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CHROMOGRANINS — NEW SENSITIVE MARKERS FOR NEUROENDOCRINE TUMORS

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Abstract

Chromogranins A, B and C, proteins that are costored and coreleased with peptides and amines, have been identified in a variety of endocrine and nervous tissues, both normal and neoplastic. We examined the secretion of chromogranin A and chromogranin A + B by hormone-producing tumors in patients with endocrine pancreatic tumors (EPT), carcinoid tumors, pheochromocytomas and small cell lung cancer (SCLC). Radioimmunoassay (RIA) of the plasma/serum concentrations of chromogranin A + B showed a greater sensitivity than RIA of chromogranin A alone. All patients with EPT, carcinoids and pheochromocytomas had increased levels of chromogranin A + B, whereas a small number of the patients (5/18 with EPT and 1/3 with pheochromocytomas) had normal levels of chromogranin A. Also in immunocytochemical stainings, our polyclonal antiserum detecting both chromogranin A and B showed a greater sensitivity than other available antisera against chromogranin A, B and C.

Key words: Neuroendocrine tumors, chromogranins A and B, radioimmunoassay.

The chromogranins represent a family of proteins with a widespread distribution in mammalian neuroendocrine tissues and tumors (1–4). They appear to be the most abundantly secreted proteins in higher organisms.

The first detected member of the family, chromogranin A, was discovered in 1965 (5) and subsequently purified in 1967 from bovine adrenal medulla (6). It was found to be a highly acidic protein with a molecular weight of approximately 75000. In 1985, a larger acidic protein with a molecular weight of 100000, belonging to the same family, was found in bovine adrenal medulla and was designated chromogranin B or secretogranin I (7). It has been shown that chromogranin B is the predominant component of rat and human chromaffin granules (7–9). Recently a third member of the family, also very acidic, with a molecular weight of about 86000, was identified in pituitary secretory granules and this protein has been named chromogranin C or secretogranin II (10, 11).

The chromogranins are supposed to be costored (12) and coreleased with peptides and amines from granules in endocrine cells, but so far no function has been attributed to these proteins.

The screening of neuroendocrine tumors and chromogranins. The Grimelius' silver staining technique has been the most outstanding procedure for verifying the neuroendocrine properties of tissues and tumors immunohistochemically. During the last few years, the role of chromogranin A as a neuroendocrine marker has been documented both immunocytochemically and in radioimmunoassays (3, 13). O'Connor et al. have shown that plasma chromogranin A is elevated in patients with peptide-producing tumors. Interestingly, a possible relationship between chromogranin A and Grimelius' argyrophilia has been postulated, on the basis of homologies in their distributive patterns (12, 14). Recently, it was proposed that the glucose part of chromogranin A might interact with other nonendocrine components to form silver-complexing sites accouting for granular argyrophilia (15). The chromogranins A, B and C make up about 87% of the total soluble proteins of chromaffin granules. The relative distribution of the 3 chromogranins varies between speices and also tissues, chromogranin A being the predominant type in bovine chromaffin granules and chromogranin B the major component in human chromaffin granules (8).

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Chromogranin-like immunoreactivity or chromogranin A + B. Our objective in the studies of chromogranins was to obtain a good 'screening' marker for neuroendocrine tumors and not necessarily a specific antiserum against one of the subtypes of chromogranins. In our preparation, we used human pheochromocytoma and a method previously described by O'Connor et al. (16) to purify chromaffin granules. The lysate from chromaffin granules was separated by gel filtration, and the void volume peak containing chromogranin (tested by immunoneutralization) was further separated by an anion exchange column, since the chromogranins are known to be acidic. We obtained several fractions (Fig. 1), which again were tested by immunoneutralization and 2 fractions, F3 and F4, completely neutralized monoclonal chromogranin A antibodies and also polyclonal antisera against chromogranin A. These partially purified antigens, F3 and F4, were used in the immunization schedule. Two antisera were obtained, one against F3 and the other against F4, which cross-reacted strongly. The latter showed superior immunoreactivity and was therefore used in subsequent immunocytochemical stainings and in the radioimmunoassay.

