ORIGINAL

Localization of chromogranin A and B, β -endorphin and enkephalins in the submandibular glands of mice

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Abstract : The localization of chromogranin A and B, β -endorphin, met-enkephalin and leu-enkephalin was immunocytochemically investigated in the submandibular salivary gland (SMG) and pancreas of male mice using different antibodies. Procedures were carried out with different immunocytochemical methods, such as peroxidase-antiperoxidase and indirect immunofluorescence methods, visualized with diaminobenzidine, 4-chloro-1-naphthol and FITC stainings. Chromogranin A immunoreactivity was located in the granular convoluted tubule cells (GCT), whereas chromogranin B immunoreactivity was located in some of the solitary cells scattered around the acini, but not in GCT of SMG. In the pancreas, different localizations were observed between chromogranin A and B. β -Endorphin and met-enkephalin immunoreactivities were also located in GCT, but in the striated duct cells as well. However, met-enkephalin, but not β -endorphin, immunoreactivity was found in the nerves around the duct system. Our results strongly suggest that chromogranin A, but not chromogranin B, may be useful as a means to differentiate the cells in the duct system of the SMG responsible for production of biologically-active factors.

Introduction

The definition of endocrine cells has recently changed greatly but new evidence has arisen indicating the existence of specific and discriminative substances, such as chromogranins, within the cells of the endocrine system¹⁻³⁾.

The localization of chromogranins, the major soluble proteins of catecholamine storage-vesicles has recently been studied in the adrenal gland⁴⁻⁶⁾, in the endocrine system^{3,5)}, in the central and peripheral nervous systems^{7,8)}, and in the endocrine cells of the gastrointestinal tract⁹⁾. Chromogranin A is a protein with a molecular weight of 68,000 daltons that was first found to be released from adrenal medulla cells^{1,4)} and later was characterized⁶⁾. More recently, several other minor

chromogranins were separated from chromogranin vesicle lysate and named B and C with the immunological reactivity similar to that of the major chromogranin A^{8,10)}. The proteins coexist with catecholamines in the storage granules of the adrenal medulla cells and sympathetic nerves, and are released together with catecholamines.

In addition, many biologically-active hormone-like factors have been discovered and investigated in the submandibular salivary glands, such as nerve growth factor (NGF)^{11,12)}, epidermal growth factor (EGF)¹³⁾ or somatostatin¹⁴⁻¹⁶⁾. Although some authors have tried to investigate endocrine-like cells of the submandibular salivary glands¹⁷⁻¹⁹⁾ using argyrophilic or argentaffin methods, no comparative studies have been carried out dealing with the colocalization of EGF, NGF, somato-

statin, endorphin and enkephalins with the newly discovered markers of the endocrine cells such as the aforementioned proteins in the submandibular salivary glands of mice. Furthermore, no information is yet available on the localization and functions of chromogranins, β -endorphin and enkephalins in the submandibular salivary glands in particular except a report⁷⁾.

The present study on the submandibular salivary glands of mice was designed to determine distribution and localization of chromogranin A and B whether they were present within the same cells as those contain endorphin and enkephalins, and to compare with chromogranin A and B in the pancreas.

Materials and Methods

Animals. Male mice of the inbred CBA/H strain were obtained from the Institute for Experimental Medicine, Military Academy, Belgrade, Yugoslavia. All animals were approximately 4 weeks of age at the time from the beginning to the end of the experiments. The animals had *ad libitum* access to rat chow and water.

Tissue preparation. The submandibular salivary glands and pancreases were collected and processed for histological and immunohistochemical stainings. Fresh tissues were fixed in saturated vapor of paraformaldehyde and para-amino-benzoquinone for 3 h at 60 °C⁹⁾ or Bouin solution, and embedded in paraffin wax, or cryo sections were made (Table 1). Serial sections (3-5 μ m, Reichert-Biovar) were collected on poly-L-lysine (PLL)-coated slide glasses and prepared for histological and immunohistochemical stainings. For light microscopy, tissues were stained with haematoxylin-eosin and analyzed under a Reichert-Biovar microscope. Immunohistochemical staining procedures and characteristics of antisera are shown in Table 2.

