American Journal of Pathology, Vol. 143, No. 1, July 1993

Immunoreactivity and Expression of Amylin in Gastroenteropancreatic Endocrine Tumors

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Amylin was isolated from human insulinomas, but there has been only preliminary data regarding whether this peptide can also be detected in other types of gastroenteropancreatic endocrine tumors. In the present study, immunohistochemical staining of 87 gastroenteropancreatic endocrine tumors demonstrated amylin immunoreactivity in 21.8% of the neoplasmas. Thirteen of 15 insulinomas, three of 21 gastrinomas, two of 29 nonfunctioning tumors, and one of 18 carcinoids were amylin-immunoreactive. Seventeen of the 19 amylin-immunoreactive tumors were primarily located in the pancreas, but two tumors were found in the intestine. Measurements of amylin messenger RNA expression in a few tumors revealed amylin synthesis in these tumors. Amylin immunoreactivity did not correlate with invasion and metastasis. However, the rate of curative resections was significantly higher in amylin-immunoreactive tumors. These results demonstrate for the first time that amylin immunoreactivity is not restricted to insulinomas and can also occur rarely in endocrine tumors of the intestine. (Am J Pathol 1993, 143:283–291)

Hyalinosis of the pancreas was first described in 1900,[1] and it has been shown that this material is amyloid.[2] Excess of amyloid occurs in the islets of Langerhans of patients with noninsulin-dependent diabetes mellitus (NIDDM), and even healthy humans and animals can store amyloid deposits.[2–5] About 50% of all insulin-producing tumors contain varying amounts of amyloid in the tumor stroma.[6] Recently, the peptide that forms the amyloid fibrils in the pancreatic islets of diabetic patients and insulinomas has been isolated.[7–9] It was termed islet amyloid polypeptide, diabetes-associated peptide, or amylin. The peptide consists of 37 amino acids and displays 46% homology with the neuropeptide calcitonin gene-related peptide.[9,10]

Amylin is normally stored in B-cell secretory granules[11–15] and co-secreted with insulin in response to glucose and other secretagogues.[16–20] Amylin expression was reported in human[7,15,21–24] and canine[25] B cell tumors, and the peptide was considered to be a specific marker for insulinomas.[26] On the other hand, amylin immunoreactivity does not only occur in the pancreas, but also in the stomach and intestine,[27–30] where B cells are lacking. Measurements with a specific radioimmunoassay revealed small amounts of amylin immunoreactivity in various types of gastroenteropancreatic endocrine tumors.[31]

Therefore, the significance of amylin as a marker of these tumors is unclear so far. In the present study, 87 gastroenteropancreatic endocrine tumors were investigated for amylin immunoreactivity by immunohistology. To demonstrate expression of the peptide by these tumors, messenger (m)RNA measurements were performed by Northern blotting and *in situ* hybridization. Parameters of tumor pathological status and clinical data of the patients were evaluated to determine the characteristics of amylin-immunoreactive endocrine tumors.

Materials and Methods

Gastroenteropancreatic Tumors

Tissue from 87 gastroenteropancreatic endocrine tumors was investigated immunohistochemically for amylin immunoreactivity. In some cases, only one sample of the primary tumor was available. In other

RE is a postdoctoral fellow of the German Society of Gastroenterology and Metabolism (Asche Stipendium).

Accepted for publication January 15, 1993.

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tumors, up to 15 tissue blocks of the primary tumor and metastases could be processed. The tissue had been removed by surgery and fixed either in Bouin's solution or 4% formaldehyde. In control experiments, immunoreaction for amylin was identical for both of these fixatives. All material was paraffin-embedded, and 5-μ sections were cut for hematoxylin and eosin staining and immunohistochemistry. The classification of the gastroenteropancreatic endocrine tumors was based on clinical symptoms and supported by serum hormone levels. Nonfunctioning tumors have either no detectable hormone secretion or the secreted hormone does not cause clinical symptoms. Amylin immunoreactivity was evaluated in 21 gastrinomas, 15 insulinomas, 18 tumors associated with carcinoid syndrome, 29 nonfunctioning tumors, three vasoactive intestinal polypeptide (VIP) tumors, and one adrenocorticotropic hormone-producing tumor.