In the continued work, our antiserum seemed to stain a higher proportion of cells, including pancreatic B-cells (Fig. 2), than other chromogranin A antisera available (O'Connor, Lloyd and Winkler). It was thus considered of interest to further purify and characterize our antigens F3 and F4. RP-HPLC was performed and both F3 and F4 eluted in seveal peaks (Fig. 3) Amino acid sequencing of 2 major peaks of F3 showed identity with the N-terminal sequence of human chromogranin A in one and identity with a midportion fragment of human chromogranin A in the other. Amino acid sequencing of the major peak of F4 showed identity with a midportion fragment of human chromogranin B. A smaller peak was identical with a midportion fragment of human chromogranin A.

Although much remains to be done in a complete characterization of our antigens and antisera, we consider it likely that the antiserum obtained against F4 is a polyclonal antiserum, which detects chromogranin A and B immunoreactivity.

In a recent study, we compared the results with 2 radioimmunoassays, one measuring plasma chromogranin A and the other plasma chromogranin A + B. We have also compared our antiserum to other available chromogranin antisera available immunocytochemically.

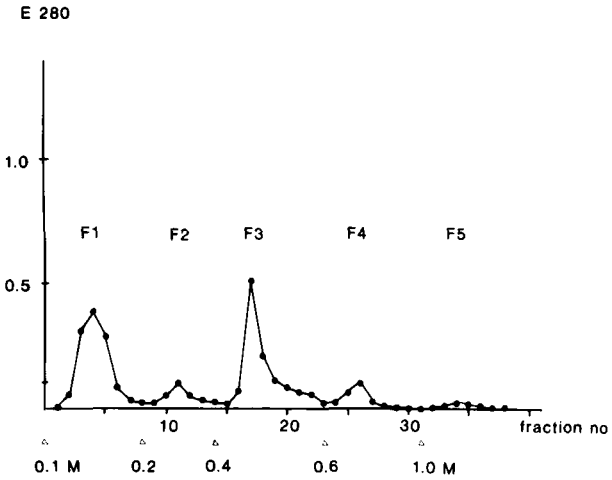

Fig. 1. Fractions obtained by ion exchange chromatography on a DEAE cellulose column, using a stepwise gradient.

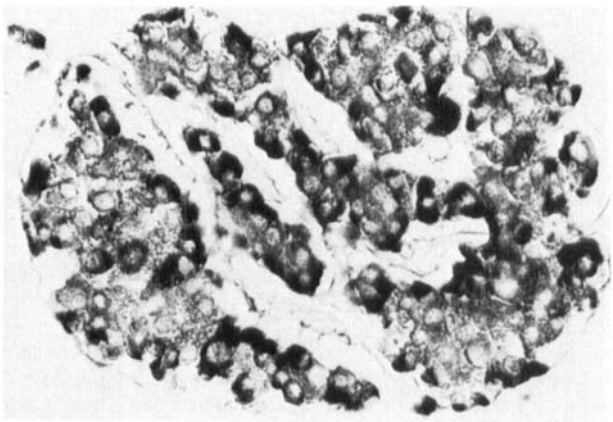

Fig. 2. Human pancreatic islet stained with the chromogranin A + B antiserum less than optimally diluted. One peripheral cell population (presumably A-glucagon cells) displays heavy cytoplasmic staining, while the centrally located cells (presumably B-insulin cells) show less pronounced immunoreactivity, ×640.

Material and Methods

Patients. Samples of plasma or serum were obtained from 39 subjects with neuroendocrine tumors. There were 19 patients with endocrine pancreatic tumors (3 had the Zollinger-Ellison syndrome, 4 had insulinomas, 5 had the watery diarrhea hypokalemia achlorhydria (WDHA) syndrome and the remaining 5 had so-called 'non-functioning' tumors, i.e. they had no specific hormone-related symptoms). Seventeen patients had metastatic carcinoid tumors of different origin (13 had midgut carcinoids, 3 had foregut carcinoids and 1 a carcinoid of unknown origin). Three patients had tumors of adrenal medullary origin (two pheochromocytomas and one ganglioneuroblastoma).

Plasma samples from 15 patients with small cell lung cancer (SCLC) all classified as WHO group II, were also analyzed for chromogranin A + B immunoreactivity by our RIA.

Plasma or serum samples were also obtained from 22 healthy controls (11 men and 11 women). Plasma chromogranin A + B levels in patients with cancer of the testis, prostate, urinary bladder and kidney were included as controls (10 patients in each tumor category).