Immunohistochemistry. Sections were immunostained by the peroxidase-antiperoxidase (PAP) procedure²⁰⁾ or by an indirect immunofluorescence method²¹⁾. Tissues were extensively washed through all procedures in 0.15 M phosphate buffer saline (PBS) at pH 7.1. Visualization of the PAP complex was achieved by the diamine-benzidine (DAB) method²²⁾ or by the 4-chloro-1-naphthol meth-

Table 1 Fixation methods and embedding media for the fresh submandibular glands and pancreas of mice

	Fixation	Embedding
Submandibular gland	1) Bouin	Paraffin wax
	2) Paraformaldehyde	Paraffin wax
	3) Para-amino-benzoquinone	Cryo section
Pancreas	1) Bouin	Paraffin wax
	2) Paraformaldehyde	Paraffin wax

Table 2 Properties of various antisera, and staining and visualization methods for the fresh submandibular gland and pancreas of mice

Antibodies to:	Types of antibodies	Dilution	Staining	Visualization
Chromogranin A	Mouse MC	1 : 200	PAP	1) Diaminobenzidine (DAB) and
	Rabbit anti-bovine	1 : 200	PAP	2) 4-chloro-1-naphthol
Chromogranin B	Rabbit anti-rat	1 : 200	PAP	
β -Endorphin	Mouse MC	1 : 200	IF and PAP	FITC, 1) and 2)
	Rabbit anti-rat	1 : 6000	IF and PAP	FITC, 1) and 2)
Met-enkephalin	Rabbit anti-rat	1 : 4000	IF and PAP	FITC, 1) and 2)
Leu-enkephalin	Rabbit anti-rat	1 : 4000	IF and PAP	FITC, 1) and 2)
	Mouse MC	1 : 200	IF and PAP	FITC, 1) and 2)

MC = monoclonal antibody

od²⁹).

After developing, the sections were counterstained with haematoxylin, dehydrated (not after 4-chloro-1-naphthol staining) through graded concentrations of alcohol to xylene, mounted in Entellan mounting media (Merck) and examined under an Olympus AH-2 microscope. The controls for immunostaining included omission of the first or second layer, or replacement of the first layer with normal sera from the same donor species, although endogenous peroxidase activity and non-specific staining due to the bridging antisera were blocked by sequential incubation in 0.03 % H₂O₂ for 30 min⁹. In some instances, antisera were absorbed with the respective antigens: 10⁻⁸ mol β -endorphin (Sigma), 5 nmol met-enkephalin (Sigma) and 5 nmol leu-enkephalin (Sigma).

Chemicals. They were used as follows: mouse monoclonal antisera to chromogranin A, β -endorphin and leu-enkephalin, polyclonal antisera of rabbit anti-bovine chromogranin A, rabbit anti-rat chromogranin B, rabbit anti-rat β -endorphin, and rabbit anti-rat met- and leu-enkephalin were used (Table 2). All these antisera were kind gifts from the Histochemical Department, Royal Postgraduate Medical School, London, England. Second layer antibodies were goat anti-mouse IgG (Miles Lab.), rabbit anti-mouse IgG (Miles Lab.), goat anti-rabbit IgG (H+L) (Miles Lab.) and rabbit anti-mouse IgG labeled with biotin (Sera Lab.). The third layer antibodies were mouse monoclonal PAP (Servac) and rabbit PAP (Miles Lab.).

Results

Chromogranin immunoreactivities in the submandibular salivary gland and pancreas

Chromogranin A immunoreactivity was located in the duct system, particularly in the granular convoluted tubule cells, of the mouse submandibular glands (Fig. 1 A), although in the pancreas it was located in various types of cells of the islets (Fig. 1 B). By fixation with paraformaldehyde (Fig. 1 A), chromogranin A-positive contents were well preserved in the granules of the granular convoluted tubule cells. Intensity of staining indicated that in numerous granules, chro-

mogranin A could be located. This chromogranin A immunostaining was predominantly located in the apical part of the cells. The immunoreactivity was almost always granular and obviously dependent on the phase of the secretory process of the cell. Some cells did not contain chromogranin A-positive granules (Fig. 1 A, arrows), even though the granular convoluted tubule cells retained their secretory granules within the cytoplasm.

The chromogranin B immunoreactivities in the submandibular gland were not in the granular convoluted tubule cells, but in some of the solitary cells scattered around the acini (Fig. 2 A). These cells were pyramidal (-like) cells in shape and immunostained homogeneously. Their nuclei were not stained. By Bouin fixation as well as by para-aminobenzoquinone fixation, the chromogranin B immunoreactivity was well preserved within the cytosol of the cells, not yet well defined (Fig. 2 A). In a similar manner, chromogranin A and B immunoreactivities were present in different locations in the pancreas, used as the controls for chromogranin A and B immunoreactivities, as well. In the pancreas, chromogranin B immunoreactivity was specifically located in the pancreatic islet cells (Fig. 2 B), different from that of chromogranin A immunoreactivity (Fig. 1 B). Chromogranin A in the pancreas was located in more cells, even in the middle or the periphery of the islets, but chromogranin B only in lesser cells at the periphery of the islets (Fig. 2 B).

