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Gastroenteropancreatic Neuroendocrine Tumours Contain a Common Set of Synaptic Vesicle Proteins and Amino Acid Neurotransmitters

G. Ahnert-Hilger, K. Grube, L. Kvols, I. Lee, E. Mönch, E.-O. Riecken, L. Schmitt and B. Wiedenmann

Human neuroendocrine tumours of the gastroenteropancreatic system contain major integral membrane proteins of small synaptic vesicles of neurons, together with characteristic membrane polypeptides of large dense-core vesicles of neurons and neuroendocrine cells. The membrane polypeptides characteristic for small synaptic and large dense-core vesicles are detected in pheochromocytomas (n = 6), functional (n = 6) and non-functional (n = 6) foregut, and midgut carcinoids (n = 17). All gastroenteropancreatic tumours contain large amounts of amino acid neurotransmitters, i.e. glycine and glutamate. γ-Aminobutyric acid, however, is only found in some foregut carcinoids. Thus, neuroendocrine gastroenteropancreatic tumours possess a vesicle type with a content and membrane composition similar to small synaptic vesicles of neurons.
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INTRODUCTION

The regulated secretion of neurotransmitters via small synaptic vesicles was considered to be restricted to neurons. Recently, a similar vesicle type was discovered in neuroendocrine (NE) cells [1, 2], which probably releases amino acid neurotransmitters such as γ-aminobutyric acid (GABA) by a regulated pathway [3, 4].

Assuming a strong relationship between neuronal small synaptic vesicles (SSV) and a similar type of vesicle in NE cells of the gastroenteropancreatic (GEP) system, major integral membrane proteins of SSV in addition to synaptophysin [5, 6] should be present together with considerable amounts of amino acid neurotransmitters like glycine, GABA, and glutamate in NE GEP tumours.

MATERIALS AND METHODS

Cells and tissues

PC 12 cells [1] and BON cells [7] derived from a rat pheochromocytoma and a human carcinoid, respectively, were cultivated as described. Human tumour tissue was obtained from patients during tumour resection and used in accordance with the standards set by the ethics committees of the University of Heidelberg and the Freie Universität Berlin.

Immunofluorescence microscopy

Cryostat sections of frozen tissues and cultured cells were processed and analysed as described [5].

Antibodies

Antibodies against various intermediate filament proteins were obtained from Boehringer Mannheim (Mannheim, Germany). Polyclonal antibodies against desmin were purchased from Bioscience Products (Emmenbrücke, Switzerland) and a monoclonal antibody against glial fibrillary acidic protein (GFAP) was obtained from Progen (Heidelberg, Germany). A monoclonal antibody SY38 against synaptophysin was used as given [1, 5]. A monoclonal antibody against protein S.V.2 was provided by Dr E. Schweitzer (UCLA Medical School, U.S.A.) [8]. Polyclonal antibodies against the synaptotagmins (p65) were provided by Dr T. Südhof (University of Texas, Dallas, U.S.A.) [9]. A monoclonal antibody against the synaptobrevins (p18) and polyclonal antibodies against protein p29 (p29) were provided by Dr R. Jahn (Yale University, New Haven, U.S.A.) [10, 11]. Polyclonal antibodies against dopamine-β-hydroxylase (DBH) were generated by immunising rabbits with purified bovine chromaffin granule membranes. Using the homogenate from bovine adrenal medulla, the antibody only reacted with a polypeptide of 75 kDa. Murine monoclonal antibody DCV against cytochrome b561 (DCV) was obtained by immunising mice with total bovine chromaffin granule membranes. In western blotting it recognises a polypeptide of $M_r \approx 26$ kDa.

Determination of amino acid neurotransmitters was as previously described [3].

RESULTS

Human pheochromocytoma tissues (n = 6), as well as the rat pheochromocytoma cell line PC 12, showed strong immunostaining for all marker polypeptides of SSV of neurons, protein S.V.2, the synaptotagmins (p65), synaptophysin (p38), protein p29 (p29) and the synaptobrevins (p18). Parallel sections also showed positive immunoreactions with antibodies against DBH and cytochrome b561, markers for large dense-core vesicles (LDCV). Foregut carcinoids (n = 12) and the human carcinoid cell line BON contained five marker polypeptides of SSV as well as the two marker polypeptides of LDCV. Among the intermediate filaments, only cytokeratins and vimentin were

Correspondence to B. Wiedenmann.
G. Ahnert-Hilger, K. Grube, E.-O. Riecken, L. Schmitt and B. Wiedenmann are at the Department of Internal Medicine, Universitätsklinikum Steglitz, Hindenburgdamm 30, Free University of Berlin, D-12200 Berlin, Germany; L. Kvols is at the Department of Medicine, Mayo-Clinic, Rochester, Minnesota, U.S.A.; I. Lee is at the Department of Pathology, Asan Medical Center, Seoul, Korea; and E. Mönch is at the Department of Pediatrics, Universitätsklinikum R. Virchow, Free University of Berlin, Germany.
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detected in the tumour cells. No differences in immunocytochemical staining patterns for polypeptides specific for SSV, LDCV and intermediate filaments were observed between functional and non-functional foregut carcinoids. Intermediate filament typing demonstrated that the majority of tumours were only positive for cytokeratins. A coexpression with vimentin and neurofilament protein was occasionally found.

A very similar immunohistochemical pattern was observed in midgut carcinoids, however, only cytokeratins were present (Table 1). The immunohistochemical data were substantiated by an immunoreplica analysis using vesicles purified by sucrose-gradient centrifugation. Vesicles from pheochromocytoma contained proteins S.V.2, p65, p38, p29 and p18. Vesicles from foregut carcinoids reacted strongly with antibodies against proteins S.V.2, p38 and p18 but weakly with antibodies against proteins p65 and p29. Vesicles from midgut carcinoids reacted strongly only with an antibody against synaptophysin and weakly with antibodies against proteins S.V.2 and the synaptotagmins (not shown).

