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Cleaved caspase-3 immunocytochemical staining for pancreatic islets and pancreatic endocrine tumors

A potential marker for biological malignancy

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Keywords: caspase-3, immunocytochemistry, pancreatic islets and pancreatic endocrine tumors

Abbreviations: C, caspase; CC-3, cleaved caspase-3; PET, pancreatic endocrine tumor; PPoma, pancreatic polypeptidoma; TUNEL, terminal deoxynucleotidy transferase-mediated dUTP nick end labeling

Aims: Involvement of caspase (C)-3 has been implicated in β-cells from diabetic subjects. This study is aimed to immunocytochemically identify cleaved caspase-3 (CC-3) positive cells in pancreatic endocrine tumors (PETs) compared with control islets.

Results: Control islets revealed some CC-3 positive cells, ranging from 3.6 to 7.3% of total islet cells. Small islets in the pseudocapsule of PETs showed higher immunopositive cells at about 9% for CC-3, suggesting an accelerated apoptosis in these compressed, elongated islets before proceeding to imminent cell death. Majority of primary PETs except 9 cases were negative for CC-3 immunostaining: five insulinomas, one somatostatinoma, one gastrinoma and one pancreatic peptidoma (PPoma) were positive for CC-3.

Methods: Using commercially available rabbit anti-CC-3, immunocytochemical staining was performed in 42 cases of PETs compared with control islets.

Conclusions/Interpretation: Majority of primary PETs (28/37, 76%) were negative for CC-3, suggesting that majority of PETs are not on apoptotic program of the normal islets. Since 21 of 24 (88%) of potentially malignant primary non-β-cell PETs were negative, whereas 5 of 12 (42%) benign insulinomas were positive for CC-3 immunostaining, CC-3 negative immunostaining may serve as a possible malignant marker for all PETs.

Introduction

Accumulating evidence has shown that an essential component in apoptosis is the C, a family of cysteine proteases, which are evolutionally conserved cysteine-aspartyl specific proteases.[1,2] Each C family protease becomes active when the precursor is cleaved into a large subset with a molecular mass of ~20 KDa and a small subunit with a molecular mass of ~10 KDa, which then forms a tetramer consisting of two large and two small units.[3] One of these cleaved Cs is present on activated C-3, an ubiquitously distributed C that is the main effector C of apoptotic cascade within the cells.[4,5] This polyclonal rabbit anti-CC-3 was produced by immunizing rabbits with a synthetic peptide (KLH coupled) corresponding to amino terminal residues adjacent to Asp 175 in human C-3,[6] which detects endogenous levels of the large (17/19 KDa) activated C-3 resulting from cleaved adjacent to Asp 175, and does not recognize full length C-3 or other CCs.[6] Recently, C-3 (CPP/yama/apopain) was implicated in both type 1 and type 2 diabetes. In type 1 diabetes, Fas-Fas legend may be critical for pancreatic β-cell destruction as apoptosis in a β-cell clone expressing the human Fas β-cell line is mediated by elevated C-3-like activity in tissue culture.[1] The frequency of β-cell apoptosis increased ten-folds in lean humans and three-folds in obese humans of type 2 diabetes compared with their respective non-diabetic control islets by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling).[7] This evidence supports the significance of C-3 in human pancreatic islets, which share a final common pathway involving IL-1β, nuclear factor-κB and Fas.[8] Immunocytochemical localization of CC-3 in the normal islets and in PETs has not been documented to our knowledge. In the current study, immunocytochemical staining for CC-3 was performed in PETs to test CC-3 as a biologically malignant marker for PETs. A preliminary study with 33 cases of PETs excluding CC-3 immunostaining of adjacent normal islets was recently reported.[9]