From these tumors, specific mRNA could be measured by Northern blotting in three insulinomas, five gastrinomas, and two nonfunctioning tumors. In situ hybridization was performed in five gastrinomas, five insulinomas, one nonfunctioning tumor, and one tumor causing carcinoid syndrome. The time of ischemia (time between devascularization and fixation or freezing respectively) varied from 5 minutes to 2 hours. For Northern blots, tissue was frozen on dry ice and stored at −80 C up to RNA extraction. For *in situ* hybridization, the tumors had been fixed primarily in 4% paraformaldehyde, dehydrated in 35% sucrose, and frozen in liquid nitrogen before cutting sections on the cryostat.

Immunohistochemistry

The avidin-biotin complex technique[32] or the alkaline phosphatase-anti-alkaline phosphatase method[33] was used for immunohistochemistry. Amylin immunoreactivity was tested with a primary polyclonal rabbit anti-human amylin serum (RAS-7321-N, Peninsula, Heidelberg, Germany). There is no cross-reactivity with calcitonin gene-related peptide, and the antibody was used in a dilution of 1:3,000 in both immunohistological techniques. Sections of the pancreas of a patient with type II diabetes mellitus with marked amyloidosis of the islets of Langerhans served as a positive control. For negative controls, the primary antiserum was replaced by nonimmune rabbit serum. The staining procedure for amylin was carried out twice and only tumors in which amylin immunoreactivity could be demonstrated in the cytoplasma of the tumor cells were considered to be positive.

All gastroenteropancreatic endocrine tumors were well-characterized immunohistochemically by a panel of antibodies (Table 1): chromogranin A, PHE 5, neuron-specific enolase, α-chain of human chorionic gonadotropin, insulin, gastrin, glucagon, somatostatin, pancreatic polypeptide, serotonin, neurotensin, and VIP.

Northern Blotting

For Northern blotting and in situ hybridization, an anti-sense RNA probe for amylin was generated by transcription of a linearized RNA expression vector, p GEM 4zfct (2775 bp). The vector contained a 676-bp insert of the DNA sequence of the human amylin gene.[22] The probes were labeled with [^{32}P]-UTP or [^{3}H]UTP (Amersham, Buckinghamshire, UK).

Total RNA was extracted according to the guanidine thiocyanate-cesium chloride method.[34] RNA was quantified by spectrophotometric analysis (OD 280/260 nm). RNA degradation was monitored by agarose gel electrophoresis; only those samples in which the ratio of 28S and 18S RNA exceeded 2:1 were processed further. From each case, 10 μg of total RNA were separated on denaturing gels con-

Table 1. *Antibodies Used for Characterizing the Gastroenteropancreatic Endocrine Tumors: Working Dilutions and Sources*

Antigen	Antibody	Working dilution	Source
Amylin	Polyclonal	1:3,000	Peninsula Lab.
α-HCG	Monoclonal	1:2,000	Peninsula Lab.
Chromogranin A	Monoclonal	1:50	Hybritech
Gastrin	Polyclonal	1:100	DAKO
Glucagon	Monoclonal	1:20	Novo Biolabs
Insulin	Monoclonal	1:500	Novo Biolabs
Neurotensin	Polyclonal	1:1,000	INCstar Corporation
Pancreatic polypeptide	Polyclonal	1:2,000	DAKO
Phe 5	Monoclonal	1:50	Enzo Diagnostics
Serotonin	Monoclonal	1:100	DAKO
Somatostatin	Monoclonal	1:500	Novo Biolabs
Vasoactive intestinal Polypeptide	Polyclonal	1:500	Milab

taining formaldehyde[35] and transferred onto nylon membranes by vacuum blotting.

