Different patterns of chromogranin A and Leu-7 (CD57) expression in gastrointestinal carcinoids: immunohistochemical and confocal laser scanning microscopy study*

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Thirty-seven carcinoids of the gastrointestinal tract were studied with immunohistochemical staining for chromogranin A (CgA) and Leu-7 (CD57). The aim of this study was to distinguish and describe the differences in patterns of distribution of immunostaining of these two non-specific neuroendocrine markers in neuroendocrine tumors of different degree of differentiation (typical, vs. atypical carcinoids) at different gastrointestinal sites. Selected 5 tumors from this group were studied in detail using confocal laser scanning microscopy (CLSM) and double immunofluorescence staining to disclose the patterns of distribution of CgA and CD57 positive granules within the individual tumor cells. Prominent differences in the patterns of immunohistochemical staining for both studied markers related to the degree of differentiation of the tumors were observed in studied neoplasms. Regular (diffuse) strongly positive immunoreaction for CgA predominated in typical carcinoids, whereas atypical tumors were characterized by irregular patchy staining. Both typical and atypical tumors displayed predominantly irregular patchy staining for CD57. The results of CLSM study indicate that different modes of CgA and CD57 expression and/or co-expression can occur in neuroendocrine tumors. Neoplastic cells that contained either CgA positive neuroendocrine granules (NEG), or Leu-7 positive NEG, were frequently observed in different areas of the tumor samples, especially in atypical carcinoids. Varying number of cells revealed co-localisation of both CgA and Leu-7 within the NEG. Similar co-localisation of CgA and CD57 was found in non-neoplastic Kultschitski cells of the mucosa of small intestine. In conclusion, our results suggest that the differences in CgA and CD57 expression in human neuroendocrine tumors are related to the degree of differentiation of the neoplasms and probably reflect the degree of maturation (functional state) of neuroendocrine granules within the neoplastic cells.

Key words: Gastrointestinal carcinoid, chromogranin A, Leu-7 (CD57), immunohistochemistry, confocal laser scanning microscopy.

Neuroendocrine tumors of gastrointestinal (GI) tract represent a group of relatively rare malignant epithelial neoplasms characterized by less aggressive biological behavior compared to GI carcinomas, distinctive histological appearance and by expression of markers suggesting neuroendocrine differentiation of neoplastic cells [8, 23]. The histogenetic background of these tumors has not been clarified yet, however, the origin of these tumors from enterochromaffine epithelial cells, which belong to amine precursor uptake and decarboxilation system (APUD) or diffuse neuroendocrine system (DNES) is widely accepted [1, 15]. Several markers of neuroendocrine differentiation are recently conventionally used in histological diagnosis and characterization of neuroendocrine tumors [6, 13, 16, 22]. Among them, chromogranin-A (CgA) and Leu-7 (CD-57), proteins, located in the core of neuroendocrine granules (NEG), have a special significance [9, 17, 20, 25]. Never-

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theless, the results of immunohistological detection of these markers in individual neuroendocrine neoplasms can be controversial [2, 3, 4, 19]. The aim of this study was to compare the patterns of distribution of immunostaining of CgA and CD57 in neuroendocrine tumors of different degree of differentiation (typical, vs. atypical carcinoids) at different gastrointestinal sites and to determine the patterns of distribution of CgA and CD57 positive granules within the individual tumor cells using confocal laser scanning microscopy (CLSM) and double immunofluorescence staining.

Material and methods

Paraffin blocks were obtained from the files of Hlava 1st Department of Pathology, 1st Faculty of Medicine, Charles University, Prague, from the period of years 1992–2000. The diagnosis was rendered by an experienced pathologist (VM) based on examination of hematoxylin and eosin (H&E) and Grimelius stains, and on immunohistochemical detection of NSE and synaptophysin.

Immunohistochemistry. Five-micron-thick representative tissue sections were deparaffinized in xylene and hydrated in graded alcohol. The slides were incubated with the following primary antibodies: 1) rabbit polyclonal antihuman CgA antibody, (DAKOPATTS A 430, DAKO, Glostrup, Denmark; diluted 1:200 withTBS (Tris buffered saline) containing 5% fetal calf serum), consequently visualized using a LSAB+ Peroxidase Kit (DAKO, Glostrup, Denmark), 2) mouse monoclonal antihuman CgA antibody, (clone DAK A3 DAKO, Glostrup, Denmark; diluted 1:50 withTBS containing 5% fetal calf serum), and 3) mouse monoclonal antihuman CD57 antibody, (clone NK-1 DAKO, Glostrup, Denmark; diluted 1:50 withTBS containing 5% fetal calf serum). The EnVision Peroxidase Kit (DAKO, Glostrup, Denmark) was used to visualize the sections incubated with primary mouse monoclonal antibodies (either anti-CgA, or anti-CD57) (Tab. 1). The chromogen 3,3-diaminobenzidine (FLUKA, Buchs, Switzerland) was applied in all sections, and counterstaining was performed with Harris hematoxylin. Appropriate positive controls were performed at the same time. Sections incubated without primary antibody were used as a negative control.