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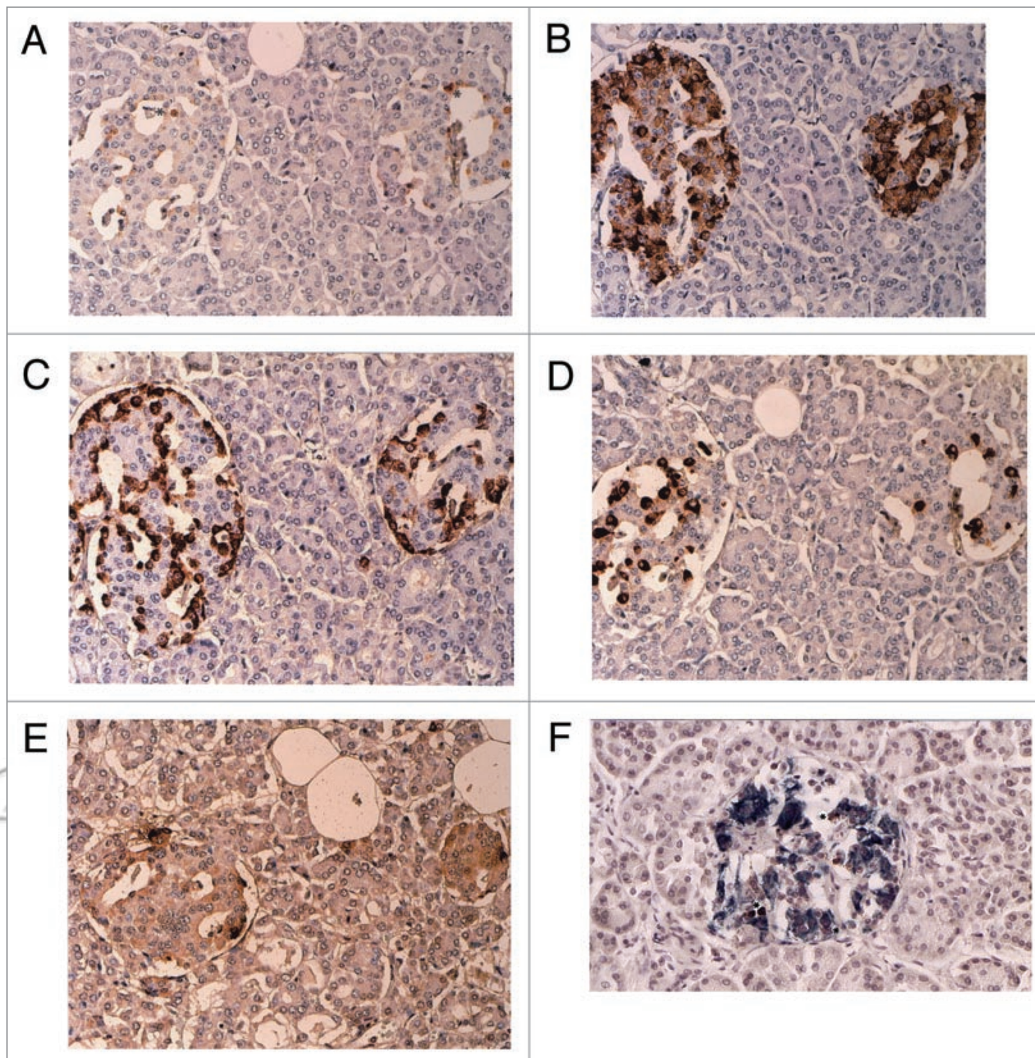

Figure 1. Normal and type 2 diabetic islets. In these islets from continuous sections, positive immunostaining for CC-3 was in the nucleus as compared with broader and stronger staining for pancreatic hormones mostly in the cytoplasm but also spreading into the nucleus especially for insulin staining. This cytoplasmic immunostaining with partial nuclear involvement was also noted for glucagon, somatostatin and PP cells to a lesser degree. There is no definite preferential immunostaining for CC-3 among four pancreatic hormone cells because a few islet cells were positive for CC-3 immunostaining, which made it difficult to match nuclear CC-3 staining with cytoplasmic hormone staining. By double immunostaining for CC-3 and insulin, an islet from a type 2 diabetic subject showed about 15% CC-3 positive nuclei in the degranulated islet cells, consistent with the degranulated insulin cells in a type 2 diabetic islet. *nuclear staining. (A) CC-3, (B) Insulin, (C) Glucagon, (D) Somatostatin, (E) PP immunostaining, (F) CC-3 and insulin double immunostaining.