Prehybridization was performed at 68 C for 6 hours with a buffer containing 50% deionized formamide, 5× standard saline citrate (SSC) (pH 7.0), 10× Denhardt's, 50 mmol/L $NaH_2PO_4/NaPO_4$ (pH 6.5), 1% sodium dodecyl sulfate (SDS), and denatured salmon sperm DNA 100 mg/ml. Hybridization was carried out at 65 C overnight with 2×10^6 cpm probe/ml buffer (50% deionized formamide, 5× SSC (pH 7.0), 2× Denhardt's, 25 mmol/L $NaH_2PO_4/NaPO_4$ (pH 6.5), 1% SDS, and denatured ssDNA 100 mg/ml). Washing was done at 65 C in a 2× SSC-0.1% SDS solution for 30 minutes, followed by two 30-minute wash steps in 0.2× SSC-1% SDS solution. Blots were exposed to x-ray films with intensifying screens at −70 C for 48 hours. Hybridization with nonspecific vector sequences and sense amylin RNA probes produced negative results. The blots were rehybridized with anti-sense ^{32}P-labeled actin probes and processed as described above.

In Situ *Hybridization*

A detailed protocol for *in situ* hybridization utilizing anti-sense RNA probes has been published previously.[36] Briefly, after pretreatment of the tissue sections with protease K and acetic anhydride, 10 µl of the hybridization mixture, containing hybridization buffer (50% formamide, 2× SSC, 10% dextran sulfate, 0.25% bovine serum albumin, 0.25% Ficoll 400, 0.25% polyvinylpyrrolidone 360, 0.5% SDS, 250 µg/ml denatured ssDNA) and the ^3H-labeled anti-sense RNA probe (5×10^5 cpm/10 µl buffer) were applied. Hybridization was carried out at 43 C for 16 hours. Nonhybridized probe was removed by several washes at 37 C and RNAse A treatment. For autoradiography, slides were dipped in NTB-2 (Eastman Kodak, NY, USA), dried, and exposed for 21 days. The autoradiographs were analyzed by bright- and dark-field microscopy. As in Northern blotting, nonspecific vector sequences and sense RNA probes served as negative controls. Tissue specimens of the pancreas of a patient with type II diabetes mellitus were used as a positive control.

Characteristics of the Tumors and Clinical Data

The following parameters of the tumors were evaluated by analyzing the charts and protocols of surgery and pathology: location of the primary tumor, tumor size, metastases, invasion, and resectability. Furthermore, we looked for clinical symptoms of the patients, especially diabetes mellitus and serum hormone levels corresponding to the endocrine tumor.

Statistical Analysis

The statistical significance was assessed according to the χ-square test.[37]

Results

Immunohistology

Amylin-Immunoreactive Tumors (Figure 1, a and b)

Nineteen of 87 (21.8%) gastrointestinal endocrine tumors showed a positive immunoreaction for amylin. The distribution of amylin-immunoreactive cells

