Rectum	2+/4#
7	Stomach	1+/3#	15	Colon	1+/4#	23	Rectum	2+/3#
8	Stomach	1+/3#	16	Colon	2+/4#	24	Rectum	–

Results of immunostainings for cystatin C (CC)
Average immunoreactivity per cell: 4+, very strong; 3+, strong; 2+, moderate; 1+, weak; –, negative. Number of positive cells: 4#, more than 50%; 3#, 25–50%; 2#, 5–25%; 1#, less than 5%

most cases, the CC staining was strong and present in virtually all tumour cells (Fig. 2B). The distribution of CC-, TTR- and CGA-positive cells as well as that of argyrophilic cells was the same in a majority of the midgut and hindgut tumours, and the granular staining patterns for these substances were also similar. The foregut carcinoids were more heterogeneous in this regard, and in each such tumour negative staining results were obtained for one or two of the four substances mentioned.

In addition to the strongly CC-immunoreactive endocrine cells, weak CC staining was also noted in the non-endocrine glandular epithelial cells of the gastrointestinal tract (Fig. 2A). The epithelial cells of the Brunner glands in the duodenum showed moderately strong CC immunoreactivity. Non-endocrine cells of the pancreatic ducts were CC negative in normal tissues. However, dilated and reactively changed ducts in the vicinity of pancreatic tumours frequently expressed a weak to moderately strong CC immunoreactivity (Fig. 1B). All three pancreatic adenocarcinomas as well as a majority of the gastrointestinal adenocarcinomas were also found to be CC immunoreactive (Table 2) (Fig. 2C). The staining intensity of these non-endocrine tumour cells was weaker than that of their endocrine counterparts and the cytoplasmic staining pattern did not appear granular. Furthermore, the CC immunoreactivity in the non-endocrine normal and neoplastic cells was frequently most strongly expressed in the apical (luminal) part of the cells, and in some areas CC staining was also noted extracellularly in the adjacent secretory fluid (Fig. 1B).

Discussion

Glucagon A cells in pancreatic islets are the only documented sites for CC immunoreactivity in the pancreas and gut (Löfberg et al. 1981b). In the present study, virtually all pancreatic islet cells were shown to be CC immunoreactive, although the most intense CC staining was noted in glucagon-producing cells. In addition, we found strongly CC-positive endocrine cells in the pancreatic ducts, and such cells were also distributed throughout the gastrointestinal tract. In agreement with our findings in normal tissues, all endocrine pancreatic tumours (glucagonomas, insulinomas, gastrinomas and non-producing tumours) as well as virtually all gut carcinoid tumours (foregut, midgut and hindgut tumours), were also shown to express strong CC immunoreactivity.

The granular cytoplasmic staining pattern obtained with CC antibodies was similar in pattern to that found with TTR and CGA antibodies and also with the argyrophilic reaction both in normal gastro-entero-pancreatic endocrine cells and in corresponding endocrine tumours. Immunocytochemical studies at the electron-microscopic level have shown that in both normal and neoplastic endocrine cells, TTR, CGA and the argyrophilic reaction are all mainly located in secretory (hormone storage) vesicles (Grimelius and Wilander 1980; Miller et al. 1984; Wilson and Lloyd 1984; Liddle et al. 1985). It is reasonable to assume that CC is also mainly located in secretory vesicles and that the protein is most likely synthesized by these neuroendocrine cells.

We found that non-endocrine epithelial cells of reactively changed pancreatic ducts as well as normal glandular epithelial cells of the gastrointestinal tract also demonstrated CC immunoreactivity, although the staining intensity of these cells was less than that of their endocrine counterparts, and a majority of ordinary adenocarcinomas from these sites contained immunoreactive CC. The fact that the CC staining was most intense in the apical parts of the glandular epithelial cells and that the protein was present in the adjacent luminal fluid suggests that CC is synthesized and secreted also by these non-endocrine cells. Indeed, preliminary results demonstrate that CC mRNA is present to the same extent in both endocrine tumours and adenocarcinomas of the pancreas and gut using northern blot analysis as well as in situ hybridization, thus further indicating CC synthesis in both tumour types (Lignelid and Jacobsson, unpublished results). The strong and granular cytoplasmic CC immunoreactivity in the endocrine cells in contrast to the weak and non-granular CC staining in the non-endocrine cells may be due to the fact that CC is stored and concentrated in secretory vesicles of endocrine cells but rapidly secreted from non-endocrine cells.

CC is a cysteine proteinase inhibitor. It has been suggested that CC may play an intracellular role in the endocrine cells as a regulator of proteinase activity re-

sponsible for excision of peptide hormones from their precursors (Grubb and Löfberg 1985). However, CC secretion from non-endocrine glandular cells probably has a different function, namely to protect the surface epithelium of the gastrointestinal tract from degradation by cysteine proteinases contained within the lumen. The expression of CC in these non-endocrine cells may be regulated by the amount of cysteine proteinases exposed to the cells, as suggested by the fact that immunoreactive CC was present in epithelial cells of dilated and reactively changed pancreatic ducts but could not be observed in the cells of normal ducts.

Cathepsin B (CB) is the most thoroughly studied of the cysteine proteinases (Sloan 1990). An increase in CB activity and also release of the enzyme have been demonstrated for a number of tumour types, including colorectal adenocarcinomas, and a correlation between CB activity and tumour malignancy has been found (Sloan 1990). Yet this correlation appears to be a qualitative rather than a quantitative one. This may reflect the fact that alterations in CB activity in malignant tumours can be due not only to synthesis of the enzyme, but also to processing and subcellular localization of the enzyme and to its regulation by endogenous inhibitors (Sloan 1990). The present investigation, demonstrating presence of CC, the main inhibitor of CB, in a majority of gastro-entero-pancreatic adenocarcinomas, is of interest in this context and further studies will determine whether this local expression of CC plays a role in tumour malignancy.

In conclusion, immunoreactive CC is strongly expressed in a majority of normal and neoplastic endocrine cells of the pancreas and gut and the protein is most likely synthesized at these sites. However, CC is not a reliable immunohistochemical marker for endocrine gastro-entero-pancreatic tumours, as the protein is expressed also in adenocarcinomas.

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