Results

In the control pancreas, practically all normal islets contained CC-3 positive cells in some of the islet cells. The strong positive immunostaining was mostly in the nucleus with less positive staining also noted in some islet cell cytoplasm, which appeared to be partially an extension from the stronger nuclear staining (**Fig. 1**). Immunostaining for pancreatic hormones was mostly in the cytoplasm but the positive staining was also found in the adjacent nucleus (**Fig. 1**). The concomitant immunostaining for four pancreatic hormones showed mostly cytoplasmic staining, especially more and broader staining for insulin whereas relatively smaller staining for glucagon, somatostatin and PP was noted. All hormone staining revealed broader cytoplasmic staining than that of narrower nuclear staining for CC-3 (**Fig. 1**). CC-3 immunostaining was predominantly in β-cells as these cells were the major component of the islets in the continuous sections (**Fig. 1**).

By double immunostaining for CC-3 and insulin, CC-3 positive nuclei were identified in the degranulated islet cells, surrounded by granulated insulin cells, the former were consistent with the degranulated insulin cells from this type 2 diabetic subject, who showed about 15% CC-3 positive nuclei (**Fig. 1**). Hormone staining was more intense including the adjacent nucleus than mostly nuclear CC-3 immunostaining (**Figs. 2A–C and 3A**). For this study, only definite nuclear positive staining for CC-3 was counted for the normal islet cells. The relative number and percentage of CC-3 positive

cells were: 2.5 cells in 69 total islet cells per large islet with 3.6% being positive, and 1.8 cells in 24.6 total islet cells per small islet with 7.3% being positive for control pancreata (**Fig. 3, Table 1**).

Using the WHO criteria for PET nomination,[12,13] 12 primary insulinomas, one glucagonoma and one somatostatinoma were well differentiated endocrine tumors with benign and uncertain behavior. Among seven primary PPomas, four cases were well differentiated endocrine tumors and one case, Case 4 in **Table 1**, was well differentiated endocrine carcinoma, which later became poorly differentiated endocrine carcinoma when the tumor involved the entire remaining pancreas with liver metastasis 4 years after the initial resection (**Table 2**). Among 12 gastrinomas, six pancreatic and two duodenal tumors were well differentiated endocrine tumors whereas three pancreatic and two duodenal tumors were well differentiated endocrine carcinomas. All three non-functioning tumors were well differentiated endocrine tumors.

Regarding the tumor sizes, twelve primary insulinomas were >1.5 x 1.5 cm including one well differentiated endocrine tumor with uncertain behavior (0.6 x 0.5 cm) which metastasized to the liver 2 years after the initial resection whereas one patient with the largest well differentiated endocrine tumor (7 x 7 cm) is alive without recurrence to date. One pancreatic glucagonoma, which was classified as a well differentiated endocrine carcinoma measured 14 x 10 x 10 cm. One somatostatinoma, measuring 11 x 6 x 5 cm, was a well differentiated endocrine tumor. Among nine pancreatic gastrinomas, six cases were >1.0 x 1.0 cm, and three cases measured 5 x 5 x 4 cm, whereas among three duodenal gastrinomas, two cases were >2.0 x 1.0 cm and one case measured 5 x 5 x 4 cm. All eight small gastrinomas were well differentiated endocrine tumors and three large tumors were well differentiated carcinomas. Among seven primary PPomas, five cases were less than 1.5 x 1.5 cm whereas one case, Case 1, measured 13 x 13 x 14 cm, involving body-tail portion, well differentiated endocrine carcinoma, which metastasized to the liver involving the entire remaining pancreas 4 years after the initial resection, presenting as poorly differentiated endocrine carcinoma of small cell type.[15] All three non-functioning tumors were >1.0 x 1.0 cm.

Among 42 PETs included in this study, 22 cases contained enough adjacent pancreatic tissue to count CC-3 positive cells, including seven insulinomas, one glucagonoma, one stomatostatinoma, four PPomas, six gastrinomas and three non-functioning tumors with the remaining 12 primary pancreatic PETs having less than 15 islets, which did not contain a sufficient number of islets and islet cells and the remaining two cases were liver and lymph node metastasis of glucagonoma and PPoma, respectively, without pancreatic tissue. Twelve cases contained only enough large islets, three cases contained only enough small islets and seven cases contained both enough large and small islets (**Table 1**). Large islets contained 2.3 CC-3 positive cells in a total of 51.8 islet cells per islet with 4.4% being positive for CC-3, which revealed relatively less total islet cells and relatively more positive percentage compared with that of controls (**Table 1**). Small islets contained 2.1 CC-3 positive cells in 22.8 total islet cells per islet with 9.2% being positive for CC-3 (**Table 1**). Thus, small