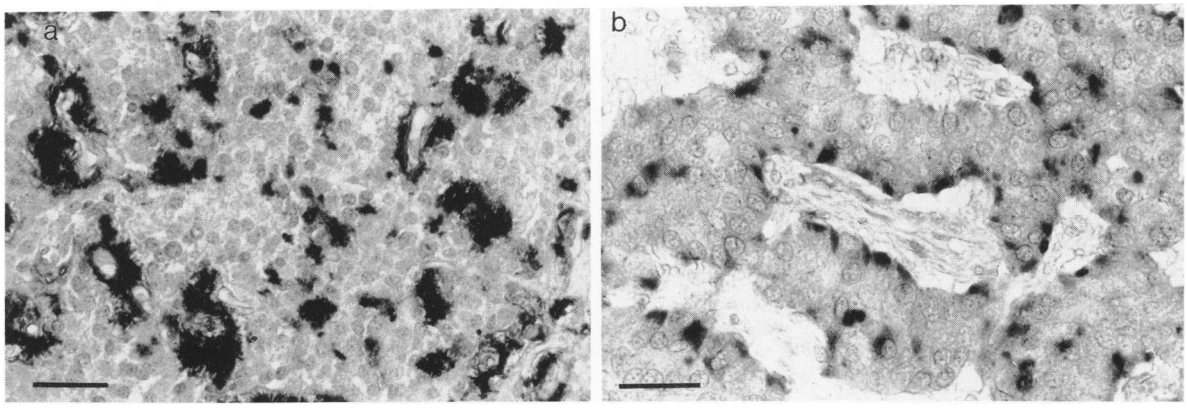

Figure 1. *Immunolocalization of amylin in different pancreatic endocrine tumors using the alkaline phosphatase-anti-alkaline phosphatase technique.* **a:** *Patient KD, insulinoma. Amylin immunoreactivity is visible both in the cytoplasma of the tumor cells and in the intercellular space (perivascularly). Magnification 1:312; scale bar: 15 µ.* **b:** *Patient HK, gastrinoma. Focal amylin deposits are located in the tumor cells close to the basement membrane. Magnification 1:500; scale bar: 10 µ.*

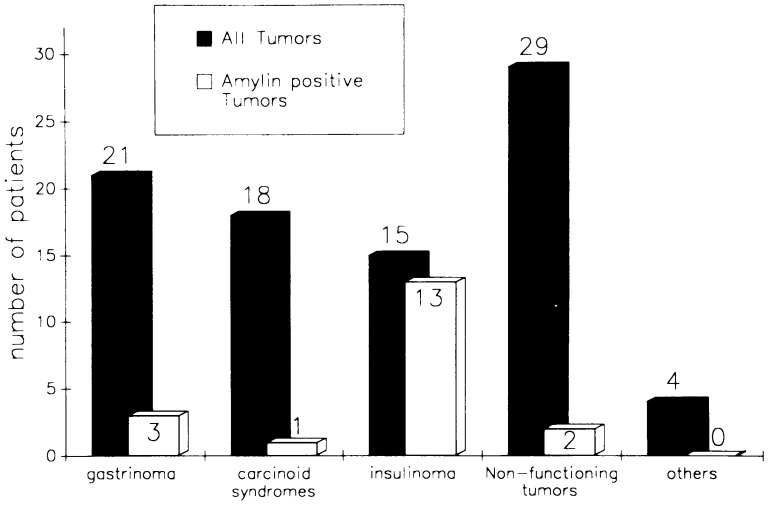

Figure 2. *Amylin immunoreactivity in different subtypes of gastroenteropancreatic endocrine tumors. The classification of the tumors was based on clinical symptoms and serum hormone levels. Others include three VIP tumors and one adenocorticotropic hormone-producing tumor.*

in these tumors was either diffuse, arranged in clusters, or only single cells were amylin-positive. Thirteen of 15 insulinomas, three of 21 gastrinomas, two of 29 nonfunctioning tumors, and one of 18 carcinoid tumors were amylin-immunoreactive (Figure 2).

Colocalization with Other Peptides

Insulin immunoreactivity could be detected immunohistologically in all amylin-positive insulinomas and in the two nonfunctioning tumors. The three amylin-immunoreactive gastrinomas and the carcinoid tumor were negative for insulin. Both amylin-immunoreactive nonfunctioning tumors showed a positive reaction for pancreatic polypeptide. All insulinomas were negative for α-HCG, whereas one gastrinoma, one nonfunctioning tumor, and the carcinoid tumor showed a positive reaction for α-HCG.

mRNA Expression of Amylin

Northern Blot Analysis (Figure 3)

Three amylin-immunoreactive insulinomas were investigated by Northern blot analysis. Amylin mRNA expression could be detected only in one tumor. Five gastrinomas, which were negative for amylin immunohistologically, were also negative in Northern blot analysis. One of the two nonfunctioning tumors showed a very strong specific signal for amylin. This tumor was reactive for amylin and insulin by immunohistology.