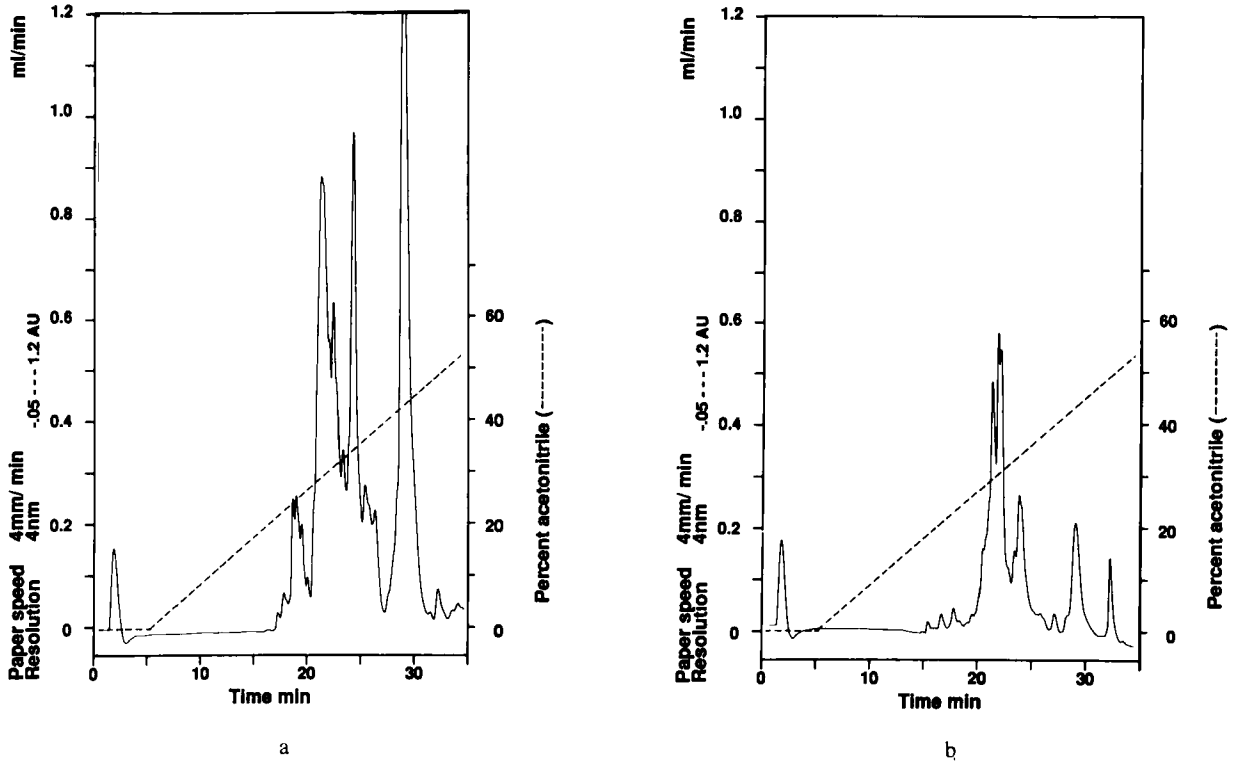

Fig. 3. a) RP-HPLC of chromogranin fraction F3 from human pheochromocytoma and b) RP-HPLC of chromogranin fraction F4 from human pheochromocytoma.

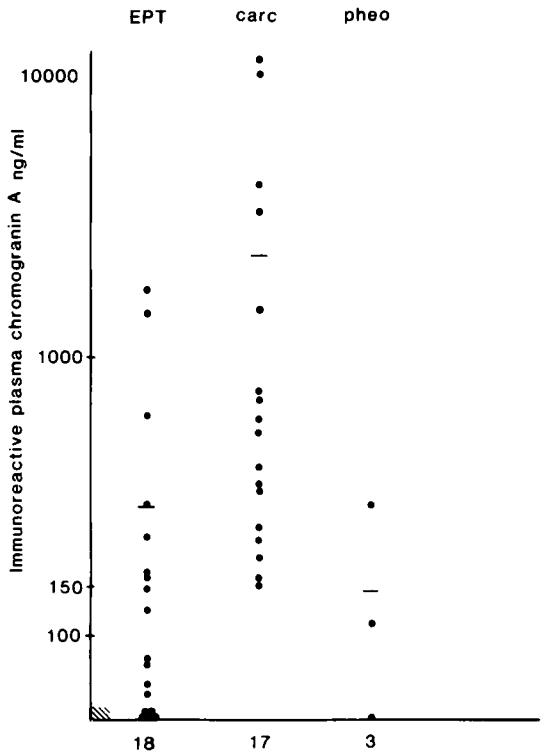

Fig. 4. Immunoreactive plasma chromogranin A in patients with endocrine pancreatic tumors (EPT), carcinoids (carc) and pheochromocytomas (pheo) as determined by O'Connor.