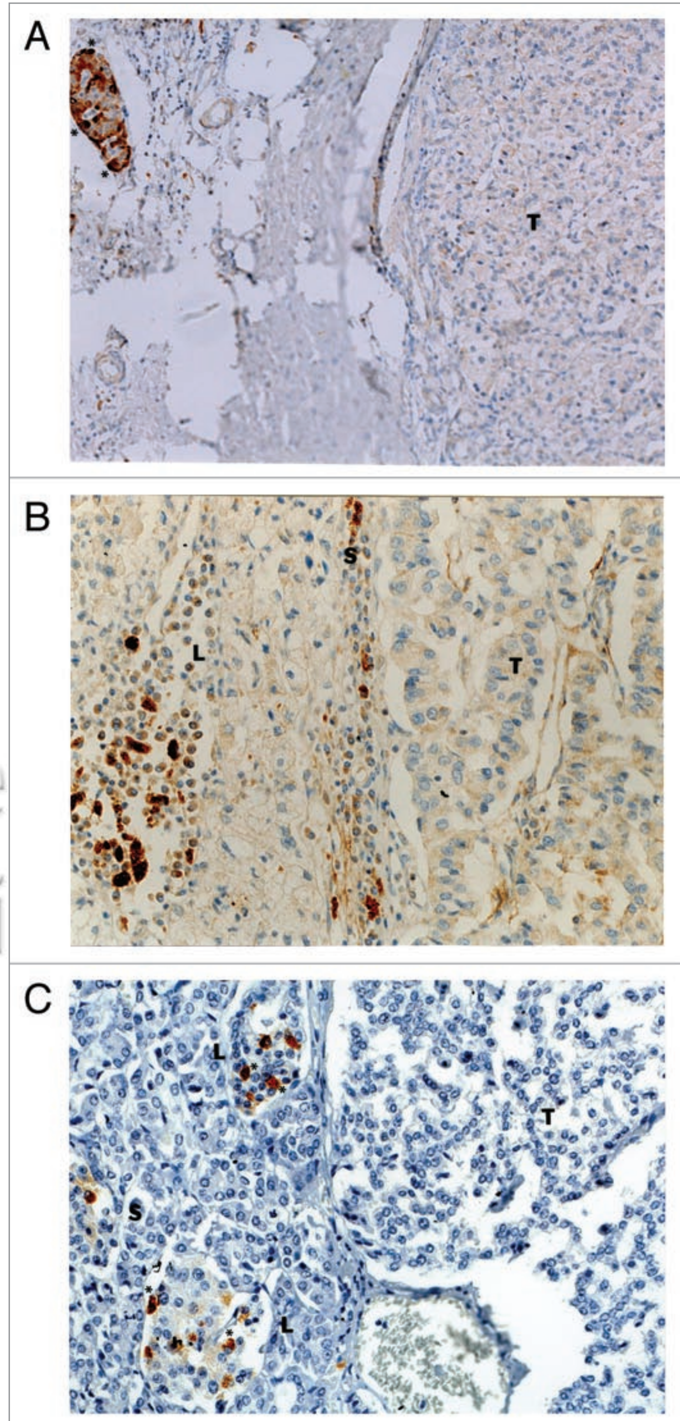

Figure 2. CC-3 negative PETs. In this glucagonoma, the tumor cells were completely negative for CC-3 whereas adjacent large islets revealed similar positive staining as control islets in Figure 1 with strong nuclear staining and weaker cytoplasmic staining adjacent to the strongly stained nuclei (A). This gastrinoma was also completely negative for CC-3 whereas adjacent large and small islets revealed predominantly nuclear positive staining (B). This PPoma was completely negative for CC-3 with normal large islets being positive predominantly for nuclei (C). (A) Glucagonoma, Case 1, (B) Gastrinoma, Case 2, (C) PPoma, Case 2.