In Situ *Hybridization (Figure 4)*

In one of five insulinomas (all reactive for amylin by immunohistochemistry), specific mRNA could be detected by *in situ* hybridization. A very strong signal for amylin mRNA could be observed in the nonfunctioning tumor that had been positive in Northern blot analysis. The five gastrinomas and one carcinoid tumor investigated by *in situ* hybridization were negative.

Characteristics of the Tumors

Seventeen of the 19 amylin-immunoreactive tumors were primarily located in the pancreas. However, one gastrinoma of the duodenum and one carcinoid tumor of the jejunum were reactive for amylin by immunohistology (Figure 5). The study included eight patients with a multiple endocrine neoplasia syndrome I. Two insulinomas of these patients were amylin-immunoreactive, whereas three gastrinomas,

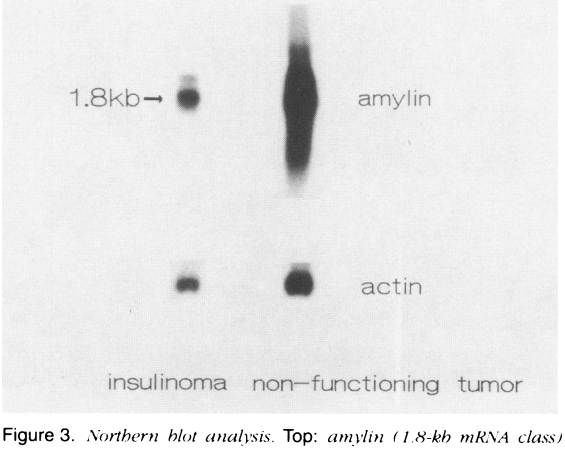

Figure 3. *Northern blot analysis.* Top: *amylin (1.8-kb mRNA class) mRNA expression in an insulinoma (patient DG) and in a nonfunctioning tumor (patient GK).* Bottom: *control hybridization for actin.*

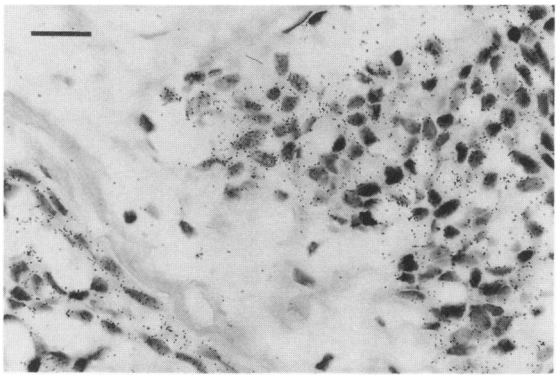

Figure 4. In situ *hybridization of a nonfunctioning tumor (patient GK): silver grains located above tumor cells after hybridization with an amylin mRNA probe labeled with ^3H. Magnification: 1:200; scale bar 20 μ.*

one nonfunctioning tumor, one VIP tumor, and one adrenocorticotropic hormone-producing tumor were negative for amylin.

No difference was found between the size of amylin-immunoreactive and amylin-negative tumors. Fifty percent of the amylin-immunoreactive and 76% of the amylin-negative tumors had metastasized (n.s.). Invasion beyond the primary organ could be seen in 23% of the amylin-immunoreactive and in 34% of the amylin-negative tumors (n.s.). Altogether, criteria of malignancy according to Klöppel and Heitz[38] were present in 50% of the amylin-immunoreactive and 79% of the amylin-negative tumors (n.s.). Eighty-five percent of the amylin-immunoreactive tumors could be curatively resected, whereas this was the case in only 30% of the amylin-negative tumors ($P < 0.001$). The three tumors in which amylin mRNA abundance could be detected were all located in the pancreas. The nonfunctioning tumor showed local lymph-node metastases. No criteria of malignancy were present in the two insulinomas. One of the insulinomas was a multiple endocrine neoplasia syndrome I tumor.