In contrast to immunohistochemistry, some SSV marker polypeptides could not always be detected within the same tumour by immunoblotting. The large content of connective tissue, characteristic of NE tumours, as well as the a high protease content of these tumours (especially of those localised in the pancreas), may be responsible. Quantitative analysis of the main SSV markers, protein S.V.2 and synaptophysin by dot blot immunoassay [12] with purified synaptic vesicles from rat brain as standard revealed that all tumours tested and the respective tumour cell lines contain measurable amounts of S.V.2 (between 0.3 and 2 ng/μg protein) and synaptophysin (between 1.4 and 4.9 ng/μg protein).

Tumour tissue of the GEP system and the two cell lines PC 12 and BON contained the neurotransmitters glutamate and glycine in larger amounts than rat brain. GABA was only present in about half of the foregut carcinoids (Table 2). In tumours of non-NE origin, only small amounts of glycine and glutamate, but no GABA, were detected (data not shown).

DISCUSSION

NE tumours of the GEP system contain a second type of regulated secretory vesicle resembling neuronal SSV:

(i) Major integral membrane proteins of SSV are expressed in 35 NE tumours of the GEP system, as demonstrated by immunohistochemical and supported by biochemical analyses. Probably all NE cells and tumours of the GEP system contain SSV membrane proteins.

(ii) NE tumour tissues of the GEP system and the pancreatic cell lines BON (human) and AR42J (rat) [3] contain large amounts of amino acid neurotransmitters. The amino acids may, at least in part, be stored in vesicles similar to SSV of neurons [4]. GABA was only detected in tumours derived from the foregut (mainly NE cells of the stomach, pancreas and duodenum), but not in tumours originating in the midgut (mainly NE cells of jejunum and ileum) or in pheochromocytomas. Accordingly, extraneuronal GABA was only found in NE cells of the foregut, namely in normal and neoplastic β-cells of the pancreas [13–15]. Thus, the synthesis of certain amino acid transmitters in

Table 1. Immunocytochemical detection of marker proteins specific for membranes of small neuroendocrine (NE) vesicles, large dense-core vesicles (LDCV), and for intermediate filaments in tumour cell lines and human tumour tissues of the GEP system

	NE vesicles					LDCV		Intermediate filaments				
	S.V.2	p65	p38	p29	p18	Dopamine-β-hydroxylase (DBH)	Cytochrome b561	Cytokeratin	Vimentin	NFP	GFAP	Desmin
A. Pheochromocytoma												
Cell line												
(PC 12)	++	+++	+++	++	++	++	+	+++	–	+	–	++
Tumour tissues	++	+	+++	–	–	+++	+	+++,–	+++	++	–	–
(n = 6 or 5)	6/6	5/6	6/6	3/5	3/6	6/6	5/6	2/6; 4/6	6/6	5/6	4/6	5/6
B. Foregut carcinoid												
Cell line												
(BON)	+	(+)	+	+	+	++	–	++	+++	–	–	–
Tumour tissues												
Functional												
Gastrinomas												
(n = 5)	+	++	+++	++	++	++	+	++	–	+	–	–
	5/5	5/5	5/5	4/5	5/5	5/5	5/5	5/5	3/5	4/5	5/5	5/5
Vipoma (n = 1)	+	+	++	–	+	–	–	+	+	–	–	–
Non-functional	+	+	+++	+	–	+	++	++	–	–	–	–
(n = 6)	6/6	5/6	6/6	3/6	4/6	4/6	5/6	6/6	5/6	4/6	6/6	5/6
C. Midgut carcinoid												
Functional	+	+	+++	++	+	+++	++	++	–	–	–	–
(n = 16)	14/16	15/16	15/16	12/16	12/16	14/16	14/16	16/16	14/16	16/16	16/16	15/16
Non-functional	+	++	+++	–	++	–	++	++	–	–	–	–
(n = 1)												

Immunofluorescence microscopy was performed as described [1, 5]. Antibodies were the same as given in Materials and Methods. The intensity of reaction was assessed as follows: +++ very strong; ++ strong; + less strong; – no positive staining. NFP, neurofilament protein.

Table 2. Neurotransmitter content of neuroendocrine tumours of the GEP system

Origin of tissues	GABA (nmol/mg protein)	Glycine (nmol/mg protein)	Glutamate (nmol/mg protein)
Rat brain	97.1	34.9	29.1
A. Pheochromocytoma			
Cell line (PC 12)	—	132.4	89.7
Tumour tissue (n = 1)	—	77.6	213.6
B. Forecut carcinoid			
Cell line (BON)	4	203.3	87.2
Tumour tissues			
Functional (n = 3)	—; 146; 30	294 ± 208	300 ± 187
Non-functional (n = 2)	—; 12.6	69; 148	83; 200
C. Midgut carcinoid			
Tumour tissues			
Functional	—	108 ± 93	214 ± 170
Non-functional (n = 9)			

Amino acids were determined in cell homogenates as given in [3].

neuroendocrine GEP tumours is presumbly restricted to specific types of NE cells, similar to the GABAergic neurons in the central nervous system.

(iii) Subcellular fractionation of synaptophysin-containing membranes of tumour tissues showed a co-sedimentation with SSV of rat brain. The variable amounts of SSV marker proteins are not reflected by the clinical symptoms, i.e. there is no obvious correlation between the polypeptide composition of SSV, content of amino acid neurotransmitters and the functional state of the NE tumour. The presence of SSV proteins together with amino acid neurotransmitters suggests a neuron-like secretory pathway in neoplastic NE cells, as found in AR42J cells [4].

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