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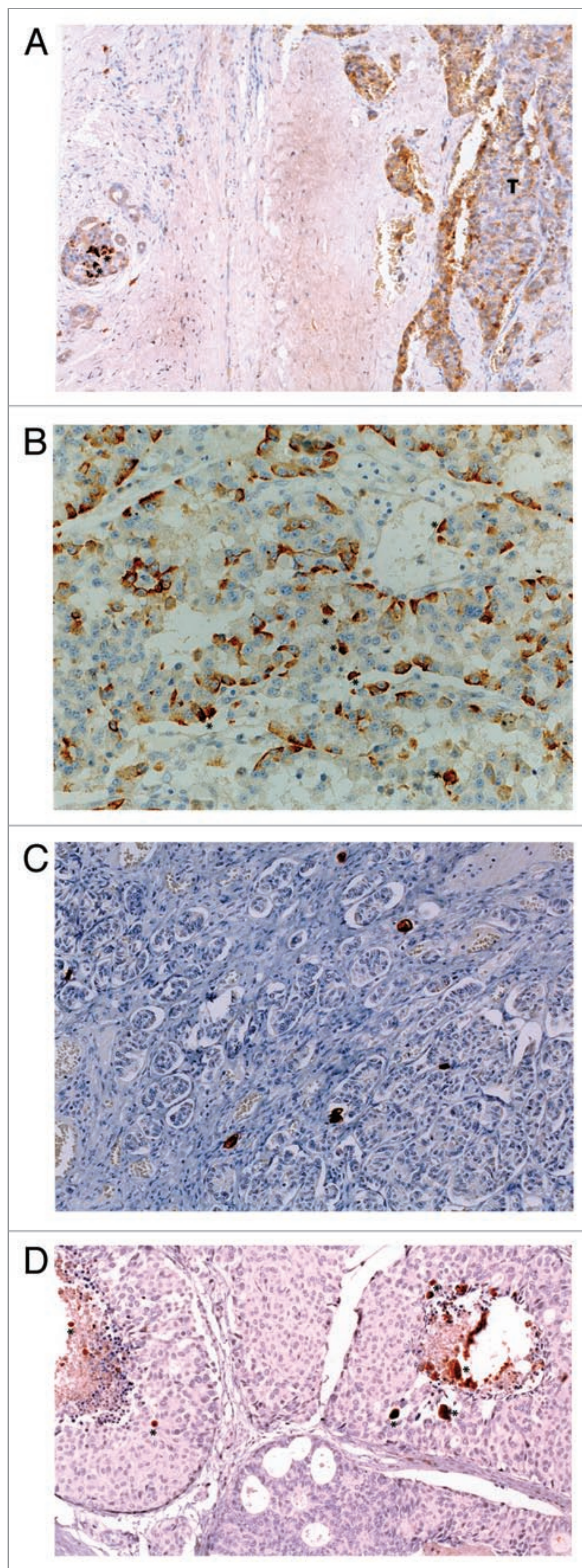

Figure 3. CC-3 positive PETs. The positive staining for this somatostatinoma was diffuse and moderate whereas strongly positive nuclear staining was noted in the adjacent islets (A). This insulinoma reveals diffuse positive staining for both nuclei and perinuclear cytoplamsms (B). This gastrinoma reveals positive immunostaining in the nuclei of both normal sizes and large spherical sizes (C). This malignant PPoma, which presented liver metastasis 2 years after the initial partial pancreatectomy, showed positive nuclear staining in central necrotic area and in mid non-necrotic tumor cells, the latter supports an imminent programmed cell death within still viable tumor cells. T: Tumor, *nuclear staining. (A) Somatostatinoma, Case 1, (B) Insulinoma, Case 3, (C) Gastrinoma, Case 3, (D) PPoma, Case 1.

elongated islets from the adjacent pancreas to the PETs contained higher percentages of CC-3 positive cells (9.2%) than those of the control pancreas (7.3%). The difference between the adjacent pancreata and the control pancreata was not statistically significant ($p < 0.5$). The immunostaining for CC-3 was mostly in the islet cell nucleus whereas acinar cells and ductal cells were almost completely negative for CC-3 immunostaining (**Fig. 2**). Majority of primary PETs were negative for CC-3 immunostaining (**Table 2** and 28/37, 76%) except in five insulinomas, one somatostatinoma, two gastrinomas and one pancreatic PPoma (**Fig. 3, Table 2**).