Comparative analysis of the locations of β -endorphin and enkephalins.

Our results showed that β -endorphin (Fig. 3) and met-enkephalin (Figs. 4 A and B) immunoreactivities were localized within the cells of the submandibular salivary glands of mice. Both peptides were predominantly localized in the granular convoluted tubule cells as well as in the cells of the striated duct (Figs. 3 and 4 B, arrows). Additionally, some immunoreactivities were observed within the solitary cells scattered around the acini (Figs. 3 and 4 B, arrow heads), although the endogenous peroxidase activity and non-specific staining were blocked and the successful absorption test was carried out, as aforementioned

Fig. 1 The localization of chromogranin A immunoreactivity in the mouse submandibular salivary glands (A) and pancreas (B), fixed with para-formaldehyde and stained with DAB (A), fixed with Bouin and stained with DAB (B). The arrows indicate lack of responses from chromogranin A immunostainings in some duct cells of the submandibular glands. Bar = 25 μ m.

in Materials and Methods. These cells, which are not defined so far, do not belong to any part of the duct system. β -Endorphin immunoreactivity was mostly in the apical parts of the cells, but not homogeneously manifested in the duct cells, although in some cells of the striated duct, absence of this peptide could be evident (Fig. 3, white arrow head). We could not determine any positive β -endorphin immunostainings in the nerves of the submandibular salivary glands. However, more cells in the duct compartment (arrows) and the solitary cells (arrow heads) scattered around the acini, showed met-enkephalin immunoreactivities (Fig. 4 B). Thus, such kinds of immunostainings can be seen in the nerves as well (Fig. 4 A). Met-enkepha-

lin-positive nerves were mostly distributed around the duct system. Neither the nerves near the secretory endpieces nor the cells belonging to the ganglion in the submandibular glands were stained with the antibodies used. Leu-enkephalin-containing cells or nerves were not detected in any compartment of the submandibular glands regardless of the monoclonal or polyclonal antibodies used.

Discussion

Chromogranin A was originally thought to be confined to adrenergic organs including sympathetic nerves^{4,6)}. Since Cohn *et al.*²⁴⁾ reported that secretory protein I, isolated from parathyroid glands, was quite similar biochemically and immunologically to chromo-

Fig. 2 The localization of chromogranin B immunoreactivity in the mouse submandibular glands (A) and pancreas (B), fixed with para-amino-benzoquinone using cryo sections and stained with 4-chloro-1-naphthol (A), fixed with Bouin and stained with 4-chloro-1-naphthol (B). Bar = 25 μ m.

granin A, distribution of chromogranin A was established in non-adrenergic organs including most of the endocrine (anterior pituitary, endocrine pancreas, C-cell of thyroid gland and entero-chromaffin (EC-) cells) and nervous tissues. This protein has been believed to be absent from the exocrine tissues³⁾ and from liver, lung and kidney^{9,10,25-27)}. However, we have found chromogranin A and B localized in specific parts of the exocrine gland, namely, the mouse submandibular gland.

Chromogranin A immunoreactivity was located in the duct system, particularly in the granular convoluted tubule cells of the submandibular gland of mice with the different localization from that of chromogranin B. This

result may suggest that the endocrine and exocrine cells can be colocalized in the granular convoluted tubule cells, since EGF as an exportable polypeptide and somatostatin-related peptides were colocalized with chromogranin A immunoreactivity, defined as a new marker of the endocrine cells⁹⁾ in other tissues. In the submandibular gland, however at present there are no reports available, that in the granular convoluted tubule cells any cells have histologically been found to have the endocrine function. These results are consistent with some reports⁷⁾, dealing with the immunohistochemical detection of chromogranin-positive cells within the bovine submandibular gland. However, our results, with more details and with different fixation

Fig. 3 The localization of β -endorphin immunoreactivity in the mouse submandibular glands, fixed with para-amino-benzoquinone and stained with 4-chloro-1-naphthol. Arrows indicate immunostainings within the duct cells, arrow heads immunoreactivities within the solitary cells scattered around the acini and a white arrow head lack of responses from the immunostainings. Bar=25 μ m.

procedures, indicated chromogranin A-positive cells within the small duct, i. e., the granular convoluted tubule cells, in contrast to those within the large interlobular duct⁷⁾. Furthermore, in the gastrointestinal tract chromogranin A-positive cells were also identified in serial sections as gastrin- or somatostatin-containing cells⁹⁾, which are consistent with our results on the location of chromogranin A as well as somatostatin within the granular convoluted tubule cells.