The stained slides were evaluated semi-quantitatively, according to the quantity and distribution of positive neoplastic cells in each tumor, using an optical microscope Nikon ECLIPSE E 400. Three different patterns of positive immunostaining were distinguished: 1) regular (diffuse) immunostaining – the majority of tumor cells showed expression of detected marker, 2) irregular (patchy) immunostaining – positive staining was present only in some areas of the tumor, prominent irregularities in the intensity of immunostaining within the tumor cells were present, and 3) dispersed (solitary) positive cells were present within the tumor the majority of cells were not stained for evaluated marker.

Table 1. Antibodies: origin and dilution

Antibody	Specification/ Clone	Dilution	Species
chromogranin-A (polyclonal)	code no. A 430	1:200	rabbit
chromogranin-A (monoclonal)	DAK-A3	1:50	mouse
leu-7 (monoclonal)	NK-1	1:50	mouse

Source of all antibodies: Dako, Glostrup, Denmark

Confocal laser scanning microscopy. Five selected paraffin blocks from different tumors were studied using confocal laser scanning microscope Leica TCS SP (Leica, Germany). Microsections were deparaffinized and pre-treated with 2.73% oxygen peroxide and 0.1% sodium azide, diluted with distilled water. Double immunolabelling was performed in each slide: Slides were at first incubated with rabbit polyclonal antihuman CgA antibody (DAKOPATTS A 430, DAKO, Glostrup, Denmark; diluted 1:800 withTBS containing 5% fetal calf serum) at 4 ˚C for 12 hours. Consequently, incubation with secondary anti-rabbit IgG antibody, conjugated with FITC (Jackson, West Grove, USA; diluted 1:100 with PBS (phosphate buffered saline)) was performed at room temperature for 30 min. At the second step, slides were incubated with mouse antihuman monoclonal antibody (clone NK-1 DAKO, Glostrup, Denmark; diluted 1:50 inTBS with 5% fetal calf serum) for 60 min at 37 ˚C. Finally, incubation of slides with anti-mouse IgG antibody, conjugated with CY-3 (Jackson, West Grove, USA; diluted 1:100 with PBS) in room temperature for 30 min was performed. The slides were mounted in Mowiol (Hoechst, Germany) and observed in confocal microscope using 40x, 63x and 100x oil immersion objectives. An argon-ion laser at a wavelength of 488-nM was used for excitation of FITC and a helium-neon laser at a wavelength of 543-nM for excitation of Cy-3, respectively. Simultaneous excitation of FITC and Cy-3 was used to evaluate double staining within the slides. Final images were obtained by projection of series of 0.285-μm optical sections on a single plane (maximum projection). Kultschitski cells of jejunal mucosa were used as a positive control for both detected markers of NEG. The slides incubated without primary or secondary antibodies were used as negative controls.

Results

Table 2 summarizes the cases, location and microscopic appearance of tumors. Four tumors originated in foregut, stomach (n=3) and duodenum (n=1), 26 neoplasms were located in midgut, small intestine (n=7), appendix (n=18) and 3 carcinoid tumors were present in hindgut (rectum).

Table 2. Results of immunohistochemical staining for chromogranin-A and Leu-7 in gastrointestinal neuroendocrine tumors