One case each of metastatic PPomas to the liver and lymph node revealed central necrosis and some of the tumor cells adjacent to the necrotic area and mid non-necrotic area were positive for CC-3 (**Fig. 3D**). Compared with adjacent normal pancreatic islets, positive CC-3 immunostaining for PETs was weakly cytoplasmic for three insulinomas and one somatostatinoma, and positive for both nucleus and perinuclear cytoplasm for one gastrinoma, and scattered nuclear positive staining for one gastrinoma and one primary pancreatic PPoma. Two metastatic PPomas also revealed CC-3 immunostaining in the central necrotic area, the latter is relatively rare for well differentiated endocrine tumors and well differentiated endocrine carcinomas (**Fig. 3**). The CC-3 positive tumor cell nucleus was of normal size as well as larger and more spherical shaped than normal islet cell nuclei (**Fig. 3**).

Discussion

There is enough evidence to support an essential role for C-3 in apoptosis.[1,2] Studies with peptide inhibitors or anti-sense C-1 constructs have shown that C-1 or C-1-like proteases are involved in Fas-mediated cell death.[18] Pro-C-3 is proteolytically activated by C-1, indicating that C-3 may lay downstream of C-1 in the apoptotic cascade.[19] Using a mouse β-cell line, which was transfected with human Fas cDNA and underwent apoptosis when exposed to anti-Fas, indicates that the mouse β-cell line has the intact machinery for Fas-mediated apoptosis.[20] Using TUNEL, Butler et al. reported increased β-cell apoptosis to 10-fold in lean and three-fold in obese subjects of type 2 diabetes compared with the respective non-diabetic islets.[7] Using commercial C protease assay kits, isolated islets from type 2 diabetics revealed a relative increase of C-3 and C-8 activities together with a relative increase of apoptosis by the Cell Death Detection ELSplus assay.[21]

Table 1. Cleaved caspase-3 immunostaining for pancreatic islets

	Large islets			Small islets		
	Positive cells	Total cells	Positive %	Positive cells	Total cells	Positive %
			Control cases (n = 7)			
Mean ± SE	2.5 ± 0.2	69.0 ± 4.1	3.6% ± 0.4	1.8 ± 0.1	24.6 ± 2.1	7.3% ± 0.8
			Insulinomas (n = 7)			
Case 1. 17/F	1.6	45.2	3.5	2.2	21.0	10.0
2. 20/F				1.7	19.7	8.6
3. 52/M				2.6	25.6	10.2
4. 68/F	1.2	41.7	5.1			
5. 68/F	2.7	47.7	5.7			
6. 71/F	2.7	47.7	3.9			
7. 81/M	2.8	57.3	5.8			
			Glucagonoma (n = 1)			
Case 1. 43/F	2.3	37.9	6.1			
			Somatostatinoma (n = 1)			
Case 1. 42/F	1.9	41.1	4.6	1.6	15.5	10.3
			PPomas (n = 4)			
Case 1. 33/M	2.9	58.4	5.0			
2. 79/M	2.7	68.5	3.8			
3. 80/M	2.3	58.1	4.0			
4. 86/F	2.2	49.7	4.4	1.8	25.0	7.2
			Gastrinomas (n = 6)			
Case 1. 31/M	1.8	46.4	3.9			
2. 47/F	2.0	56.6	3.5			
3. 54/M	2.4	70.7	3.4			
4. 59/M				1.8	24.1	7.5
5. 60/F	2.6	72.5	3.6	1.8	24.1	11.6
6. 68/M	1.2	41.7	5.1			
			Non-functioning tumors (n = 3)			
Case 1. 48/M	3.0	52.7	5.7	2.5	24.8	10.1
2. 56/F	2.5	43.6	5.7	2.7	21.9	12.6
3. 66/M	2.1	45.4	4.6	2.9	27.0	8.9
Total, mean ± SE	2.3 ± 0.12	51.8 ± 2.52	4.4% ± 0.21	2.1 ± 0.13	22.8 ± 1.08	9.2% ± 0.53