Table 1

Plasma chromogranin A and A + B in patients with endocrine pancreatic tumors

Diagnosis	Chromogranin A (ng/ml)	Chromogranin A + B (ng/ml)
Zollinger-Ellison (n=3) Mean ± SEM	713±374	2 989±691
Insulinoma (n=4) Mean ± SEM	507±411	4 541±2 193
WDHA syndrome (n=5) Mean ± SEM	69±6	886±84
Somatostatinoma (n=1)	296	3 352
Non-functioning (n=5) Mean ± SEM	108±32	1 398±315
Mean ± SEM of the group	297.5±114	2 444±677
Upper normal level	50	330

Radioimmunoassay with chromogranin A antiserum. The plasma concentration of chromogranin A was determined by a radioimmunoassay performed by O'Connor (13, 17). The upper reference level was set at 50 ng/ml. In this RIA, 13 out of 18 patients (72%) with endocrine pancreatic tumors (EPT) had levels above the upper reference limit (Fig. 4, Table 1). Two patients with insulinomas, one with the WDHA syndrome and two with non-

Table 2

Plasma chromogranin A and A + B in patients with carcinoid tumors

Diagnosis	Chromogranin A (ng/ml)	Chromogranin A + B (ng/ml)
Midgut carcinoid (n=13)		
Mean ± SEM	2 721±1 231	7 713±1 579
Foregut carcinoid (n=3)		
Mean ± SEM	800±345	6 844±2 411
Unknown origin (n=1)	193	2 731
Mean ± SEM of the group	2 233±960	7 245±1 287

Table 3

Plasma chromogranin A and A + B in patients with adrenal medullary tumors

Diagnosis	Chromogranin A (ng/ml)	Chromogranin A + B (ng/ml)
Pheochromocytoma (n=2)		
Mean ± SEM	66.9±44	928±236
Ganglioneuroblastoma (n=1)	296	3 784
Mean ± SEM of the group	143±80.6	1 880±962

Table 4

Demonstration of heterogeneity of different chromogranin antisera

		Pancreas homo		Pancreas rat	
		Fo	Bo	Fo	Bo
A	Lloyd	++	++	(+)	–
A	O'Connor	(+)	++	–	–
A	Winkler	(+)	+	++	++
B	Winkler	(+)	–	++	++
C	Winkler	–	–	(+)	–
A + B		++	++	++	++

(+) = very weak, + = weak, ++ = strong, Fo = formalin-fixed, Bo = Bouin-fixed.

functioning tumors had normal levels. The highest level was found in a patient with an insulinoma, 1733 ng/ml. The mean (± SEM) for the whole group of patients with EPT was 297 ± 114 ng/ml. All 17 patients with malignant carcinoid tumors had an increased level, with a mean of 2333 ± 960 ng/ml. The patients with the midgut carcinoids displayed the highest levels and there was a wide variation within the group (Fig. 4, Table 2). Two of three patients with adrenal medullary tumors showed slightly increased concentrations with a mean of 143±80 ng/ml.

Radioimmunoassay with a polyclonal antiserum against chromogranin A + B. To establish a reference range for our RIA, plasma samples from routine health check-ups were collected. Their mean concentration of