Tumor site	No.	Chromogranin-A mouse	Chromogranin-A rabbit	Leu-7	Histology
stomach	1.	R	R	I	t
	2.	R	R	R	a
	3.	neg	D	I	a
duodenum	4.*	neg	I	I	a
small intestine	5.	R!	R!	R!	t
	6.	I	R	I	t
	7.*	R!	R!	R!	t
	8.	R!	R!	R!	t
	9.	R	R	R	t
	10.	I	I	I	a
	11.	neg	neg	R	a
appendix	12.*	I	R	I	t
	13.*	R!	R	I	t
	14.	R!	R!	I	t
	15.	R!	R!	I	t
	16.	R!	R!	I	t
	17.	neg	R	I	t
	18.	D	R	R	t
	19.	R!	R!	I	t
	20.	R!	R!	I	t
	21.	R!	R!	I	t
	22.	R!	R!	I	t
	23.	R!	R!	I	t
	24.	I	R	I	t
	25.	R	R	I	t
	26.	neg	R	I	t
	27.	R!	R	I	t
	28.	R!	R!	I	t
	29.	R!	R!	I	t
rectum	30.	D	R	I	t
	31.	I	R	I	t
	32.	I	R	I	t
pancreas	33.	D	I	D	t
	34.	R	R	R	t
	35.	neg	R	I	a
	36.	R	I	I	a
	37.	I	I	I	a

R – regular (diffuse) positivity, I – irregular (patchy) positivity, D – dispersed positive cells, neg – negative staining, t – typical tumor, a – atypical tumor, No. – number of case, ! – pronounced immunostaining at the periphery of nests of tumor cells, * – case investigated with CLSM.

Additionally, we also included 5 primary neuroendocrine tumors of pancreas. All studied tumors revealed characteristic morphology of neuroendocrine neoplasms with solid nests, ribbon-like or small tubular structures. Twenty-nine tumors were classified as typical, 8 tumors showed solid arrangement with more prominent cytological irregularities and were classified as atypical.

Immunohistochemical study. The results are summarized in Table 2. All 4 tumors located in the foregut showed strong granular staining for CgA with polyclonal rabbit antibody (A 430). Two of these tumors morphologically classified as typical showed CgA-reactivity with monoclonal mouse antibody (DAK-A3), while two of foregut tumors classified as atypical, were negative for CgA with this antibody. Positive staining for CD57 was observed in all foregut tumors in predominantly irregular pattern that did not correspond with the distribution of positive staining for CgA.

The results of immunoreactivity differed in the midgut tumors depending on the localization and on the degree of differentiation of the tumor cells. Regular (diffuse) strongly positive immunoreaction predominated in typical carcinoids of small intestine (Fig. 1A–C), whereas irregular patchy staining for all three markers or negative immunoreaction for CgA was found in atypical carcinoids (Fig. 1D–F). One atypical tumor of small intestine revealed negative immunoreaction for CgA with both antibodies, however, this tumor was positive for CD57. The majority of appendical carcinoids revealed regular (diffuse) positive staining for CgA using both antibodies, whereas irregular patchy staining for CD57 was found in 17 of 18 of these tumors. Typical carcinoids of the midgut revealed frequently characteristic phenomenon of pronounced immunostaining of CgA at the periphery of solid nests of tumor cells (Fig. 1A–B).

Three rectal carcinoids representing hindgut tumors were characterized by irregular patchy immunoreactivity for CgA detected by monoclonal antibody (DAK A3) and for CD57, and by regular diffuse positive staining for CgA using rabbit antihuman polyclonal antibody (A 430).

Immunostaining pattern of both studied markers was predominantly irregular in pancreatic neuroendocrine neoplasms. All tumors that were classified as positive showed granular cytoplasmatic positivity of CgA and CD57.

Typical strong granular cytoplasmic positivity of both these markers was observed in Kultschitski cells of intestinal mucosa.

CLSM study. Three modes of the staining of NEG were identified in merged images within the cells revealing neuroendocrine differentiation: Green CgA-positive granules (FITC positivity), red CD57-positive granules (CY-3 positivity), and yellow granules containing both detected antigens (summation of FITC and CY-3 positivity). Numerous yellow granules showing immunoreactivity for both markers predominated within the cytoplasm at abluminal compartment of Kultschitski cells. Several NEG positive only for either CgA or CD57 were found within the cytoplasm at the luminal pole of Kultschitski cells (Fig. 2A–C).

Detailed analysis of the tumor samples discovered the presence of four cell types present within the tumors, depending on the immunostaining of NEG. The first type, resembling Kultschitski cells, contained predominantly yellow granules (positive for both markers – CgA and CD57)

Figure 1. Immunohistochemical detection of CgA and CD57 in gastrointestinal carcinoids. A,B and C – typical carcinoid of small intestine. Diffuse immunostaining for CgA accentuated at the periphery of nests of tumor cells: polyclonal rabbit antibody (A), monoclonal mouse antibody (B). Strong diffuse immunostaining for CD57 (C). D,E and F – atypical carcinoid of small intestine. Irregular positivity for CgA: polyclonal rabbit antibody (D), weak immunostaining with monoclonal mouse antibody (E). Irregular patchy immunostaining for CD57 (F); original magnification, 100x.