Chromogranin B can be now clearly differentiated from chromogranin A²⁵⁾ and has also a widespread distribution, somewhat different from that of chromogranin A. By means of immunohistochemistry, we detected chromogranin B immunoreactivity in some of the solitary cells scattered around the acini, but not in any duct cells, in the submandibular glands of mice, particularly of younger mice during postnatal development (publication in preparation).

This dissimilar pattern of the chromogranin A and B localizations seems to occur in the pancreas, used for the immunostaining controls of chromogranins, as well. These results are consistent with several reports on the distribution of chromogranin A in 5-hy-

droxytryptamine-producing argentaffin cells, intestinal non-EC-cells, gastric ELC-cells, pancreatic glucagon (A-) cells, thyroid C-cells and other organs^{10,26)}, although insulin-producing cells have the weakest immunoreactivity²⁶⁾. However, chromogranin B immunoreactivity has been localized only in the glucagon (A-) cells of the pancreas¹⁰⁾, within the sympathetic ganglia, anterior hypophysis and EC-cells of the upper intestinal tract²⁷⁾. Our results that no exocrine acini in the pancreas of mice have shown any chromogranin A- or B-positive reactivity, were consistent with several reports⁵⁾. As successful results on the distribution of chromogranin A and B immunoreactivities in the pancreas were obtained and our main aim was to compare the localization between chromogranin A and B, we used polyclonal antisera (1 : 200 dilution) for the detection of chromogranins in the submandibular salivary glands of mice. In particular, no good results on the chromogranin B immunostaining were obtained using the polyclonal antisera (1 : 1000 dilution).

Opioid peptides can be divided into two or three groups, such as enkephalins and endorphins²⁸⁾. Leu- and met-enkephalins are pentapeptides isolated from adrenal medulla²⁹⁻³¹⁾.

Fig. 4 The localization of met-enkephalin immunoreactivity of the nerves (A) and the granular convoluted tubule cells or the striated duct cells (B) in the mouse submandibular glands, fixed with para-amino-benzoquinone (A and B) and stained with the indirect immunofluorescence method (A) and with 4-chloro-1-naphthol (B). Arrows indicate immunostainings within the duct cells and arrow heads within the scattered cells. Bar=25 μ m.

These peptides can be found in the central nervous system^{32,33}, in the spinal cord³⁴ and also in the gut. Enkephalin immunoreactivity can be found in stomach and duodenum³⁵, pancreas³⁶ and mesenteric plexus in the gut³⁷. Although Yang *et al.*³⁸ emphasized the significance of the coexistence of catecholamines and enkephalin-like peptides, the localization of enkephalin-like immunoreactivity in the submandibular glands has

not been reported in any systemic study. β -Endorphin-like immunoreactivity has also been reported in the hypophysis³⁹, pancreas⁴⁰ and stomach⁴¹.

No reports can be found on the β -endorphin and met-enkephalin content in the submandibular glands. However, recent data indicated that catecholamines and enkephalin-like peptides coexist in the nerves of the central nervous system⁴² and in the nerve terminals

of the sympathetic ganglia, and in adrenal medullary (gland) cells^{38,43)}. In addition, so far, it is well-known that catecholamine-containing nerves are abundant in the submandibular salivary glands and following such findings, it is logical to presume the existence of enkephalin-like peptides in the nerves of the submandibular glands. Results from moderate to weak immunostainings also indicate coexistence of these peptides, probably with some other transmitters. However, further investigations are necessary to elucidate these co-existences.

From these results, we can conclude that chromogranins, especially chromogranin A,

can be visualized particularly in the duct system in the submandibular glands of mice. Chromogranin A may also be useful as a means to differentiate the specific types of cells in the duct system of the submandibular salivary glands involved in the production of biologically-active factors.

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抄録：4週齢の CBA/H 系雄マウスの顎下腺および脾臓を用いて、クロモグラニンAとB、 β -エンドルフィン、Met- および Leu- エンケファリンの局在を各種のモノおよびポリクロナール抗体を用いた PAP および間接蛍光抗体法から比較検索した。クロモグラニンA陽性物質は顎下腺の顆粒管に局在していたが、クロモグラニンB陽性物質は顆粒管には存在せず、腺房細胞の周囲に散在する Solitary 細胞に局在していた。 β - エンドルフィンおよび Met- エンケファリン陽性物質は顆粒管だけでなく、線条部にも存在していた。Leu- エンケファリン陽性物質は顎下腺には検出できなかった。しかし、Met- エンケフェリン陽性物質は、 β - エンドルフィンと異なり、導管系周囲の神経にも局在していた。この実験から、クロモグラニンAは、クロモグラニンBとは異なり、特に顎下腺の生理活性物質を産生する導管系の細胞を鑑別する手段に用いることができると思われた。

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