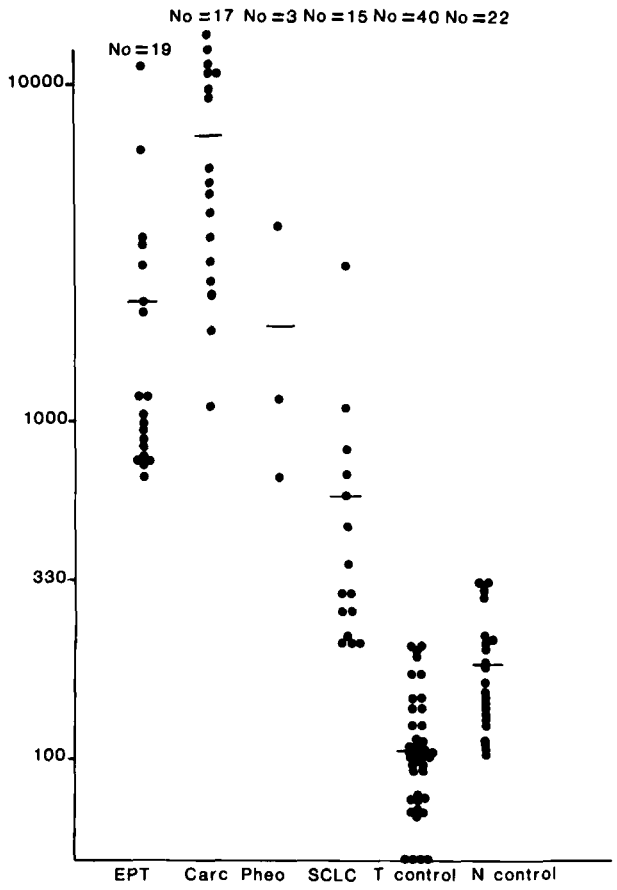

Fig. 5. Immunoreactive plasma chromogranin A + B (ng/ml) in patients with endocrine pancreatic tumors (EPT), carcinoids (carc), pheochromocytomas (pheo), small cell lung cancer (SLCL), other non-endocrine tumor patients (T control) and healthy controls (N control).

plasma chromogranin A + B was 181±33 ng/ml (range 102–322) (Fig. 5). Other patients with malignant non-endocrine tumors were included as controls (tumor controls). None of the patients with testicular (n=10), prostatic (n=10), bladder (n=10) or renal cancer (n=10) had levels above our upper reference limit, which was set at 330 ng/ml. Plasma chromogranin A + B in the tumor controls ranged between 26 and 221 ng/ml.

All 19 patients with EPT showed increased plasma levels of chromogranin A + B immunoreactivity, with a mean of 2440±677 ng/ml (Fig. 5, Table 1). The patients with insulinomas and gastrinomas had the highest levels. Patients with nonfunctioning tumors also had clearly elevated values with a mean of 1398±315 ng/ml. Somewhat lower but still clearly elevated levels were found in patients with the WDHA-syndrome.

Plasma samples from carcinoid patients showed the most extreme elevations with a mean value of 7245±1287 ng/ml. Similar to patients with EPT, there was a wide range, with the highest value found in a patient with a midgut tumor, 19054 ng/ml. The patients with midgut and

foregut carcinoids had mean values in the same order. All 3 patients with adrenal tumors had elevated levels of chromogranin A + B, with a mean of 1880±961 ng/ml. The highest concentration, 3784 ng/ml, was observed in the ganglioneuroblastoma patient (Table 3).

Since small cell lung cancer (SCLC) can exhibit neuroendocrine properties and hormone production, it was considered of interest to examine a number of patients with this type of tumor. Not unexpectedly, half of these patients, 7/15 (45%) showed increased plasma levels of chromogranin A + B (mean 596±183 ng/ml).

Immunocytochemistry. In immunocytochemical stainings, our polyclonal antiserum was superior to all the other chromogranin antisera available. It could be used in high dilutions (1:3200) and it reacted with pancreatic B-cells, which are not stained by the monoclonal antibody produced by Lloyd. Table 4 illustrates the heterogeneity in the staining properties of chromogranin antisera and also the sensitivity of our antiserum.

Discussion

Chromogranin A, B and C are at present attracting great interest. Several investigators have demonstrated the presence of these glucoproteins in neuroendocrine tissues in the body. O'Connor & Deftos (13) found elevated levels of chromogranin A in patients with peptide-producing tumors. However, by using a polyclonal antiserum raised against a mixture of chromogranin A and B in the present study, an even better distinction between normal subjects, patients with nonendocrine tumors and those with neuroendocrine tumors was achieved. Immunocytochemically, there was also a difference between our chromogranin A + B antiserum and pure chromogranin A antisera, in that our antiserum seemed to stain a higher proportion of cells and could be used in higher dilutions. All the patients with EPT, carcinoids and pheochromocytomas had active disease, although not necessarily advanced. It will be very important to find out whether chromogranin A + B could be helpful in the early diagnosis and screening for endocrine neoplasia, especially in patients with MEN-1. Several studies of the amino acid sequences of chromogranin A have shown the presence of basic pairs which are potential cleavage sites for the generation of smaller polypeptides (18). A role for chromogranin A and B as precursors for other peptides has been suggested, since a homology has been found between chromogranin A and pancreastatin (19), a newly characterized smaller peptide (20).

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