Clinical Parameters

No differences of age and sex were found between amylin-immunoreactive and amylin-negative tumors. The clinical pictures of the patients were characterized by the effect of the dominating hormone (e.g., insulin or gastrin) or by nonspecific symptoms. Retrospective exploration of the charts revealed no additional and specific symptoms for patients with amylin-immunoreactive tumors. Of the six patients with amylin-reactive noninsulinoma tumors, only one patient had a type II diabetes mellitus. Serum gastrin levels of amylin-immunoreactive gastrinomas were below the median of all gastrinomas (850 pg/ml): patient I.M. 280 pg/ml, L.T. 300 pg/ml, H.K. no data. Unfortunately, no data was available on the serum insulin levels of the two patients with amylin-negative insulinomas.

Discussion

Amylin was first isolated from a human insulinoma in 1986.[7] Since that time, amylin immunoreactivity and mRNA have been found in a few pancreatic B-cell tumors.[7,15,21,22,24] Recently, a more comprehensive study was published in which amylin immunoreactivity was investigated in 21 endocrine pancreatic tumors by immunohistology.[23] All insulinomas (n = 10) were amylin-immunoreactive. Additionally, seven nonfunctioning tumors, one gastrinoma, one glucagonoma, and one VIP tumor were reactive both for amylin and insulin. In this study, amylin immunoreactivity was consistently found in insulin-immunoreactive B cells and was closely related to insulin expression.

Our investigations confirm the view that most insulinomas are amylin-immunoreactive (13 of 15 tumors). Two nonfunctioning tumors of the pancreas revealed amylin and insulin immunoreactivity. However, two insulinomas and several other types of pancreatic endocrine tumors were negative for amylin but positive for insulin by immunohistology. The density of amylin- and insulin-immunoreactive cells varied among the different tumors. In our study, no close relationship existed between the two peptides: expression of insulin in pancreatic endocrine tumors was not necessarily accompanied by expression of amylin.

There is good evidence that amylin is mainly synthesized in B cells,[11–15,39,40] and it is disputed whether this peptide plays an important role in the pathogenesis of type II diabetes.[41–45] Therefore, it was not surprising to detect amylin immunoreactivity in most B cell tumors (13 of 15 insulinomas). However, two pancreatic gastrinomas that were negative for insulin stained positive for amylin in the present study. In addition, amylin-immunoreactive endocrine tumors have been found in the intestine: one gastrinoma of the duodenum and one tumor associated with carcinoid syndrome of the jejunum. Gastroenteropancreatic endocrine tumors are often composed of various cell types with multiple hormone expression.[46] Our results confirm these observations. It has been shown that phenotypically distinct islet cells arise from a common endodermal precursor.[47] Therefore, neoplastic transformation of

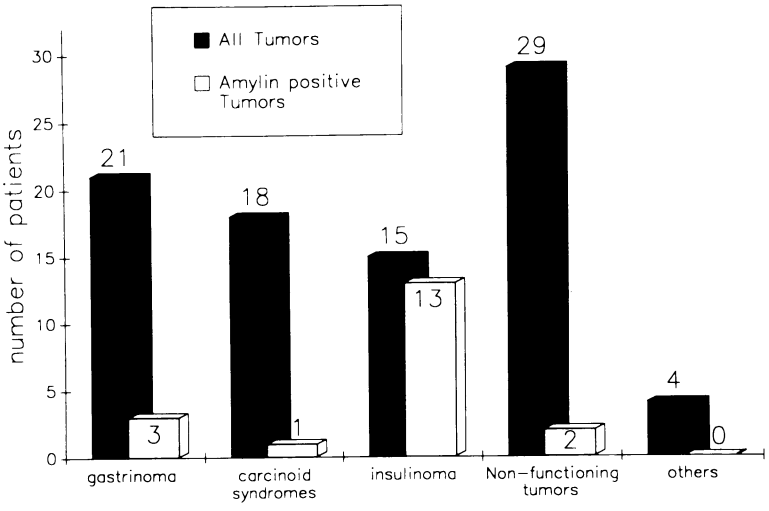

Figure 5. *Occurrence of amylin-immunoreactive and amylin-negative tumors in different parts of the intestine and pancreas.*

these precursor cells might be associated by coexpression of the amylin gene with other genes of peptide hormones in the same tumor.