From the above information, we have pursued immunocytochemical staining for CC-3 staining in pancreatic islets and this immunostaining revealed exclusively in islet cells with practically no immunostaining in exocrine pancreatic acinar cells and ductal cells (**Figs. 1–3**). In control islets, CC-3 immunostaining was almost exclusively located in the islet cell nucleus of 3.6% of islet cells for large islets and 7.3% of islet cells for small islets (**Table 1**). This specific nuclear staining for CC-3 is also supported by nuclear staining in the cultured KB human carcinoma cells using the same antibody as ours by confocal immuofluorescent images.[6]

Twenty-two of 42 PETs contained enough adjacent pancreatic tissue for counting immuostaining for CC-3 in normal islets. Large islets revealed 2.3 CC-3 positive cells in a total of 51.8 cells per islet with 4.4% being positive, whereas small islets revealed 2.1 CC-3 positive cells in a total of 22.8 islet cells per islet with 9.2% being positive (**Table 1**). The small islets in PET cases were located in the pseudocapsule adjacent to the PET and showed a higher percentage of CC-3 positive cells than control islets and large islets from the adjacent pancreas to the PETs (**Fig. 3**). The former were slender, atrophic and compressed by the pushing tumor margin and appeared to be in a process of apoptosis and imminent cell death with more percentage of CC-3 immunostaining. All primary PETs except nine cases were negatively stained for CC-3, which may implicate that the majority of primary pancreatic PETs are not on the same apoptotic program as that of control islet cells or below the detectable levels of CC-3 by this immunocytochemical staining. Eight of nine CC-3 positive PETs showed fewer nuclear staining than the control islets, which may also suggest lesser CC3 presence in these PETs than in the control islets. One remaining case revealed more numerous CC-3 immunostianing in the tumor cell nucleus and perinuclear

Table 2. Cleaved caspase-3 immunostaining for pancreatic endocrine tumors

Types of PETs	Total numbers	Locations	Immunostaining	
			Positive	Negative
Insulinomas	15	Pancreas—13 Liver meta—2	WDET—5	WDET—8 WDEC—2
Glucagonomas	2	Pancreas—1 Liver meta—1		WDET—1 WDEC—1
Somatostatinoma	1	Pancreas—1	WDET—1	
PPomas	9	Pancreas—7	WDET—1	WDET— 4 WDEC—1 PDEC—1
		Liver meta—1	WDEC—1	
		LN meta—1	WDEC—1	
Gastrinomas	12	Pancreas—9	WDET—1	WDET—5 WDEC—3
		Duodenum—3	WDET—1	WDEC—2
Non-functioning	3	Pancreas—3		WDET—3

LN, Lymph node; meta, metastasis.

cytoplasm than control islet cells (**Fig. 3**), suggesting an accelerated apoptosis in this insulinoma.

Central necrosis was observed in two metastatic cases of malignant PPoma, one in liver and another in lymph node with some CC-3 positive cells in the central necrotic area and mid non-necrotic tumor area. The latter supports programmed cell death in the CC-3 positive viable tumor cells in the metastatic PPomas (**Fig. 3D**). In primary PETs, necrosis is a useful parameter for high-grade cancers but is a rare finding in low-grade tumors.[22,23]

Since five of 12 (42%) benign insulinomas were positive for CC-3 whereas one malignant insulinoma and 19 of 23 (87%) potentially malignant primary non-β-cell PETs were negative for CC-3, CC-3 negative immunostaining may serve as a possible malignant marker in all PETs.

A concurrent study with human type 1 and type 2 diabetic pancreatic islets has also revealed relatively increased immunostaining for CC-3, about 16% for type 1 diabetes and about 9% for type 2 diabetes compared with non-diabetic total control pancreatic islets of about 5% at our hand (unpublished report), which corresponds roughly to those of C-3 activity assays performed in isolated pancreatic islets from diabetic subjects.[7]