with the admixture of sparse CgA-positive (green) or CD57-positive (red) granules. The second cell type was characterized by the presence of relatively big portion of CgA-positive (green) granules located in one pole of the cell with the admixture of CD57-positive (red), and both CgA and CD57-positive (yellow) granules located in the opposite pole of the cell. The third cell type represented the cells containing either CgA-positive (green), or CD57-positive (red) granules. The fourth cell type was negative for both studied markers. Typical carcinoid tumor was composed predominantly of the first and the second cell types. The majority of cells revealed polarization with either green or yellow granules in the pole near the stroma and red granules in the pole oriented to the center of the nest of tumor cells (Fig. 2D, E). Cells of the third type (positive either for CgA, or for CD57) randomly distributed within the cells of the fourth type (negative for both markers) were present in samples of atypical carcinoid (Fig. 2F). The mixture of cells of the second, third and fourth types predominated in pancreatic neuroendocrine tumor (Fig. 2G).

Discussion

This study attempted to characterize the differences in immunostaining patterns of two non-specific neuroendocrine markers, CgA and CD57 in neuroendocrine tumors of different degree of differentiation (typical, vs. atypical

Figure 2. CLSM results. A,B and C – Kultschitski cell. A, CgA positive granules (green), B, CD57 positive granules (red), C, summation of positive staining for both markers (yellow) suggesting the presence of both CgA and CD57 within the granules (original magnification, 1000x). D,E,F and G – tumor cells of neuroendocrine neoplasms. D, nest of small intestinal typical carcinoid tumor: granules positive for CgA (green) or for both CgA and CD57 (yellow) at the peripheral pole of tumor cells, CD57 positive (red) granules are located at the opposite pole of these cells (original magnification, 400x). E, different cell subtypes within a small intestinal typical carcinoid tumor: cells containing predominantly CgA positive (green) granules, and cells containing granules positive for both markers (yellow). Scattered CD57 positive granules (red) randomly distributed within the cells (original magnification, 1000x). F, irregularly distributed cells containing either CgA positive (green), or CD57 positive (red) granules in a nest of atypical small intestinal carcinoid. The majority of cells are negative for both markers (original magnification 630x). G, a mixture of CgA positive (green), CD57 positive (red) and both CgA and CD57 positive (yellow) granules within a cell of atypical pancreatic neuroendocrine tumor (original magnification, 1000x).

carcinoids) at different gastrointestinal sites and to disclose the patterns of distribution of CgA and CD57 positive granules within the individual tumor cells. To our knowledge, the relation between CgA and CD57 positive cells within neuroendocrine tumors, the differences in immunostaining of these markers in individual neoplastic cells and the distribution of neurosecretory granules positive for CgA and CD57 within the cytoplasm of non-neoplastic Kultschitski cells and neoplastic cells of neuroendocrine tumors have not been reported so far.

The diagnosis of carcinoid tumors is based on morphologic features, nevertheless, confirmation of the diagnosis relies on the detection of markers of neuroendocrine differentiation of tumor cells [8, 23]. Among them, chromogranin A and Leu-7 (CD57), being the markers of neurosecretory granules, are widely used [2, 3, 4, 17, 20, 24].

Chromogranins represent a group of acidic soluble polypeptides that are considered the main protein component of neurosecretory granules in different types of endocrine cells [21]. The function of chromogranins has not been fully elucidated yet, however, they have been reported to play an important role as the storage and precursor proteins for smaller hormone peptides like pancreastatin [17, 18, 25]. Chromogranin also helps to stabilize the soluble portion of the secretory granule through interaction with ATP and catecholamines [5]. Presence of CgA has been detected in many different human cells, including the cells of APUD/DNES system [10, 24] and carcinoid tumors [2, 3, 4, 6, 7, 14]. Various peptide fragments of CgA responsible for different immunohistochemical reactions with antibodies raised against their epitopes were described in laboratory conditions [9], as well as in different human neuroendocrine tumors [17]. In the present study 31 of 37 tumors were positive for CgA with mouse monoclonal antibody, while 36 of 37

tumors showed immunoreactivity with rabbit polyclonal antibody to CgA. This observation suggests that wider spectrum of CgA epitopes is covered by the polyclonal antibody and may explain the differences in CgA immunoreactivity described in previous studies in neuroendocrine tumors [2, 3, 4, 19], and in different animal non-neoplastic tissues [10]. In the present study, we observed strong CgA immunoreaction predominantly in typical carcinoids. The staining was usually regular and diffuse, occasionally accentuated at the periphery of solid nests of tumor cells, suggesting different degree of cellular maturation within the tumor. Our study showed that the majority of tumors negative for CgA were atypical carcinoids. Negative CgA immunoreactivity was not related to the site of primary tumor.