Up to now, no amylin-immunoreactive endocrine tumors outside of the pancreas have been described. Medullary C cell carcinomas are characterized by amyloid deposition mainly consisting of procalcitonin and calcitonin.[48] Characterization of the amyloid of a human somatostatinoma did not reveal amylin immunoreactivity.[49] Amylin immunoreactivity in endocrine tumors of the intestine occurs only rarely: two of 31 neoplasmas of the intestine were amylin-immunoreactive. Staining for insulin was completely negative in both tumors. Examinations by ultrasound and computer tomography before surgery, as well as exploration of the pancreas during surgery, ruled out a pancreatic tumor in these two cases. There is no doubt that these tumors originated from the intestine. Unfortunately, there was no adequate tissue available to measure amylin mRNA in these cases. Because there are no B cells in the intestine, it is likely that these two tumors were derived precursor cells or other types of endocrine cells of the intestinal mucosa. Recently, radioimmunoassay measurements of extracts of several parts of the gastrointestinal tract showed amylin immunoreactivity in the stomach and intestine.[28,30] Amylin-immunoreactive endocrine cells were found in the normal mucosa of the stomach, duodenum, and rectum by immunohistochemistry.[29] In the rat, specific amylin mRNA could be detected by Northern blotting in the stomach and even the lung.[31]

After determination of the amino acid sequence of amylin, it was possible to clone the complementary DNA and the chromosomal gene encoding this polypeptide.[21,22,50–56] Complementary DNAs of amylin precursors from humans and other mammals have shown these to be relatively small proteins comprising about 90 amino acids.[22,40,55] The human amylin gene seems to be a single copy gene, and it has been localized to the p12.3 region of chromosome 12.[21,56] Several mRNAs from 0.9 to 2.1 kb have been determined in insulinomas and human B cells.[21,52,57] In the present study, a mRNA of 1.8 kb was seen in Northern blot analysis of one insulinoma and one nonfunctioning tumor. *In situ* hybridization revealed amylin mRNA in the same nonfunctioning tumor and another insulinoma. As RNA is very unstable, the varying time from resection of the tumor until fixation or freezing (5 minutes to 2 hours) may be the reason why only single gastroenteropancreatic endocrine tumors showed mRNA abundance. There is no doubt that amylin is expressed in a proportion of these tumors, even in other tumors than insulinomas.

The number of curative resections was significantly higher in amylin-immunoreactive tumors. This may be due to the fact that most amylin-positive tumors are insulinomas. Insulinomas are often benign, grow slowly, and cause symptoms of hypoglycemia in an early stage of the disease.[58] It seems questionable whether amylin itself is a marker for a high differentiation of gastroenteropancreatic endocrine tumors and a better prognosis.

Amylin is thought to act as a hormone but its physiological role is still unclear. We could not find any specific symptoms in patients with amylin-immunoreactive tumors. Clinical symptoms were nonspecific or caused by the dominating (and secreted) hormone, e.g., insulin or gastrin. From the six patients with amylin-immunoreactive tumors, which were not insulinomas, only one patient had a

type II diabetes mellitus. The others had no impaired blood glucose levels.

In summary, amylin is a frequent marker of pancreatic insulinomas. However, 31% of all amylin-immunoreactive tumors are noninsulinomas of the gastroenteropancreatic endocrine system. These tumors may be located in the intestine.

Acknowledgments

The skillful technical assistance of Mrs. B. Pütz and Mrs. I. Höpner is gratefully appreciated. The complementary DNA probe was generously supplied by Dr. D.F. Steiner (Department of Biochemistry and Molecular Biology, University of Chicago).

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