Pancreatic islets are an essential and special organ for sustaining life by regulating blood glucose levels through paracrine secretion of insulin, glucagon, somatostatin, pancreatic polypeptide and amylin,[22,23] and also through many other mechanisms, that tightly regulate blood glucose, the major fuel of the body.[24] The preliminary study with intestinal carcinoids for CC-3 staining revealed negative staining in carcinoids, which makes a phenotypic difference between benign PETs and intestinal carcinoids, also supporting potential malignancy of intestinal carcinoids (unpublished data). The current results support the dynamic process of remodeling islets through apoptotic cascade mediated by C-3. The active and continuous remodeling of islets is also supported by the exclusive presence of zinc-containing matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) in normal islets and in some PETs, as MMPs and TIMPs comprise one of the main components of tissue remodeling mechanisms.[10,11,27-30]

Finally, archival paraffin tissue blocks represent a potential source of information that may be employed to study many disease processes for semi quantitative study,[30] including tumorigenesis, and thus may increase interest in developing methods that may specifically demonstrate apoptotic cells in tumor cells using formalin-fixed and paraffin-embedded tissue sections.[5]

Materials and Methods

All cases of PETs were from the University of Kansas Medical Center and collected between 1975 and 2001 during my tenure at the Medical Center. A total of 42 cases were included for this study, in which CC-3 immunocytochemical staining was revealed in the islets from the adjacent pancreas. Included were 15 insulinomas, two glucagonomas, one somatostatinoma, nine PPomas, 12 gastrinomas and three non-functioning tumors, the majority of which were previously reported by us.[9-11] The WHO classification of PETs includes insulinoma, gastrinoma, glucagonoma, VIPoma, somatostatinoma and non-functioning tumors, the latter also include PPoma with no obvious clinical symptoms attributed to PP hypersecretion.[12,13] Our PPoma cases were extensively studied for serum and tumor tissue PP levels together with four pancreatic hormone levels by radioimmuno-assay using tissue extracts in addition to immunocytochemical staining.[14,15] The PET of predominantly PP cell type should be recognized as PPoma in our opinion as also supported by Hruban et al.[16] The histopathological criteria of PETs were employed according to the WHO criteria, including well differentiated endocrine tumor with benign or uncertain behavior, well differentiated endocrine carcinoma and poorly differentiated endocrine carcinoma.[12,13] All the tissue was routinely fixed in buffered formalin and embedded in paraffin. Deparaffinized sections were treated with antigen retrieval procedure using citrate buffer pH 6.2. All

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the staining procedures were the same as previously reported including immunostaining for insulin, glucagon, somatostatin and PP[10,11] except for CC-3 immunostaining, in which rabbit anti-CC-3 (Cell Signaling Technologies, Beverly, MA, Cat. # 9661) was used at 1:300 dilution. This antibody is produced by immunizing rabbits with a synthetic peptide (KLH coupled) corresponding to amino terminal residues adjacent to (Asp 175) in human C-3.[6] Double immunostaining for CC-3 and insulin was also performed: the sections were initially stained for CC-3 using diaminobenzidine tetrahydrochloride for brown color, then the same sections were stained for insulin using Vector SG (SK-4700, Vector Lab, Burlingame, CA) for blue color. The counting of CC-3 positive islet cells was performed by systematically examining immunostained microscopic slides observing 15 to 20 large islets and small islets containing more than 10 islet cells, respectively, using microscopic stage at 10 x 20 = x200 magnification. In all PET cases, where normal pancreatic tissue was present, 15 to 20 islets were counted for CC-3 positive cells and for total islet cell numbers per islet for large islets and small islets, respectively. The islets within abundant margins of normal pancreas consisted of mostly large islets of more than 34 islet cells and the compressed pushing margin by the tumor contained elongated small islets of less than 34 islet cells with relatively more CC-3 positive cells, thus the number and percentage of CC-3 positive cells were counted for large islets and small islets, respectively, excluding smaller islets containing less than 10 islet cells as these smaller islets tended to reveal much more various percentage of positive immunostaining from 0% to 60% for CC-3, which makes it difficult and impractical to include smaller islets for statistical analysis. The control pancreas consisted of 7 non-diabetic cases, 35 to 65 years of age, with which 15 to 20 large and small islets were counted for CC-3 immunostaining, respectively. All PET tissues together with normal non-neoplastic pancreatic tissues to PETs and control pancreatic tissues were systematically immunostained for four pancreatic hormones including insulin, glucagon, somatostatin and PP as previously reported.[10,11]

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