Monoclonal antibody HNK-1 (known as anti-Leu-7 or anti-CD57) was initially described as capable to detect human natural killer cells. Lately, immunoreactivity in a wide variety of non-lymphoid tissues was reported. In neural tissues, immunoreactivity of myelin-associated glycoprotein (MAG) with this antibody was shown [12]. It was suggested that anti-Leu-7 antibody recognizes neural cell adhesion molecule (NCAM) [21]. Carcinoid tumors showed immunopositivity for CD57 with HNK-1 antibody. This antibody reacts with intracellular protein of MW 75 KD that is localized within NEG matrix [11, 20]. Very little is known about the physiological role of this protein, nevertheless, it was proven that the protein is different from both CgA and MAG [20]. Anti Leu-7 antibodies are widely used in carcinoid diagnostics [2, 3, 4, 7, 11, 19].

In the present study all tumors showed immunoreactivity for CD57, predominantly in irregular (patchy) fashion. Strong regular positivity of this marker was obsedved in the majority of small intestinal carcinoids, while patchy staining was found in the majority of appendical and rectal tumors. Variable CD57 positivity and scattered isolated cells were also described in appendical carcinoids in a previous study [3]. Two disparate immunostaining patterns suggesting different degree of cellular maturation and/or differentiation within gastrointestinal carcinoids have been already reported [11].

In our study we found differential staining patterns in tumors showing irregular positivity for CgA and CD57, predominantly in atypical tumors. The majority of cells showed either CgA, or CD57 immunoreactivity in these tumors. This finding is consistent with previous reports describing tumors containing CD57 areas negative for chromogranin in some duodenal, colonic and rectal carcinoids [2, 7]. Disparities of immunostaining in gastrointestinal carcinoids suggesting several subsets of neurosecretory granules were mentioned in previous studies [11, 20].

Cytoplasmic distribution of NEG within the cells showing neuroendocrine differentiation has not been studied in detail so far. Kultschitski cells reveal strong diffuse cytoplasmic immunoreactivity for CgA and for CD57 as well. In carcinoid tumors, accumulation of CgA positive NEG was immunohistochemically detected in the periphery or in the basal part of cytoplasm in carcinoid tumor cells [2, 3]. In the present series of carcinoids, we observed similar phenomenon especially in solid nests of typical carcinoids located in the midgut.

The most important part of our study is the description of distribution pattern of CgA and/or CD57 positive NEG within the cells showing neuroendocrine differentiation. Merged images of double immunofluorescence staining obtained by CLSM enabled us to distinguish either CgA or CD57 positive granules from the granules revealing immunopositivity for both these markers. Our results indicate that the majority of granules located within the basal (abluminal) and perinuclear compartments of non-neoplastic Kultschitski cells co-express CgA and Leu-7. Scattered granules containing CD57 are present within the cytoplasm at the luminal pole of these cells.

Neuroendocrine tumors are composed of at least four cell subtypes. The first one resembling Kultschitski cells contains NEG that co-express CgA and Leu-7 and scattered granules expressing either CgA, or CD57. The second subtype is characterized by the predominance of CgA positive NEG, some of these cells contain also CD57 positive NEG. The third cell subtype contains CD57 positive NEG and in the fourth cell subtype no NEG were detected. The first and second subtypes, usually revealing the signs of polarization, predominate in typical carcinoids. The presence of the second, third and fourth types was characteristic for atypical tumors. This observation suggests impaired properties of NEG in neuroendocrine tumors related to the degree of differentiation of neoplastic cells and could explain the differences in the expression of neuroendocrine markers in individual tumors.

In summary, we found that different cell subtypes characterized by NEG containing CgA and/or CD57 are present in neuroendocrine tumors. The pattern of NEG distribution within the cytoplasm and the signs of polarization of neoplastic cells in typical carcinoids resemble to some extent Kultschitski cells. In contrary, atypical tumors are composed of less differentiated cells containing either CgA or CD57 positive granules and of larger proportion of cells negative for both these markers. Further studies will clarify the significance of our findings in the explanation of mechanisms involved in differentiation of neuroendocrine cells and in maturation of NEG.

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